ANALYTICAL METHODS FOR FOOD SAFETY BY MASS SPECTROMETRY

VOLUME II VETERINARY DRUGS

GUO-FANG PANG

Chemical Industry Press

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Volume II Veterinary Drugs

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Preface

Today more and more requirements and restrictions are being placed on the residues of pesticides and veterinary drugs in edible animal and plant-derived agricultural products all over the world, and more and more kinds of pesticides must be controlled. Meanwhile, the index of maximum residue limit (MRL) for pesticides is getting lower, so the barriers to international trade of edible agricultural products are becoming higher.

The research team of Guo-Fang Pang took the lead in research focusing on the strategic development of food safety. They met the challenge, and a series of low-cost and highly efficient detection methods have been established.

In the field of pesticide residues determination, the authors conducted a systematic study of the detection of multiclasses and multitypes of pesticide residues in different edible agricultural products, and 20 high-throughput sample preparation and analytical methods were developed for simultaneous extraction, separation, enrichment and determination of 400-500 pesticide residues with a once-for-all sample preparation. These methods are applicable to the simultaneous determination of multipesticide residues in animal and plant-derived agricultural products, such as fruit juices, vegetable juices, fruit wines, fruits, vegetables, cereals, tea, Chinese medicinal herbs (such as ramulus mori, honeysuckle, Chinese wolfberry, and lotus leaf), edible fungi (such as mushrooms), honey, milk, milk powder, aquatic products (such as globefish, eel, and prawn), animal muscle tissues, and drinking water. A high-selectivity, high-sensitivity, high-resolution, and high-throughput analysis methodology for the detection of more than 1000 pesticide residues has been established. These methods are considered to be the international leaders in the type and quantity of pesticides that can be detected simultaneously.

In veterinary drug residue detection, the authors have established 65 high-selectivity, high-sensitivity veterinary drug residue detection methods for the determination of 20 types of nearly 200 commonly used veterinary drugs that may be residual in edible animal-derived agricultural products, and the methods are applicable to samples with complicated bases, such as edible animal tissue (such as muscle, liver, kidney, and fat), milk, milk powder, bee products (such as honey, royal jelly, and its lyophilized powder), aquatic products (such as globefish, eel, and prawn), and animal urine.

This book is a systemic summary of the research of Guo-Fang Pang's team on the theory and applied practice of detection technology of pesticide residues

over the past 20 years. The innovative research achievements at an internationally advanced level in this field are fully demonstrated in this work. In addition, the progress of food safety detection technology has been promoted. This is mainly reflected in the following aspects:

1. High-throughput sample pretreatment technology: A series of technical problems such as the extraction of pesticide and veterinary drug residues at the level of micrograms per kilogram in various complex matrix samples and the effective purification of the CO-extracted disrupting chemicals have been overcome, and high-throughput sample preparation and purification technology at an international leading level has been developed. Implementation of the detection of 400–500 pesticide residues simultaneously using one sample preparation has been achieved.
2. The most comprehensive database available of pesticide mass spectrometry has been constructed: gas chromatography-(tandem) mass spectrometry and liquid chromatography-(tandem) mass spectrometry characteristics of 1000 commonly used pesticides worldwide were systematically studied and a database with tens of thousands of mass spectra has been constructed. The database can provide a qualitative and quantitative basis and has laid the foundation for research and development of high-throughput detection technology.
3. A new technique of group detection by time intervals using chromatography and mass spectrometry is proposed: pesticides with similar chemical properties and retention times are divided into several groups in turn; thus, the selectivity of the method can be enhanced. Each group of pesticides is detected according to the peak order and the time intervals, so the selectivity of the sensitivity of the methods can be improved and the monitoring range expanded.

Furthermore, academician Pang's team also cooperated fully with units from the quality inspection system of China, institutions of higher learning, and scientific research institutes for collaborative verification of the reliability and applicability of the methods, thus leading to the formation of Chinese national standards (GB/T series standards). These methods have been widely used in the detection of pesticide and veterinary drug residues in edible animal and plant-derived agricultural products and they have made a great contribution to the guarantee of food safety in China. We believe these methods will provide a useful reference value for edible animal and plant-derived agricultural product safety detection all over the world.

The book is suitable for scientific researchers in the residues testing areas and technicians working in agricultural products testing and inspection, as well as teachers and scholars of higher learning institutions as their reading material or reference literature.

October 10, 2017

Brief Introduction

Food safety is a major security issue necessary to ensure the sound development of human society. The widespread use of pesticides, veterinary drugs, and other agricultural chemicals have contributed to widespread contamination in agricultural products. The long-term consumption of food with high residues of pesticides and veterinary drugs will cause both acute and chronic toxicity in humans and induce resistant strains, thus resulting in allergies, cancer, mutation, and teratogenicity. In order to ensure human health and food safety, countries all over the world have issued strict food safety and hygiene standards. The People's Republic of China (PRC) has implemented strict monitoring of pesticide and veterinary drug residues, such as maximum residue limits for veterinary drugs in food of animal origin (Announcement No. 235 of the Ministry of Agriculture of the People's Republic of China) and national food safety standards: Maximum residue limits for pesticides in food (GB 2763-2016). The commonly used pesticides and veterinary drugs approved in China and the main agricultural products of urban residents' daily consumption are almost all covered.

The book is a systematic summary of the high-throughput chromatography-mass spectrometry technique for the analysis of multipesticides, veterinary drugs, and other agricultural chemical residues in agriculture products of plant and animal origin. Its technical characteristics are mainly embodied in the simultaneous determination of hundreds of compounds, as well as low cost and high efficiency.

This book is divided into two volumes. In Volume 1, pesticides and related chemicals are selected as the main research object, while gas chromatography-mass spectrometry (GC-MS), gas chromatography-tandem mass spectrometry (GC-MS-MS), and liquid chromatography-tandem mass spectrometry (LC-MS-MS) are concerned. This volume includes more than 20 analytical methods for the high-throughput analysis of multipesticide residues, and more than 1000 pesticides and related chemicals in more than 10 kinds of agricultural products and drinking water can be detected, and 400–500 kinds of pesticide residues can be detected simultaneously with the pretreatment undertaken just once. The methods for the analysis of 793 pesticides and related chemical residues in foods of plant origin applicable to fruit juices, vegetable juices, fruit wines, fruits, vegetables, cereals, tea, Chinese medicinal herbs (ramulus mori, honeysuckle, Chinese wolfberry, and lotus leaf), and edible fungi (mushroom) are introduced in Part 1; the methods for the analysis of 790 pesticides and related chemical residues in foods of animal origin applicable to honey, milk, milk

powder, aquatic products (globefish, eel, and prawn), and animal muscle tissues are presented in Part 2; while the methods for the analysis of 450 pesticides and related chemical residues in drinking water are introduced in Part 3. Furthermore, the three major parameter databases adopted in the analysis of more than 1000 pesticides and related chemicals are also included in this volume: ①chromatography-mass spectrometry characteristic parameters include retention time, quantitative and qualitative ions, fragment voltage and collision energy, etc.; ②performance parameters of the methods include linear equation, linear range and correlation coefficient, etc.; and ③gel permeation chromatography (GPC) purification analysis parameters.

In Volume 2, the methods for the analysis of 20 species (nearly 200 kinds) of veterinary drug residues in edible animal tissues (muscle, liver, kidney, and fat), dairies (milk and milk powder), bee products (honey, royal jelly, and its lyophilized powder), aquatic products (globefish, eel, and roasted eel) and animal urine are selected as main subjects of study. The 65 analytical methods for the analysis of multiveterinary drug residues are described in different chapters by category (sulfonamides, β-adrenergic agonists, aminoglycosides, chloramphenicols, β-lactams, macrolides, nitrofurans, anabolic steroids, nonsteroidal anabolic steroids, glucocorticoids, fluoroquinolones, tetracyclines, sedatives, pyrazolones, quinoxalines, nitromidazoles, benzimidazoles, levamisole, thiourea pyrimidines, and polyethers), and 90% of the methods are LC-MS-MS. Meanwhile the physical and chemical properties, efficacy, side effects, and maximum allowable residual limit of all the compounds are also provided.

In short, this book is the summary of the work of the author team who, for more than 20 years, engaged in the research and practice of detection technology of pesticides and veterinary drug residues. These methods for the analysis of pesticides and veterinary drugs are innovative research results based on the international frontier of pesticides and veterinary drug residue analysis, and the analytical techniques adopted are new technologies widely concerned in the field of residue analysis in the world today. The performance indexes of the methods can meet the requirements of the Codex Alimentarius Commission and the world's major developed countries; meanwhile the methods are in conformity with the developing trend of international residue analysis and they are advanced in the world. In addition, the methods established in this book have become the current effective national standard methods in China and they can be used as the detection basis of relevant testing institutions and the law basis of the relevant government departments. Nevertheless, due to the limitations of the level, there may be unavoidable errors. We would kindly ask the users of this publication to provide feedback to the authors so that subsequent editions may be improved upon.

Guo-Fang Pang

Chapter 1

Sulfonamides

1.1

Curative Effects and Side Effects of Sulfonamides

Sulfonamides are a family of antibiotics used to treat a wide range of bacterial infections. They inhibit bacterial synthesis of dihydrofolic acid by preventing the condensation of pteridine with aminobenzoic acid through competitive inhibition of the enzyme dihydropteroate synthetase. Resistant strains have altered dihydropteroate synthetase with reduced affinity for sulfonamides or produce increased quantities of aminobenzoic acid. Topically applied sulfonamides are considered active against susceptible strains of the following common bacterial eye pathogens: *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus* (viridans group), *Haemophilus influenzae*, *Klebsiella* species, and *Enterobacter* species. Topically applied sulfonamides do not provide adequate coverage against *Neisseria* species, *Serratia marcescens* and *Pseudomonas aeruginosa*. A significant percentage of staphylococcal isolates are completely resistant to sulfa drugs.

The extensive use of sulfonamides in animal husbandry has been associated with the presence of sulfonamide residue in meat and meat products. The presence of sulfonamide residue in food is of concern because some of the compounds are known to be carcinogenic and they generally enhance the risk of developing antibiotic resistance, which makes the therapeutic use of similar drugs inefficient. Recent evidence has implicated sulfamethazine as a possible thyroid carcinogenic agent. Sulfonamide residues in food and animal tissues may be present in minute concentrations but may pose a health threat to consumers. Therefore, monitoring of these compounds has attracted interest from the scientific communities.

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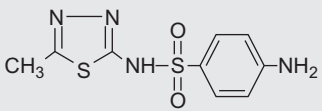
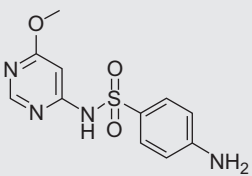
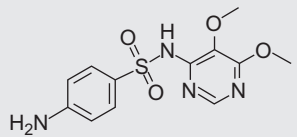
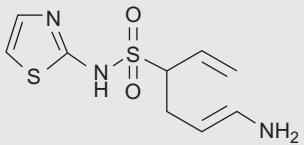
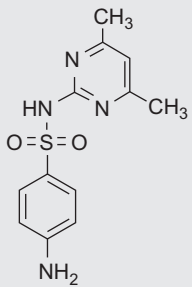
1.2

Chemical Structures and Maximum Residue Limits for Sulfonamide Drugs

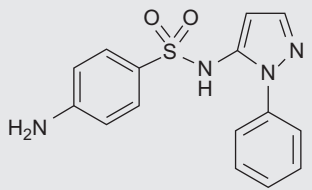
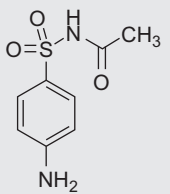
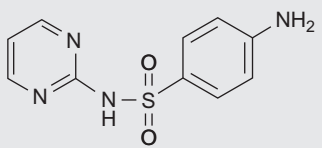
TABLE 1.1 Chemical Structures and Maximum Residue Limits for Sulfonamide Drugs

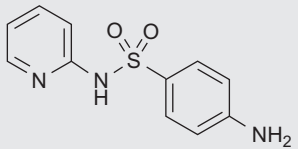
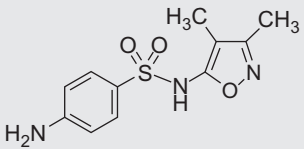
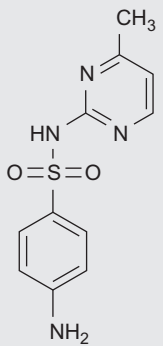
Compounds	Structure	Molecular Weight	Cas.No.	MRL (μg/kg)
Sulfamethizole		270.33	144-82-1	US: 100; CA: 100; EU: 100
Sulfamonomethoxine		280.30	1220-83-3	US: 100; CA: 100; EU: 100 Chicken: 100 Cattle muscle: 10 Pig muscle: 20 Cattle and pig kidney, fat, liver: 50

Sulfadoxine		310.32	2447-57-6	US: 100; CA: 100; EU: 100 Milk: 60
Sulfathiazole		255.32	72-14-0	US: 100; CA: 100; EU: 100
Sulfamethazine		278.33	57-68-1	US: 100; CA: 100; EU: 100 Pig, edible offal excluding liver: 20 Milk: 60

Continued

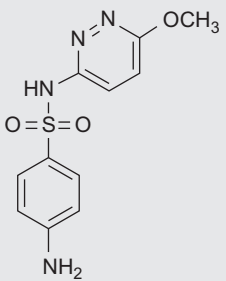
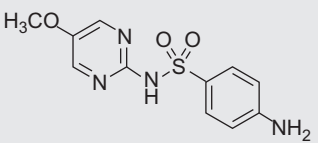
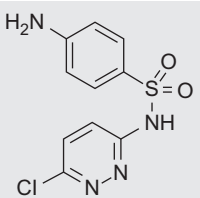
TABLE 1.1 Chemical Structures and Maximum Residue Limits for Sulfonamide Drugs—cont'd

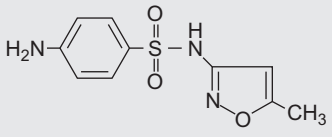
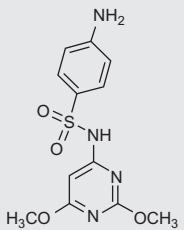
Compounds	Structure	Molecular Weight	Cas.No.	MRL (μg/kg)
Sulfaphenazole		314.36	526-08-9	US: 100; CA: 100; EU: 100
Sulfacetamide		214.24	144-80-9	USA: 100; CHI: 100; EU: 100 Milk: 10
Sulfadiazine		250.28	68-35-9	US: 100; CA: 100; EU: 100 Milk: 70 Poultry eggs: 20

Sulfapyridine		249.29	144-83-2	US: 100; CA: 100; EU: 100 Milk: 10
Sulfisoxazole		267.30	127-69-5	US: 100; CA: 100; EU: 100 Salmoniformes, Perciformes, fish: 100
Sulfamerazine		264.3	127-79-7	US: 100; CA: 100; EU: 100 JP: 100

Continued

TABLE 1.1 Chemical Structures and Maximum Residue Limits for Sulfonamide Drugs—cont'd

Compounds	Structure	Molecular Weight	Cas.No.	MRL (μg/kg)
Sulfamethoxypyridazine		280.30	80-35-3	US: 100; CA: 100; EU: 100 Pig muscle: 30 Pig fat, liver, kidney: 50
Sulfameter		280.30	651-06-9	US: 100; CA: 100; EU: 100
Sulfachloropyridazine		284.72	80-32-0	US: 100; CA: 100; EU: 100 Cattle: 100 Pig: 50

Sulfamethoxazole		253.28	723-46-6	US: 100; CA: 100; EU: 100 Pig: 20 Chicken fat: 50 Chicken muscle, kidney, liver: 20
Sulfadimethoxine		310.33	122-11-2	US: 100; CA: 100; EU: 100 Pig muscle, liver: 200 Chicken: 50 Milk: 20

1.3

Determination of 16 Sulfonamide Residues in Livestock and Poultry Muscles—LC-MS-MS Method (GB/T 20759-2006)

1.3.1 SCOPE

This method is applicable to the determination of 16 sulfonamide residues in bovine, mutton, porcine, chicken, and rabbit muscles.

The limit of determination of this method for sulfamethizole is 2.5 μg/kg; for sulfacetamide, sulfadiazine, sulfapyridine, sulfisoxazole, sulfamerazine, sulfachloropyridazine, sulfamonomethoxine, sulfadoxin, sulfamethoxazole is 5.0 μg/kg; for sulfathiazole, sulfathiazole, sulfadimethoxine is 10.0 μg/kg; for sulfameter, sulfamethazine is 20.0 μg/kg; for sulfamethazine is 40.0 μg/kg.

1.3.2 PRINCIPLE

The drugs are extracted from livestock and poultry muscle with acetonitrile. After centrifugation, the supernatant is evaporated to dryness in a rotary evaporator, after which it is defatted with hexane. It is dissolved in mobile phase and the solution is analyzed by LC-MS-MS, using an external standard.

1.3.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be reagent grade; "water" is deionized water.

Acetonitrile: HPLC grade; Isopropanol; n-Hexane; Ammonium acetate; Anhydrous sodium sulfate: heated at 650°C for 4 h, placed in drying oven until used; Mobile phase: acetonitrile 0.01 mol/L Ammonium acetate solution (12+88); Filter membrane: 0.2 μm.

Sulfonamide standards: purity $\geq$99%.

Standard stock solutions of sulfonamides: 0.1 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the standard stock solutions of 0.1 mg/mL in concentration. The standard stock solutions are stored 2 months at 4°C.

Standard working solutions of 16 sulfonamides: Depending upon the sensitivity of each sulfonamide and instrument linear range, prepare the standard

working solutions of different concentrations for 16 sulfonamides with livestock and poultry muscle control sample extract. The standard working solutions are stored 1 week at 4°C.

1.3.4 APPARATUS

LC-MS-MS: Equipped with ESI source; Homogenizer; Rotary evaporator; Vortex mixer; Centrifuger; Analytical balance: Capable of weighing from 0.1 mg to 0.01 g; Pipette: 1 mL, 2 mL; Pear-shaped flask: 100 mL; Screw vial: 2.0 mL, with screw caps and PTFE septa; Reservoirs and adapters to fit SPE cartridge: 50 mL.

1.3.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

The representative sample, ca. 1 kg, is taken from whole sample, vigorously stirred, thoroughly mixed, divided into two equal parts, and then put in a clean container, which is sealed and labeled, respectively. In the course of sampling and sample preparation, caution should be taken to avoid contamination or any factors that may cause a change of residue content. The test samples should be stored at –18°C.

(2) Extraction

Weigh 5 g test sample (accurate to 0.01 g) into 50-mL centrifuge tube. Add 20 g anhydrous Na_2SO4 and 25 mL acetonitrile to each sample. Homogenize 2 min. Centrifuge the sample solution at 3000 rpm for 3 min. Decant the supernatant into a 100-mL pear-shaped flask. Add 20 mL acetonitrile into the dregs and repeat the operation. Combine the supernatants. Add 10 mL isopropanol into the pear-shaped flask. Evaporate to dryness on a rotary evaporator with vacuum in a water bath at 50°C.

(3) Clean-up

Accurately add 1 mL mobile phase and 1 mL hexane to drugs. Transfer this to a 5-mL centrifuge tube and mix 1 min. Centrifuge the sample solution at 3000 rpm for 3 min. Pipette supernatant hexane and discard; then add 1 mL hexane. Repeat the operation, until the lower water phase turns clear. Extract five animal muscle samples, as described in the procedure of preparing working standard mixed solutions in matrix. Pipette the lower phase; after passing through a 0.2-μm filter membrane, it is ready for determination by LC-MS-MS.

1.3.6 DETERMINATION

(1) Operation conditions

Chromatographic column: Lichrospher 100 PR-18 5 μm, 4.6 × 250 mm, or equivalent; Mobile phase: acetonitrile 0.01 mol/L Ammonium acetate

solution (12+88); Flow rate: 0.8 mL/min; Column temperature: 35°C; Injection volumes: 40 μL; Split ratio: 1:3.

Ion source: ESI source; Scan mode: Positive scan; Monitor mode: Multiple reaction monitor; Ionspray voltage: 5500 V; Nebulizer gas: 0.055 MPa; Curtain gas: 0.079 MPa; Turbo ionspray gas rate: 6 L/min; Source temperature: 400°C; MRM transitions for precursor/product ion, Quantifying for precursor/product ion, declustering potential, collision energy, declustering potential: see Table 1.2.

(2) LC-MS-MS analysis

The standard working solutions of different concentrations for 16 sulfonamides are prepared with animal muscle control sample extract on base. Then inject 20 μL of the different concentration working standard solutions, respectively, in duplication under LC and MS conditions. Draw the seven-point standard curves of each sulfonamide (peak area vs. sulfonamide concentration). Calculate the concentrations of the corresponding content from the working curve. The responses of 16 sulfonamides in the standard working solution and sample solution should be in the linear range of the instrumental detection. Under LC and MS conditions, the reference retention times of the 16 sulfonamides are seen in Table 1.3. The total ion chromatograms (TICs) of sulfonamide standards are shown in Fig. 1.1.

1.3.7 PRECISION

The precision data of the method has been determined in accordance with the stipulations of GB/T 6379 and GB/T 6379.2. The values of repeatability and reproducibility were obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for 16 sulfonamide drugs in livestock and poultry muscle are shown in Table 1.4.

If the difference in the values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations shall be reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for 16 sulfonamide drugs in livestock and poultry muscle are shown in Table 1.4.

TABLE 1.2 MRM Transitions for Precursor/Product Ion, Quantifying for Precursor/Product Ion, Collision Energy, Declustering Potential of 16 Sulfonamides

Analytes	MRM Transitions for Precursor/ Product Ion (*m*/*z*)	Quantifying for Precursor/ Product Ion (*m*/*z*)	Collision Energy (V)	Declustering Potential (V)
Sulfacetamide	215/156; 215/108	215/156	18; 28	40; 45
Sulfamethizole	271/156; 271/107	271/156	20; 32	50; 50
Sulfisoxazole	268/156; 268/113	268/156	20; 23	45; 45
Sulfachloropyridazine	285/156; 285/108	285/156	23; 35	50; 50
Sulfadiazine	251/156; 251/185	251/156	23; 27	55; 50
Sulfamethoxazole	254/156; 254/147	254/156	23; 22	50; 45
Sulfathiazole	256/156; 256/107	256/156	22; 32	55; 47
Sulfamonomethoxine	281/156; 281/215	281/156	25; 25	65; 50
Sulfamerazine	265/156; 265/172	265/156	25; 24	50; 60
Sulfadoxine	311/156; 311/108	311/156	31; 35	70; 55
Sulfapyridine	250/156; 250/184	250/156	25; 25	50; 60
Sulfameter	281/156; 281/215	281/156	25; 25	65; 50
Sulfamethoxypyridazine	281/156; 281/215	281/156	25; 25	65; 50
Sulfamethazine	279/156; 279/204	279/156	22; 20	55; 60
Sulfaphenazole	315/156; 315/160	315/156	32; 35	55; 55
Sulfadimethoxine	311/156; 311/218	311/156	31; 27	70; 70

TABLE 1.3 Retention Times of 16 Sulfonamides

Analytes	Retention Time (min)	Analytes	Retention Time (min)
Sulfacetamide	2.61	Sulfamerazine	9.93
Sulfamethizole	4.54	Sulfadoxine	11.29
Sulfisoxazole	4.91	Sulfapyridine	11.62
Sulfadiazine	5.20	Sulfameter	12.66
Sulfachloropyridazine	6.54	Sulfamethoxypyridazine	17.28
Sulfamethoxazole	8.41	Sulfamethazine	17.95
Sulfathiazole	9.13	Sulfaphenazole	22.29
Sulfamonomethoxine	9.48	Sulfadimethoxine	28.97

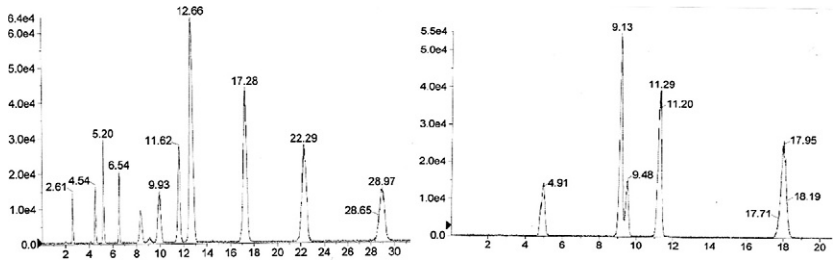


FIG. 1.1 TICs of sulfonamide standards.

1.3.8 RECOVERY

Under optimized conditions, the recoveries of 16 sulfonamides using this method are listed in Table 1.5.

RESEARCHERS

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TABLE 1.4 Content Range and Repeatability and Reproducibility

Analytes	Content Range (μg/kg)	Repeatability (r)	Reproducibility (R)
Sulfacetamide	5.0–100.0	$\lg r = 0.8332 \lg m - 0.8908$	$\lg R = 0.8867 \lg m - 0.4736$
Sulfamethizole	2.5–50.0	$\lg r = 1.1482 \lg m - 1.2062$	$\lg R = 0.8720 \lg \mathrm{m} - 0.6719$
Sulfisoxazole	5.0–100.0	$\lg r = 0.9169 \lg m - 0.9498$	$\lg R = 0.9721 \lg m - 0.7648$
Sulfachloropyridazine	5.0–100.0	$\lg r = 1.0370 \lg m - 1.1040$	$\lg R = 0.7629 \lg m - 0.4811$
Sulfadiazine	5.0–100.0	$\lg r = 1.0066 \lg m - 1.0967$	$\lg R = 0.8626 \lg m - 0.7077$
Sulfamethoxazole	5.0–100.0	$\lg r = 1.0039 \lg m - 1.1020$	$\lg R = 0.7669 \lg m - 0.4725$
Sulfathiazole	4.0–100.0	$\lg r = 0.8958 \lg m - 0.8754$	$\lg R = 0.7792 \lg m - 0.5137$
Sulfamonomethoxine	2.0–50.0	$\lg r = 0.8156 \lg m - 0.7523$	$\lg R = 0.8422 \lg m - 0.6139$
Sulfamerazine	2.0–50.0	$\lg r = 1.2468 \lg m - 1.4415$	$\lg R = 0.9169 \lg m - 0.7024$
Sulfadoxine	2.0–50.0	$\lg r = 1.1848 \lg m - 1.4131$	$\lg R = 0.8869 \lg m - 0.6944$
Sulfapyridine	2.0–50.0	$\lg r = 0.9672 \lg m - 1.0260$	$\lg R = 0.8551 \lg m - 0.6716$
Sulfameter	8.0–200.0	$\lg r = 0.7789 \lg m - 0.5842$	$\lg R = 0.7880 \lg m - 0.4602$
Sulfamethoxypyridazine	4.0–100.0	$\lg r = 0.8173 \lg m - 0.8225$	$\lg R = 0.7385 \lg m - 0.4498$
Sulfamethazine	8.0–200.0	$\lg r = 0.9702 \lg m - 0.9970$	$\lg R = 0.8554 \lg m - 0.5892$
Sulfaphenazole	12.0–300.0	$\lg r = 1.0839 \lg m - 1.1994$	$\lg R = 1.0431 \lg m - 0.8233$
Sulfadimethoxine	4.0–100.0	$\lg r = 1.0697 \lg m - 1.3020$	$\lg R = 0.7637 \lg m - 0.4218$

Note: m *is average value of parallel test results.*

TABLE 1.5 The Recoveries of Sulfonamides

Analytes	Fortifying Concentration (μg/kg)	Average Recovery (%)
Sulfacetamide	2.0	77.1
	5.0	77.7
	10.0	77.3
	50.0	78.7
Sulfamethizole	1.0	78.7
	2.5	80.0
	5.0	79.3
	25.0	77.9
Sulfisoxazole	2.0	90.2
	5.0	75.5
	10.0	76.9
	50.0	87.5
Sulfachloropyridazine	2.0	82.1
	5.0	85.7
	10.0	77.2
	50.0	78.6
Sulfadiazine	2.0	84.7
	5.0	86.6
	10.0	86.4
	50.0	77.5
Sulfamethoxazole	2.0	83.0
	5.0	87.8
	10.0	97.8
	50.0	80.5
Sulfathiazole	4.0	85.3
	10.0	70.9
	20.0	76.5
	100.0	86.7
Sulfamonomethoxine	2.0	95.6
	5.0	88.9
	10.0	94.8
	50.0	102.5

TABLE 1.5 The Recoveries of Sulfonamides—cont'd

Analytes	Fortifying Concentration (μg/kg)	Average Recovery (%)
Sulfamerazine	2.0	81.8
	5.0	83.3
	10.0	89.4
	50.0	81.6
Sulfadoxine	2.0	97.4
	5.0	86.5
	10.0	92.1
	50.0	95.4
Sulfapyridine	2.0	87.9
	5.0	85.0
	10.0	84.1
	50.0	77.9
Sulfameter	8.0	91.3
	20.0	84.2
	40.0	88.9
	200.0	76.4
Sulfamethoxypyridazine	4.0	89.8
	10.0	92.0
	20.0	80.2
	100.0	75.2
Sulfamethazine	8.0	95.0
	20.0	82.4
	40.0	92.4
	200.0	94.8
Sulfaphenazole	12.0	85.0
	30.0	83.8
	60.0	86.4
	300.0	77.6
Sulfadimethoxine	4.0	90.4
	10.0	81.9
	20.0	89.8
	100.0	74.4

FURTHER READING

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Chapter 1.4

Determination of 16 Sulfonamide Residues in Milk and Milk Powder—LC-MS-MS Method (GB/T 22966-2008)

1.4.1 SCOPE

This method is applicable to the determination of 16 sulfonamide residues in milk and milk powder.

The limits of determination for this standard are 1.0 μg/kg for sulfacetamide, sulfamerazine, sulfapyridine, sulfamethoxypyridazine, sulfamethoxydiazine, sulfachloropyridazine, sulfamethoxazole, sulfadimoxine, sulfamonomethoxine, sulfamethazine, sulfaquinoxaline, sulfadiazine, sulfathiazole, sulfamethizole, trimethoprim and sulfisoxazole, respectively, in milk; the limits of determination in milk powder are 4.0 μg/kg for sulfacetamide, sulfamerazine, sulfapyridine, sulfamethoxypyridazine, sulfamethoxydiazine, sulfachloropyridazine, sulfamethoxazole, sulfadimoxine, sulfamonomethoxine, sulfamethazine, sulfaquinoxaline, sulfadiazine, sulfathiazole, sulfamethizole, trimethoprim, and sulfisoxazole, respectively.

1.4.2 PRINCIPLE

Sulfonamide residues in the test sample are extracted by perchloric acid solution, cleaned up using Oasis HLB SPE cartridge, and eluted by methanol. Determination is carried out by LC-MS-MS, using an external standard method.

1.4.3 REAGENTS AND MATERIALS

Unless specifically noted, all reagents used should be of G.R grade; "water" is fhe first grade water prescribed by GB/T 6682.

Perchloric acid.
Methanol: HPLC grade.
Ethyl acid: HPLC grade.
Perchloric acid solution: pH = 2, add 1 mL perchloric acid to 1000 mL water, adjust to pH = 2 with water.
0.1% ethyl acid solution: add 0.1 mL ethyl acid to 100 mL water.
Methanol–ethyl acid (5+95): add 50 mL methanol to 950 mL ethyl acid solution and mix thoroughly.
Sulfacetamide, sulfamerazine, sulfapyridine, sulfamethoxypyridazine, sulfamethoxydiazine, sulfachloropyridazine, sulfamethoxazole, sulfadimoxine, sulfamonomethoxine, sulfamethazine, sulfaquinoxaline, sulfadiazine, sulfathiazole, sulfamethizole, trimethoprim and sulfisoxazole standard substance, purity $\geq$95%.
0.1 mg/mL 16 sulfonamide stock standard solution: Accurately weigh a suitable amount of sulfonamide standards, respectively; dissolve in methanol to make 0.1 mg/mL single stock standard solution. The solutions should be stored at 4°C.
1 μg/mL sulfonamide mixed standard medium working solution: Pipette 1 mL stock standard solution respectively to 100-mL volumetric flask; dilute with methanol to 100 mL and mix to homogeneity. The solutions should be stored at 4°C.
Substrate standard working solution: according to the content of analyzed matter, accurately pipette an adequate volume of mixed standard medium working solution, diluting with blank sample extraction. The solutions should be stored at 4°C.
Column of HLB or equivalent, 500 mg, 6 mL: put a small cotton into the column and rinse the column of HLB with 3 mL methanol and 5 mL perchloric acid solution. Keep the column wet.

1.4.4 APPARATUS

Liquid chromatography–mass spectrometry, equipped with electrospray ion source.

Analytic balance: able to weigh accurately from 0.1 mg to 0.01 g.
Ultrasonic bath.
Vortex shaker.
Solid phase extraction equipment.
Nitrogen concentration equipment.
Vacuum pump.
pH meter: sensibility is $\pm$0.02 pH.
Reservoir tube: attach to the Oasis HLB column.
0.22-μm filter.

1.4.5 SAMPLE PRETREATMENT

(1) Sample preparation

Take representative test sample of milk or milk powder, mix thoroughly to get a homogeneous sample, and label.

Milk should be stored at −18°C in the dark. Milk powder should be sealed and stored at room temperature.

(2) Extraction

Milk

Weigh ca. 2 g of the test sample, accurate to 0.01 g, in a 50-mL tube. Add 25 mL perchloric acid solution and shake 1 min on a vortex shaker; extract 10 min in the ultrasonic bath.

Milk powder

Weigh ca. 0.5 g of the test sample, accurate to 0.01 g, in a 50-mL tube. Add 25 mL perchloric acid solution; shake 1 min on a vortex shaker, and extract 10 min in the ultrasonic bath.

(3) Clean-up

Transfer all the solution into the reservoir tube attached to the Oasis HLB column. Add 5 mL perchloric acid solution to rinse the 50-mL tube and combine in the reservoir tube. Adjust the pressure in such a way as to make the extraction pass through the column at a flow rate of ca. 1 mL/min. Then rinse the column with 5 mL water and discard all the effluence, making the column dry. Elute the column with 3 mL methanol. Collect all the elution in a 10-mL volumetric tube. Evaporate the elution not less than 0.2 mL under a stream of nitrogen at 40°C. Make up to 1.0 mL with methanol–ethyl acid solution and vortex to homogeneous. After being filtrated with a 0.22-μm filter, the final solution is ready for analysis by HPLC-MS-MS.

(4) Blank sample solution preparation

Weigh ca. 2 g (accurate to 0.01 g) negative milk, and weigh ca. 0.5 g (accurate to 0.01 g) negative milk powder, using the same procedure as the sample solution described above.

1.4.6 DETERMINATION

(1) HPLC operating conditions

Column: C_{18} 150 mm × 2.1 mm (i.d.), 5-μm particle size or equivalent;
Column temperature: 30°C;
Flow rate: 200 μL/min;
Injection volume: 10 μL.
Mobile phase: for elution gradient of LC, see Table 1.6.

(2) MS conditions:

Ionization mode: Electron Spray Ion Source (ESI+);
Scan mode: MRM;
Sheath gas: 15 unit;
Auxiliary gas: 20 unit;

TABLE 1.6 Elution Gradient of LC

Time (min)	Flow Rate (μL/min)	0.1% Acetic Acid Solution (%)	Methanol (%)
0.00	200	75	25
5.00	200	55	45
8.00	200	20	80
10.00	200	20	80
10.10	200	75	25
13.00	200	75	25

Ion spay voltage (IS): 4000 V;
Capillary temperature: 320°C;
Source CID: 10 V;
$Q1 = 0.2$, $Q3 = 0.7$;
Impact gas: high pure Ar;
Impact gas pressure:1.5 mTorr;
Other MS conditions see Table 1.7

(3) LC/MS/MS determination

Qualitative analysis

Under the previous determination, for the same analysis batch and the same compound, the variation of the ion ratio between the two daughter ions for the unknown sample and the standard working solution at similar concentrations cannot be out of range of Table 1.8; if not, then the corresponding analyte must be present in the sample.

Quantitative analysis

According to the approximate concentration of analyte in the test sample solution, select the standard working solution with similar responses to those of the sample solution. The responses of the analyte in the standard working solution and the sample solution should be within the linear range of the instrument detection.The mixed standard working solution should be injected randomly in between the injections of the sample solution of equal volume. Under the preceding operating conditions, the chromatogram of the standard can be found in Fig. 1.2.

1.4.7 PRECISION

The precision data in this part is determined according to GB/T 6379.1 and GB/T 6379.2. The values of repeatability and reproducibility are calculated at a 95% reliability level.

TABLE 1.7 CAS No., Qualitative Ion, Quantitative Ion, and Collision Energy of 16 Sulfonamides

Compound	Retention time (min)	CAS No.	Ion Pairs (*m*/*z*)	Quantitative Ion Pair (*m*/*z*)	Collision Energy (eV)
Sulfacetamide	3.74	144-80-9	215.05/155.91	251.05/155.91	10
			215.05/107.98		22
Trimethoprim	4.66	23256-42-0	291.14/229.94	291.14/229.94	25
			291.14/260.90		21
Sulfadiazine	4.56	68-35-9	251.05/155.96	251.05/155.96	18
			251.05/107.97		25
Sulfathiazole	4.84	72-14-0	256.02/155.99	256.02/155.99	16
			256.02/108.05		22
Sulfapyridine	5.09	144-83-2	250.07/155.98	250.07/155.98	16
			250.07/184.01		19
Sulfamerazine	5.61	127-79-7	265.06/155.91	265.06/155.91	18
			265.06/171.94		14
Sulfamethoxydiazine	6.62	651-06-9	281.06/155.90	281.06/155.90	17
			281.06/107.91		27
Sulfamethizole	6.60	144-82-1	271.02/155.86	271.02/155.86	12
			271.02/107.96		21

Sulfamethazine	6.67	57-68-1	279.08/185.98	279.08/185.98	16
			279.08/155.99		18
Sulfamethoxypyridazine	7.26	80-35-3	281.07/155.98	281.07/155.98	17
			281.07/107.98		22
Sulfamethoxazole	7.49	127-69-5	254.05/155.99	254.05/155.99	16
			254.05/107.91		24
Sulfamonomethoxine	7.68	1220-83-3	281.06/155.94	281.06/155.94	19
			281.06/107.90		28
Sulfisoxazole	7.93	127-69-5	268.06/155.92	268.06/155.92	12
			268.06/107.94		26
Sulfachloropyridazine	8.03	80-32-0	285.02/156.00	285.02/156.00	16
			285.02/107.97		25
Sulfadimoxine	9.13	2447-57-6	311.08/155.96	311.08/155.96	20
			311.08/107.98		32
Sulfaquinoxaline	9.40	967-80-6	301.07/155.99	301.07/155.99	17
			301.07/107.91		20

Note: For different MS equipment, the parameters may be different, and the MS parameters should be optimized before analysis.

TABLE 1.8 Maximum Permitted Tolerances for Relative Ion Intensities While Confirming

Relative intensity (k)	$k > 50$	$20 < k \leq 50$	$10 < k \leq 20$	$k \leq 10$
Permitted tolerance	±20	±25	±30	±50

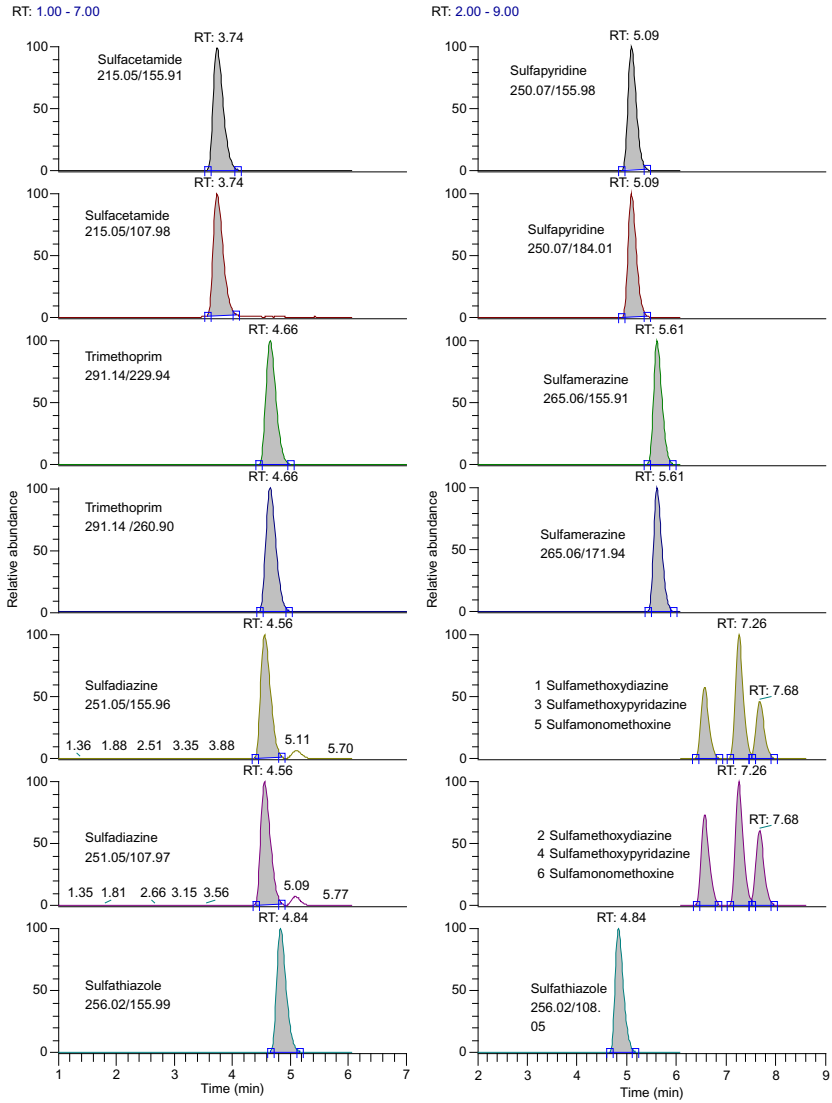

FIG. 1.2 MRM chromatogram of 16 sulfonamide standard working solutions. (Note 1: The former is qualitative, and the other is quantitative; Note 2: 1 281.06/155.90, 2 281.06/107.91, 3 281.07/155.98, 4 281.07/107.98, 5 281.06/155.94, 6 281.06/107.90.)

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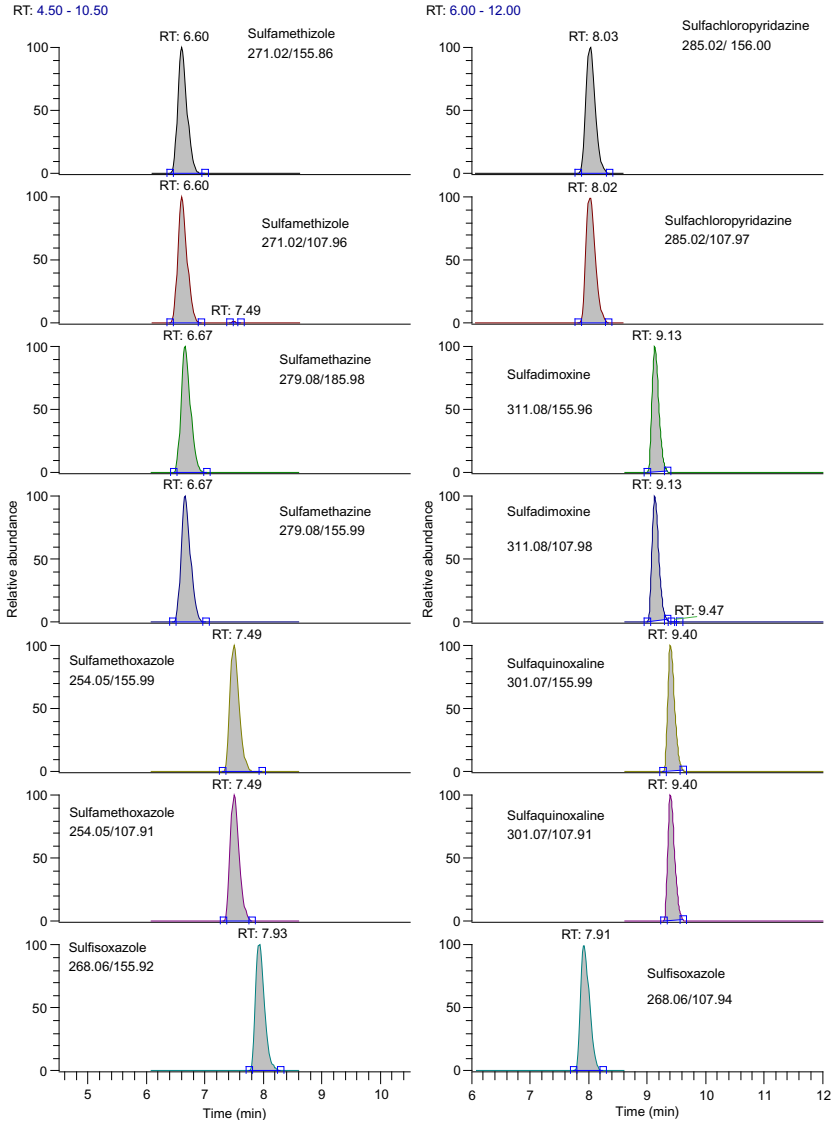

FIG. 1.2, Cont'd

(a) Repeatability

Under the repeatability conditions, the difference of absolute value of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equation of 16 sulfonamide residue in milk and milk powder are shown in Tables 1.9 and 1.10.

TABLE 1.9 Content Ranges and the Repeatability and Reproducibility Equations of 16 Sulfonamides for Milk Sample

Analyte	Content Ranges (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Sulfacetamide	1.0–10.0	$r = 0.1246m - 0.0258$	$\lg R = 0.9901 \lg m - 0.7530$
Trimethoprim	1.0–10.0	$r = 0.1256m - 0.0120$	$\lg R = 1.0445 \lg m - 0.7378$
Sulfadiazine	1.0–10.0	$r = 0.0539 \lg m + 0.0761$	$\lg R = 0.9231 \lg m - 0.6967$
Sulfathiazole	1.0–10.0	$r = 0.0887m + 0.0917$	$\lg R = 1.0098 \lg m - 0.7388$
Sulfapyridine	1.0–10.0	$r = 0.0780m - 0.0200$	$R = 0.1148\ m + 0.1102$
Sulfamerazine	1.0–10.0	$r = 0.1441m - 0.0111$	$\lg R = 1.0873 \lg m - 0.8579$
Sulfamethoxydiazine	1.0–10.0	$r = 0.08071m + 0.0311$	$R = 0.1432m + 0.0450$
Sulfamethizole	1.0–10.0	$r = 0.05701m + 0.0927$	$\lg R = 0.8796 \lg m - 0.7162$
Sulfamethazine	1.0–10.0	$r = 0.1295m - 0.0419$	$\lg R = 1.0005 \lg m - 0.7305$
Sulfamethoxypyridazine	1.0–10.0	$r = 0.0931m + 0.0568$	$\lg R = 0.9650 \lg m - 0.7389$
Sulfamethoxazole	1.0–10.0	$r = 0.1916m - 0.1244$	$R = 0.2223\ m - 0.1158$
Sulfamonomethoxine	1.0–10.0	$r = 0.1120m + 0.1091$	$R = 0.1974\ m + 0.0107$
Sulfisoxazole	1.0–10.0	$r = 0.1140m + 0.0009$	$\lg R = 0.9369 \lg m - 0.6713$
Sulfachloropyridazine	1.0–10.0	$r = 0.1032m + 0.0174$	$\lg R = 0.9275 \lg m - 0.6757$
Sulfadimoxine	1.0–10.0	$r = 0.1024m + 0.0568$	$R = 0.1950m + 0.0190$
Sulfaquinoxaline	1.0–10.0	$r = 0.0868m + 0.0562$	$\lg R = 0.8876 \lg m - 0.6692$

Note: m *is the mean determination result of the duplicate test.*

TABLE 1.10 Content Ranges and the Repeatability and Reproducibility Equation of 16 Sulfonamides for Milk Powder Sample

Analyte	Content Ranges (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Sulfacetamide	4.0–40.0	$r = 0.0724m - 0.0506$	$R = 0.1401m - 0.0420$
Trimethoprim	4.0–40.0	$r = 0.0420m + 0.2797$	$\lg R = 1.0080\lg m - 0.8965$
Sulfadiazine	4.0–40.0	$r = 0.0350m + 0.1973$	$R = 0.1098m + 0.3870$
Sulfathiazole	4.0–40.0	$\lg r = 0.9093\lg m - 0.9891$	$\lg R = 0.9854\lg m - 0.7468$
Sulfapyridine	4.0–40.0	$r = 0.0765m - 0.0391$	$\lg R = 0.8938\lg m - 0.7084$
Sulfamerazine	4.0–40.0	$r = 0.0820m - 0.0366$	$R = 0.1537m - 0.0274$
Sulfamethoxydiazine	4.0–40.0	$r = 0.0797m + 0.1291$	$R = 0.1095m + 0.5516$
Sulfamethizole	4.0–40.0	$r = 0.0733m + 0.0625$	$R = 0.1192m + 0.2682$
Sulfamethazine	4.0–40.0	$r = 0.0806m + 0.2465$	$R = 0.1302m - 0.3570$
Sulfamethoxypyridazine	4.0–40.0	$r = 0.0903m + 0.3444$	$\lg R = 0.9325\lg m - 0.6883$
Sulfamethoxazole	4.0–40.0	$r = 0.0859m + 0.1110$	$R = 0.1723m + 0.2209$
Sulfamonomethoxine	4.0–40.0	$r = 0.0987m + 0.2212$	$\lg R = 0.9198\lg m - 0.6099$
Sulfisoxazole	4.0–40.0	$r = 0.1079m + 0.3761$	$\lg R = 1.0344\lg m - 0.7879$
Sulfachloropyridazine	4.0–40.0	$r = 0.1066m + 0.4361$	$\lg R = 0.9201\lg m - 0.6035$
Sulfadimoxine	4.0–40.0	$r = 0.1004m + 0.1214$	$\lg R = 0983\lg m - 0.7116$
Sulfaquinoxaline	4.0–40.0	$r = 0.1185m - 0.0554$	$R = 0.1395m + 0.1706$

Note: m *is the mean determination result of the duplicate test.*

TABLE 1.11 Spiked Level and Recovery Range of 16 Sulfonamides in Milk and Milk Powder

Compound	Milk		Milk Powder	
	Spiked Level (μg/kg)	Recovery Range (%)	Spiked Level (μg/kg)	Recovery Range (%)
Sulfacetamide	1	81.0–93.0	4	83.0–94.4
	2	90.5–103.6	8	92.6–100.6
	5	76.3–98.6	20	78.3–94.4
	10	85.2–94.5	40	86.0–97.0
Trimethoprim	1	76.6–88.0	4	78.0–89.0
	2	84.4–100.8	8	88.1–99.3
	5	83.6–100.3	20	83.4–101.7
	10	80.4–90.4	40	79.2–89.3
Sulfadiazine	1	69.1–86.0	4	71.5–89.0
	2	76.4–97.8	8	81.4–93.5
	5	73.6–87.9	20	76.7–91.8
	10	78.1–87.7	40	78.8–97.0
Sulfathiazole	1	67.8–79.0	4	76.7–89.1
	2	78.2–96.1	8	84.5–98.7
	5	77.3–99.0	20	79.3–97.1
	10	79.1–89.8	40	80.3–93.5
Sulfapyridine	1	69.2–92.2	4	72.2–94.1
	2	76.8–93.1	8	73.2–92.6
	5	77.4–90.3	20	76.6–93.1
	10	81.5–93.2	40	78.8–94.3
Sulfamerazine	1	72.5–92.6	4	76.8–90.1
	2	81.7–99.1	8	83.6–98.3
	5	76.9–93.9	20	76.9–87.6
	10	79.3–90.9	40	79.5–89.3
Sulfamethoxydiazine	1	81.0–99.6	4	80.7–97.6
	2	73.5–93.1	8	78.5–98.2
	5	73.9–91.4	20	71.9–89.9
	10	78.5–91.7	40	78.8–90.8

TABLE 1.11 Spiked Level and Recovery Range of 16 Sulfonamides in Milk and Milk Powder—cont'd

Compound	Milk		Milk Powder	
	Spiked Level (μg/kg)	Recovery Range (%)	Spiked Level (μg/kg)	Recovery Range (%)
Sulfamethizole	1	72.6–91.6	4	74.1–91.6
	2	76.7–93.9	8	79.4–94.2
	5	79.2–96.7	20	77.2–91.8
	10	81.5–92.2	40	77.8–90.8
Sulfamethazine	1	84.0–103.2	4	80.0–100.3
	2	84.5–98.5	8	84.0–97.6
	5	77.4–97.0	20	78.0–95.3
	10	81.2–93.5	40	79.8–93.3
Sulfamethoxypyridazine	1	70.2–88.2	4	75.5–86.0
	2	71.1–90.7	8	76.1–91.1
	5	75.6–93.1	20	77.6–91.2
	10	80.7–89.9	40	78.0–90.8
Sulfamethoxazole	1	77.1–97.9	4	79.2–97.1
	2	82.9–101.4	8	83.2–91.3
	5	76.6–95.5	20	77.5–93.7
	10	83.8–96.6	40	77.3–90.8
Sulfamonomethoxine	1	74.3–92.0	4	77.3–92.0
	2	78.4–99.8	8	80.7–97.4
	5	73.1–91.6	20	76.2–95.0
	10	81.6–93.8	40	79.5–92.0
Sulfisoxazole	1	69.0–87.0	4	78.8–91.0
	2	76.5–97.7	8	81.5–96.0
	5	71.7–90.8	20	71.3–87.5
	10	74.9–88.9	40	79.3–91.3
Sulfachloropyridazine	1	72.6–89.0	4	75.7–89.1
	2	78.1–97.5	8	82.4–98.3
	5	64.8–78.3	20	72.9–91.5
	10	88.1–99.9	40	85.0–93.5

Continued

TABLE 1.11 Spiked Level and Recovery Range of 16 Sulfonamides in Milk and Milk Powder—cont'd

Compound	Milk		Milk Powder	
	Spiked Level (μg/kg)	Recovery Range (%)	Spiked Level (μg/kg)	Recovery Range (%)
Sulfadimoxine	1	66.4–87.3	4	76.4–89.0
	2	75.5–97.9	8	78.2–95.5
	5	75.4–98.5	20	78.9–96.7
	10	83.4–95.1	40	81.5–91.0
Sulfaquinoxaline	1	69.0–86.2	4	73.0–89.4
	2	72.1–93.5	8	79.2–97.8
	5	74.7–92.1	20	74.7–94.1
	10	75.3–89.9	40	78.3–89.5

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned, and two independent experiments should be done.

(b) Reproducibility

Under the reproducibility condition, the difference of absolute value of two independent results is not above the reproducibility limit (R). The content ranges and the reproducibility equation of 16 sulfonamide residues in milk and milk powder are shown in Tables 1.9 and 1.10.

1.4.8 RECOVERY

Under optimized conditions, the recoveries of 16 sulfonamides using this method are listed in Table 1.11.

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1.5

Determination of 18 Sulfonamide Residues in Fugu and Eel—LC-MS-MS Method (GB/T 22951-2008)

1.5.1 SCOPE

This method is applicable to the determination of 18 sulfonamide residues in fugu and eel.

The limit of determination of this method of 18 sulfonamides is 5.0 μg/kg.

1.5.2 PRINCIPLE

The drugs are extracted from fugu and eel with acetonitrile. After being centrifuged, the supernatant is dehydrated with anhydrous sodium sulfate; the supernatant is then blown to dryness on a nitrogen evaporator. Dissolve the drugs in acetonitrile-0.01 mol/L ammonium acetate solution, after which been diminished fatty with hexane, and then put through a 0.2-μm filter membrane. The solution is then ready for determination by LC-MS-MS, using an internal standard method.

1.5.3 REAGENTS AND MATERIALS

Water: GB/T6682, First-level.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Formic acid: HPLC grade.
n-Hexane: HPLC grade.
Ammonium acetate: G.R.
Anhydrous sodium sulfate: heated at 650°C for 4 h, and put in the water extractor spare
0.01 mol/L Ammonium acetate solution: Weigh 0.77 g ammonium acetate dissolved in 1000 mL water.
Dissolve solution: Acetonitrile-0.01 mol/L ammonium acetate solution (3+22, V/V). Mix 12 mL acetonitrile and 88 mL 0.01 mol/L ammonium acetate solution.
0.1% formic acid solution: Dilute 1.0 mL formic acid to 1000 mL with water.

18 Sulfonamide standards: sulfadiazine (CAS 68-35-9), sulfathiazole (CAS 72-14-0), sulfapyridine (CAS 144-83-2), sulfamethoxypyridazine (CAS 127-79-7), sulfamonomethoxine (CAS 1220-83-3), sulfamethizole(CAS 144-82-1), sulfamethazine (CAS 57-68-1), sulfamethoxypyridazine (CAS 80-35-3), sulfameter (CAS 651-06-9), sulfachloropyridazine (CAS 80-32-0), sulfamethoxazole (CAS 723-46-6), sulfadoxine (CAS 2447-57-6), sulfisoxazole (CAS 127-69-5), sulfabenzamide (CAS 127-71-9), sulfachloropyrazine (CAS 102-65-8), sulfaphenazole (CAS 526-08-9), sulfadimethoxine (CAS 1037-50-9), sulfaquinoxaline (CAS 59-40-5): purity ≥99%.

Standard stock solutions of sulfonamides: 0.1 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volume of methanol, then dilute with methanol separately to prepare the standard stock solutions of 0.1 mg/mL in concentration. The standard stock solutions are stored at 4°C.

Mixed standard working solutions of 18 sulfonamides: 5.0 μg/mL. Pipette 0.5 mL each standard stock solutions into 10-mL volumetric flasks, respectively. Dilute the standard solutions to volume with methanol. The standard working solutions are stored at 4°C.

Sulfonamide internal standards: sulfamethizole-D4, sulfadiazine-D4, sulfathiazole-D4: purity ≥99%.

Internal standard stock solutions of sulfonamide: 0.1 mg/mL. Accurately weigh appropriate amount of each internal standard, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the internal standard stock solutions of 0.1 mg/mL in concentration. The internal standard stock solutions are stored at 4°C.

Mixed internal standard working solutions of sulfonamides: 5.0 μg/mL. Pipette 0.5 mL each internal standard stock solution into 10-mL volumetric flasks respectively, Dilute the internal standards solution to volume with methanol. The internal standard working solutions are stored at 4°C.

Base standard working solutions: Pipette different volume mixed standard working solutions of 18 sulfonamides and 4 μL mixed internal standard working solutions of sulfonamides. Prepare the base standard working solutions of 5.0, 10.0, 20.0, 50.0, 100.0 ng/mL different concentrations for sulfonamides with fugu and eel control sample extract. Prepare fresh daily.

Filter membrane: 0.2 μm.

1.5.4 APPARATUS

LC-MS-MS: Equipped with ESI source.

Analytical balance: capable of weighing from 0.1 mg to 0.01 g.

Centrifuge tube: 50 mL.

Homogenizer.

Vortex mixer.

Centrifuge.
Nitrogen evaporator.
Conical tube: 10 mL.

1.5.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

The representative sample, ca. 1 kg, is taken from whole sample, vigorously stirred, thoroughly mixed, divided into two equal parts, and then put in a clean container, which is sealed and labeled. In the course of sampling and sample preparation, precaution should be taken to avoid contamination or any factors that may cause a change of residue content.

The test samples should be stored at −18°C.

(2) Extraction and Clean-up

Weigh 10-g test sample (accurate to 0.01 g) into 50-mL centrifuge tube, add 20 μL internal standard working solutions, 20 g anhydrous sodium sulfate, and 25 mL acetonitrile to each sample. Homogenize 2 min; centrifuge the sample solution at 3000 rpm for 3 min. Decant the supernatant into 50 mL volumetric flasks, add 20 mL acetonitrile into the dregs, and repeat the operation. Combine the supernatants, diluted to volume with acetonitrile. Pipette 10 mL supernatant into a conical tube. Blow the supernatant to dryness on a nitrogen evaporator at 45°C. Then add 1 mL dissolved solution and 1 mL hexane to the drugs, mix 1 min, and centrifuge the sample solution at 3000 rpm for 3 min. Pipette supernatant hexane and discard; then add 1 mL hexane. Repeat the operation until the lower water phase turns clear. Pipette the lower phase, and then put it through a 0.2-μm filter membrane. It is then ready for determination by LC-MS-MS.

According to the preceding steps, prepare the extraction of a blank sample.

1.5.6 DETERMINATION

(1) LC operation conditions:

Chromatographic column: Atlantis C18, 3 μm, 150 mm × 2.1 mm i.d. or equivalent;
Mobile phase: A: Acetonitrile, B: 0.1% formic acid solution, C: Methanol. For gradient elution condition see Table 1.12;
Flow: 0.2 mL/min;
Column temperature: 35°C;
Injection volume: 20 μL;

(2) MS operating conditions:

Ion source: ESI source;
Scan mode: Positive scan;

TABLE 1.12 Gradient Elution Conditions

Time (min)	A (%)	B (%)	C (%)
0.00	10.0	80.0	10.0
3.00	20.0	70.0	10.0
8.00	35.0	55.0	10.0
12.00	70.0	20.0	10.0
12.01	10.0	80.0	10.0
17.00	10.0	80.0	10.0

Monitor mode: Multiple reaction monitor;
Ionspray voltage: 5500 V;
Nebulizer gas: 0.069 MPa;
Curtain gas: 0.069 MPa;
Turbo ionspray gas rate: 6 L/min;
Source temperature: 350°C;
Precursor/product ion combinations, declustering potential, collision energy: see Table 1.13.

(3) LC-MS-MS Determination

Qualitative determination

Select one precursor and more than two daughters. In the same conditions, if the ratio error of the chromatographic retention time of the analyte and the standard corresponds in ±2.5% compared with the matrix standard solutions, the relative ion intensity of the sulfonamide in the sample and matrix standard solutions accord with Table 1.8; this sample has the sulfonamide.

Quantitation determination

Prepare the base standard working solutions of different concentrations of 18 sulfonamides with fugu and eel control sample extract on base. Then inject 20 μL of the different concentration working standard solution, respectively, in duplication under LC and MS conditions. Draw the five-point standard curves of each sulfonamide (peak area vs. sulfonamide concentration). Calculate the concentrations of the corresponding content from the working curve. The responses of 18 sulfonamides in the standard working solution and sample solution should be in the linear range of the instrumental detection. Under LC and MS conditions, for the reference retention times of the 18 sulfonamides, see Table 1.14. For MRM chromatograms of the mixed standard working solution of 18 sulfonamides, see Fig. 1.3.

TABLE 1.13 18 Sulfonamide Precursor/Product Ion Combinations, Declustering Potential, Collision Energy

Drug	Precursor Ion Combinations (*m*/*z*)	Production Ion Combinations (*m*/*z*)	Collision Energy (V)	Declustering Potential (V)
Sulfadiazine	251/156 251/185	251/156	23 27	55 50
Sulfathiazole	256/156 256/107	256/156	22 32	55 47
Sulfapyridine	250/156 250/184	250/156	25 25	50 60
Sulfamethoxypyridazine	265/156 265/172	265/156	25 24	50 60
Sulfamonomethoxine	281/156 281/215	281/156	25 25	65 50
Sulfamethizole	271/156 271/107	271/156	20 32	50 50
Sulfamethazine	279/156 279/204	279/156	22 20	55 60
Sulfamethoxypyridazine	281/156 281/215	281/156	25 25	65 50
Sulfameter	281/156 281/215	281/156	25 25	65 50

Continued

TABLE 1.13 18 Sulfonamide Precursor/Product Ion Combinations, Declustering Potential, Collision Energy—cont'd

Drug	Precursor Ion Combinations (*m*/*z*)	Production Ion Combinations (*m*/*z*)	Collision Energy (V)	Declustering Potential (V)
Sulfachloropyridazine	285/156 285/108	285/156	23 35	50 50
Sulfamethoxazole	254/156 254/147	254/156	23 22	50 45
Sulfadoxine	311/156 311/108	311/156	31 35	70 55
Sulfisoxazole	268/156 268/113	268/156	20 23	45 45
Sulfabenzamide	277/156 277/108	277/156	20 35	85 85
Sulfachloropyrazine	285/156 285/108	285/156	24 37	53 53
Sulfaphenazole	315/156 315/160	315/156	32 35	55 55
Sulfadimethoxine	311/156 311/218	311/156	31 27	70 70
Sulfaquinoxaline	301/156 301/208	301/156	25 28	42 42

TABLE 1.14 Retention Times of 18 Sulfonamides

Drug	Retention Time (min)	Drug	Retention Time (min)
Sulfadiazine	4.45	Sulfachloropyridazine	8.91
Sulfathiazole	4.77	Sulfamethoxazole	9.29
Sulfapyridine	5.39	Sulfadoxine	9.29
Sulfamethoxypyridazine	6.20	Sulfisoxazole	9.72
Sulfamonomethoxine	7.75	Sulfabenzamide	10.50
Sulfamethizole	7.76	Sulfachloropyrazine	10.85
Sulfamethazine	7.83	Sulfaphenazole	11.11
Sulfamethoxypyridazine	8.01	Sulfadimethoxine	11.12
Sulfameter	8.75	Sulfaquinoxaline	11.22

1.5.7 PRECISION

The precision data of the method for this standard have been determined in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for 18 sulfonamides in fugu and eel are shown in Table 1.15.

If the difference of values exceeds the limit of repeatability, the testing results must be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for 18 sulfonamides in fugu and eel are shown in Table 1.15.

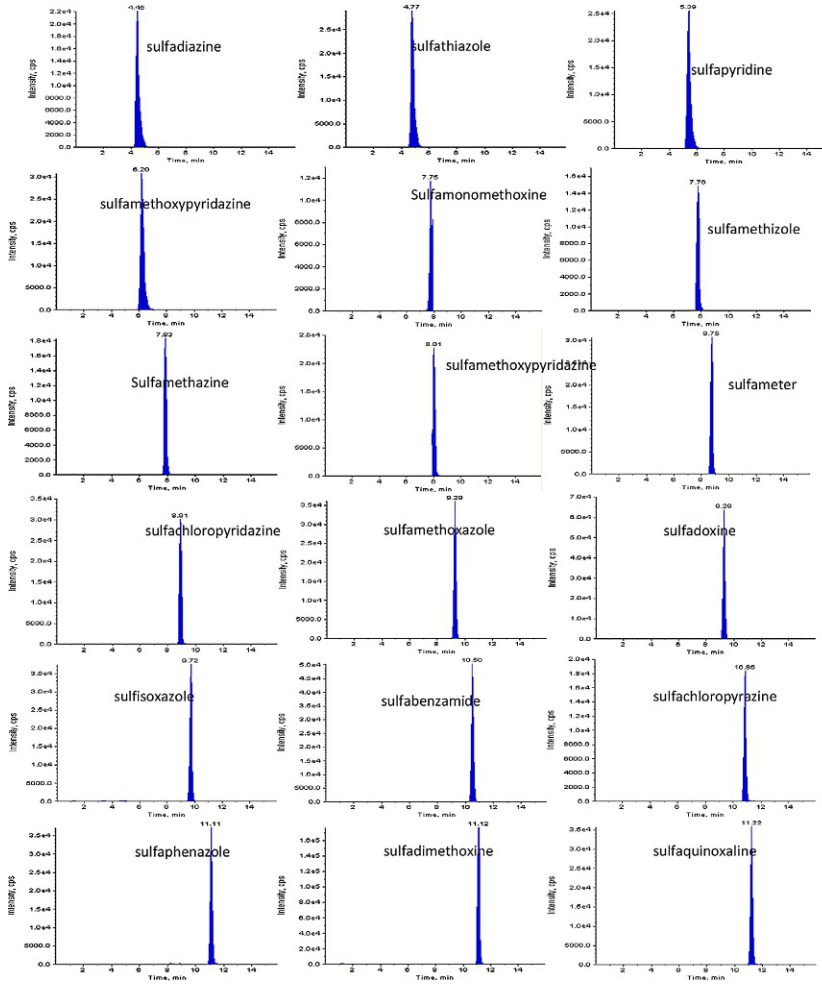

FIG. 1.3 MRM chromatograms of 18 sulfonamide standards.

1.5.8 RECOVERY

Under optimized condition, the recoveries of 18 sulfonamides in fugu using this method are listed in Table 1.16.

TABLE 1.15 Content Range, Repeatability, and Reproducibility (Units μg/kg)

Drug	Fortifying Concentration Range	Repeatability (r)	Reproducibility (R)
Sulfadiazine	5.0–50.0	$\lg r = 0.9134 \lg m - 1.2486$	$\lg R = 0.7576 \lg m - 0.6647$
Sulfathiazole	5.0–50.0	$\lg r = 0.4987 \lg m - 0.7916$	$\lg R = 0.7604 \lg m - 0.5415$
Sulfapyridine	5.0–50.0	$\lg r = 1.0801 \lg m - 1.4641$	$\lg R = 1.0237 \lg m - 0.9181$
Sulfamethoxypyridazine	5.0–50.0	$\lg r = 0.7337 \lg m - 1.1786$	$\lg R = 0.7223 \lg m - 0.3664$
Sulfamonomethoxine	5.0–50.0	$\lg r = 0.8785 \lg m - 0.9986$	$\lg R = 0.6006 \lg m - 0.3779$
Sulfamethizole	5.0–50.0	$\lg r = 1.0598 \lg m - 1.3411$	$\lg R = 0.8516 \lg m - 0.5966$
Sulfamethazine	5.0–50.0	$\lg r = 0.8247 \lg m - 1.1906$	$\lg R = 1.0778 \lg m - 1.1102$
Sulfamethoxypyridazine	5.0–50.0	$\lg r = 1.2441 \lg m - 1.7816$	$\lg R = 0.7558 \lg m - 0.5215$
Sulfameter	5.0–50.0	$\lg r = 0.8356 \lg m - 1.1511$	$\lg R = 0.9473 \lg m - 1.0112$
Sulfachloropyridazine	5.0–50.0	$\lg r = 0.4952 \lg m - 0.7540$	$\lg R = 0.6980 \lg m - 0.5740$
Sulfamethoxazole	5.0–50.0	$\lg r = 0.8986 \lg m - 0.8891$	$\lg R = 0.9931 \lg m - 0.9022$
Sulfadoxine	5.0–50.0	$\lg r = 0.8167 \lg m - 1.2076$	$\lg R = 0.8500 \lg m - 0.7679$
Sulfisoxazole	5.0–50.0	$\lg r = 1.0607 \lg m - 1.3448$	$\lg R = 1.0283 \lg m - 1.0395$
Sulfabenzamide	5.0–50.0	$\lg r = 0.7389 \lg m - 1.0551$	$\lg R = 0.7702 \lg m - 0.6410$
Sulfachloropyrazine	5.0–50.0	$\lg r = 0.9811 \lg m - 1.1361$	$\lg R = 1.0456 \lg m - 0.9163$
Sulfaphenazole	5.0–50.0	$\lg r = 0.9412 \lg m - 1.1662$	$\lg R = 0.6812 \lg m - 0.5496$
Sulfadimethoxine	5.0–50.0	$\lg r = 0.7920 \lg m - 0.9668$	$\lg R = 1.1192 \lg m - 1.2056$
Sulfaquinoxaline	5.0–50.0	$\lg r = 1.0760 \lg m - 1.4935$	$\lg R = 0.8271 \lg m - 0.8028$

Note: *The* m *is average value of parallel test results.*

TABLE 1.16 The Data of 18 Sulfonamide Fortifying Concentrations and Recovery

Drugs	Fortifying Concentration (μg/kg)	Average Recovery (%)
Sulfadiazine	5.0	92.2
	10.0	88.0
	20.0	93.5
	50.0	86.2
Sulfathiazole	5.0	91.9
	10.0	87.9
	20.0	91.7
	50.0	92.7
Sulfapyridine	5.0	91.4
	10.0	94.0
	20.0	97.1
	50.0	90.8
Sulfamethoxypyridazine	5.0	88.5
	10.0	94.9
	20.0	97.0
	50.0	90.2
Sulfamonomethoxine	5.0	88.3
	10.0	91.3
	20.0	86.4
	50.0	91.8
Sulfamethizole	5.0	89.1
	10.0	95.9
	20.0	84.5
	50.0	88.9
Sulfamethazine	5.0	95.6
	10.0	91.5
	20.0	92.3
	50.0	93.6

TABLE 1.16 The Data of 18 Sulfonamide Fortifying Concentrations and Recovery—cont'd

Drugs	Fortifying Concentration (μg/kg)	Average Recovery (%)
Sulfamethoxypyridazine	5.0	93.1
	10.0	93.4
	20.0	93.0
	50.0	93.4
Sulfameter	5.0	93.1
	10.0	91.1
	20.0	93.2
	50.0	95.0
Sulfachloropyridazine	5.0	87.3
	10.0	93.9
	20.0	87.9
	50.0	88.4
Sulfamethoxazole	5.0	96.0
	10.0	92.4
	20.0	90.0
	50.0	87.0
Sulfadoxine	5.0	86.8
	10.0	88.3
	20.0	89.4
	50.0	90.8
Sulfisoxazole	5.0	88.3
	10.0	91.3
	20.0	86.4
	50.0	91.8
Sulfabenzamide	5.0	94.5
	10.0	87.6
	20.0	89.1
	50.0	90.0

Continued

TABLE 1.16 The Data of 18 Sulfonamide Fortifying Concentrations and Recovery—cont'd

Drugs	Fortifying Concentration (μg/kg)	Average Recovery (%)
Sulfachloropyrazine	5.0	88.0
	10.0	89.0
	20.0	88.3
	50.0	90.8
Sulfaphenazole	5.0	93.1
	10.0	89.1
	20.0	95.8
	50.0	97.1
Sulfadimethoxine	5.0	87.0
	10.0	87.3
	20.0	94.1
	50.0	94.7
Sulfaquinoxaline	5.0	89.7
	10.0	95.5
	20.0	91.3
	50.0	90.5

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1.6

Determination of 8 Sulfonamides residues in Honey—LC-FLD Method (GB/T 18932.5-2002)

1.6.1 SCOPE

This method is applicable to the determination of sulfacetamide, sulfapyridine, sulfamerazine, sulfamethoxypyridazine, sulfameter, sulfachloropyridazine, sulfamethoxazole, and sulfadimethoxine residues in honey.

The limit of determination of this method for sulfonamide drugs is 0.010 mg/kg.

1.6.2 PRINCIPLE

The drugs are extracted from honey with phosphoric acid solution (pH = 2). After filtration, the honey solution is cleaned up via cation exchange and Oasis HLB or equivalent extraction. Elute with methanol until dry. Dissolve the dregs in 0.1 mol/L HCl. Upon derivatization, the sample is analyzed by HPLC with fluorescence detection. Calculate the results by comparing peak height of the sample with corresponding peak heights in the standard solution.

1.6.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be reagent grade; "water" is deionized water.

Methanol, acetonitrile: HPLC grade; Phosphoric acid: G.R.; 1-Heptanesulfonic acid sodium salt: $C_7H_{15}NaO_3S.H_2O$, HPLC grade; Hydrochloric acid; Sodium acetate; Acetone; Fluorescamine; Potassium dihydrogen phosphate; Dipotassium hydrogen phosphate; Phosphoric acid solution: pH = 2. Dilution of phosphoric acid with water;

Phosphate buffer: 0.2 M, pH = 8. Place 1.05 g potassium dihydrogen phosphate and 33.46 g dipotassium hydrogen phosphate into 1000 mL volumetric flask, and dissolve in H_2O. Dilute solution to volume with H_2O;

1-Heptanesulfonic acid solution: 0.5 M. Place 11 g 1-heptanesulfonic acid, sodium salt into 100 mL volumetric flask and dissolve in H_2O. Dilute solution to volume with H_2O;

Hydrochloric acid solution: 0.1 M. Pipette 8.25 mL HCl, dilute to 1000 mL with H_2O;

Acetate buffer: 0.6 M. Place 4.92 g sodium acetate diluted to 100 mL with water and adjusted to pH 3.0 with concentrated HCl;
Fluorescamine solution: 0.02%. Dissolve 10 mg fluorescamine and dilute to 50 mL with acetone;
Cation exchange column: Aromatic sulfonic acid, 500 mg, 3 mL. Condition each column with 5 mL methanol followed by 10 mL water before use; Oasis HLB solid extraction columns or equivalent: 60 mg, 3 mL. Condition each column with 3 mL methanol followed by 6 mL water before use.
Sulfonamide standards: purity $\geq$99%.
Standard solutions of sulfonamide: 0.1 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volume of methanol, then dilute with methanol separately to prepare the standard stock solutions of 0.1 mg/mL in concentration. According to the requirements, prepare the mixed standard working solution of appropriate concentrations.

1.6.4 APPARATUS

Liquid chromatograph: Equipped with flourescence detector (FLD); Solid phase extraction vacuum apparatus; Rotary evaporator; Vortex mixer; Analytical balance: Capable of weighing from 0.1 mg to 0.01 g; Thermo bath: 18 ± 0.5° C; Vacuum pump; Micro-syringes: 25 μL, 100 μL; Pipette: 0.2 mL, 1 mL, 2 mL; Pear-shaped flask: 150 mL; Screw vial: 2.0 mL, with screw caps and PTFE septa; Reservoirs and adapters to fit SPE columns: 50 mL; pH Meter: Capable of measuring $\pm$0.02 unit.

1.6.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath, warm at $\leq$60°C with occasional shaking until liquefied, mix thoroughly and promptly cool to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label. The test samples should be stored at 20°C.

(2) Extraction

Weigh 5 g test sample (accurate to 0.01 g) into 150-mL Erlenmeyer flask. Add 25 mL phosphoric acid solution to each sample. Mix vigorously 1 min on vortex mixer, until honey is completely dissolved.

(3) Clean-up

Connect a reservoir with glass wool plug to cation exchange cartridge. The honey sample solution is decanted into the reservoir, and let solution pass through the cation exchange cartridge at reduced press, adjusting the flow rate to $\leq$3 mL/min. Wait until sample solution has thoroughly drained, and then rinse reservoir and cartridge respectively with 5 mL phosphoric

acid solution and 5 mL water. Discard all the effluents. Finally, elute sulfonamides with 40 mL phosphate buffer into the 100-mL flat-bottom flask. Add 1.5 mL 1-heptanesulfonic acid solution to the eluate and adjust it to pH = 6 by drop-wise addition of phosphoric acid.

The sample solution is made to pass through the Oasis HLB cartridge or equivalent in the same way as mentioned previously, and the flow rate is adjusted to $\leq$3 mL/min. Wait until the sample solution has thoroughly drained, and then rinse the cartridge with 3 mL water and discard all the effluents. Dry the cartridge by drawing air through it for 5 min under 65 kPa vacuum. Finally, elute sulfonamides with 10 mL methanol into 150-mL pear-shaped flask. Evaporate to dryness on a rotary evaporator with vacuum in a water bath at 45°C. Add accurately 1 mL hydrochloric acid solution to drugs and wait for derivatization.

(4) Derivatization

Transfer 0.5 mL solution with 1-mL pipette into a 2-mL screw vial. Add 0.5 mL acetate buffer into it. Screw vial cap tightly and shake well, and then add 0.2 mL fluorescamine solution into it. Screw the cap tightly and shake well again. Place it into a water bath at 18°C for a 30-min derivatization. Use it for determination by HPLC-fluorescent detector when taken out.

1.6.6 DETERMINATION

(1) Operating conditions

Chromatographic column: symmetry shield PR18, 5 μm, 3.9 × 150 mm, or equivalent; Column temperature: 55°C; Mobile phase: acetonitrile-0.01 mol/L Potassium dihydrogen phosphate solution (27 + 73); Flow rate of mobile phase: 1.0 mL/min; Wavelength: λ_{ex} = 405 nm, λ_{em} = 495 nm; Sample size: 100 μL.

(2) LC determination

According to the appropriate concentrations of the sulfonamide drugs in the sample solution, select the corresponding mixed standard working solution with similar peak heights to those of the sample solution. The responses of the sulfonamide drugs in the standard working solution and sample solution should be in the linear range of the instrumental detection. The standard working solution should be injected in between the injections of the sample solution of equal volume, under the previously described LC conditions; the reference retention times of the sulfonamide drugs are shown in Table 1.17, and the chromatogram of the sulfonamide drugs is shown in Fig. 1.4.

1.6.7 PRECISION

The precision data of the method for this standard have been determined from the four fortification samples tested by 13 laboratories in accordance with the

TABLE 1.17 Retention Times of 8 Sulfonamides

Analytes	Retention time (min)
Sulfacetamide	9.1
Sulfapyridine	9.9
Sulfamerazine	11.1
Sulfamethoxypyridazine	13.5
Sulfameter	15.3
Sulfachloropyridazine	16.6
Sulfamethoxazole	20.4
Sulfadimethoxine	29.4

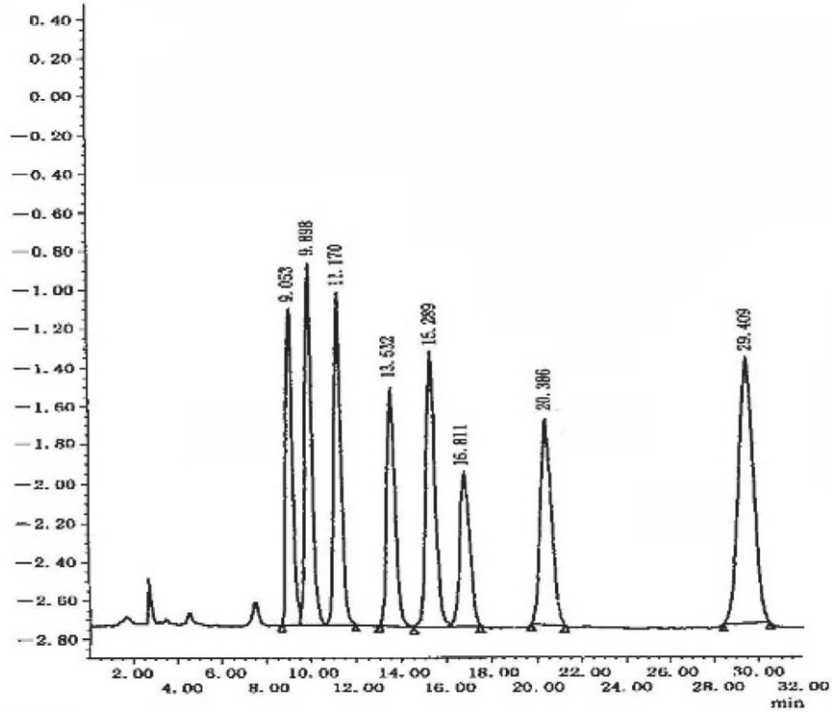

FIG. 1.4 Chromatogram of the sulfonamide drugs.

stipulations of GB/T6379. The values of repeatability and reproducibility have been obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for sulfonamide drugs in honey are shown in Table 1.18.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations shall be reconducted, and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for sulfonamide drugs in honey are shown in Table 1.18.

TABLE 1.18 Analytical Range, Repeatability, and Reproducibility

Analytes	Range (mg/kg)	Repeatability (r)	Reproducibility (R)
Sulfacetamide	0.010–0.20	$\lg r = 1.0167 \lg m - 0.9052$	$R = 0.2520m + 0.0013$
Sulfapyridine	0.010–0.20	$\lg r = 0.8512 \lg m - 1.1582$	$\lg R = 0.8783 \lg m - 0.9910$
Sulfamerazine	0.010–0.20	$\lg r = 0.6858 \lg m - 1.4476$	$\lg R = 0.8217 \lg m - 1.0422$
Sulfametho-xypyridazine	0.010–0.20	$\lg r = 1.0516 \lg m - 0.8977$	$\lg R = 0.7816 \lg m - 0.9891$
Sulfameter	0.010–0.20	$\lg r = 0.9547 \lg m - 0.9971$	$\lg R = 0.7902 \lg m - 0.9530$
Sulfachloropyridazine	0.010–0.20	$\lg r = 0.6151 \lg m - 1.3940$	$R = 0.1464m + 0.0015$
Sulfamethoxazole	0.010–0.20	$\lg r = 0.6742 \lg m - 1.2860$	$\lg R = 0.8268 \lg m - 0.8362$
Sulfadimethoxine	0.010–0.20	$\lg r = 0.9998 \lg m - 0.9143$	$\lg R = 0.9109 \lg m - 0.8915$

Note: *The* m *is average value of parallel test results.*

1.6.8 RECOVERY

Under optimized conditions, the average recoveries of this method are listed in Table 1.19.

TABLE 1.19 The Recoveries of Sulfonamide Drugs in Honey

Analytes	Fortifying Concentration (mg/kg)	Recovery (%)
Sulfacetamide	0.010	81.4
	0.050	88.6
	0.20	88.2
Sulfapyridine	0.010	81.1
	0.050	85.4
	0.20	86.7
Sulfamerazine	0.010	81.6
	0.050	84.8
	0.20	88.8
Sulfamethoxypyridazine	0.010	84.2
	0.050	84.6
	0.20	83.7
Sulfameter	0.010	80.9
	0.050	86.2
	0.20	87.8
Sulfachloropyridazine	0.010	78.0
	0.050	82.4
	0.20	84.6
Sulfamethoxazole	0.010	84.4
	0.050	89.2
	0.20	85.3
Sulfadimethoxine	0.010	79.9
	0.050	85.4
	0.20	85.2

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1.7

Determination of 16 Sulfonamide Residues in Honey—LC-MS-MS Method (GB/T 18932.17-2003)

1.7.1 SCOPE

This method specifies the determination of 16 sulfonamide residues in honey.

The limit of determination of this method for sulfamethizole: 1.0 μg/kg; sulfacetamide, sulfadiazine, sulfapyridine, sulfisoxazole, sulfamerazine, sulfachloropyridazine, sulfamonomethoxine, sulfadoxine, sulfamethoxazole: 2.0 μg/kg; sulfathiazole, sulfamethoxypyridazine, sulfadimethoxine: 4.0 μg/kg; sulfameter, sulfamethazine: 8.0 μg/kg; sulfaphenazole: 12.0 μg/kg.

1.7.2 PRINCIPLE

The drugs are extracted from honey with phosphoric acid solution (pH = 2). After filtration, the honey solution is cleaned up via cation exchange and Oasis HLB cartridges. It is eluted with methanol and dried. The residue is dissolved in acetonitrile-0.01 mol/L ammonium solutions (12 + 88), for analysis by LC-MS/MS using an external standard.

1.7.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be reagent grade; "water" is deionized water.

Methanol, Acetonitrile: HPLC grade; Phosphoric acid: G.R.; 1-Heptanesulfonic acid sodium salt: $C_7H_{15}NaO_3S{\cdot}H_2O$, HPLC grade; Ammonium acetate; Potassium dihydrogen phosphate; Dipotassium hydrogen phosphate; Phosphoric acid solution: pH = 2. Dilution of phosphoric acid with water; Phosphate buffer: 0.2 mol/L, pH = 8. Place 1.05 g potassium dihydrogen phosphate and 33.46 g dipotassium hydrogen phosphate into 1000-mL volumetric flask, and dissolve in H_2O. Dilute solution to volume with H_2O; 1-Heptanesulfonic acid solution: 0.5 mol/L. Place 11 g 1-heptanesulfonic acid, sodium salt into 100-mL volumetric flask and dissolve in H_2O. Dilute solution to volume with H_2O; Cation exchange column: Aromatic sulfonic acid, 500 mg, 3 mL. Condition each column with 5 mL methanol followed by 10 mL water before use; Oasis HLB solid extraction columns or equivalent: 60 mg, 3 mL. Condition each column with 3 mL methanol followed by 6 mL water before use.

16 Sulfonamide standards: purity $\geq$99%.

Standard stock solutions of sulfonamide: 0.1 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volume of methanol, then dilute with methanol separately to prepare the standard stock solutions of 0.1 mg/mL in concentration. The standard stock solutions are stored 2 months at 4°C.

Standard working solutions of 16 sulfonamides: Depending upon sensitivity of each sulfonamide and the instrument linear range, prepare the standard working solutions of different concentration for 16 sulfonamides with honey control sample extract. The standard working solutions are stored 1 week at 4°C.

1.7.4 APPARATUS

LC-MS-MS: Equipped with ESI source; Solid phase extraction vacuum apparatus; Rotary evaporator; Vortex mixer; Analytical balance: Capable of weighing from 0.1 mg to 0.01 g; Vacuum pump; Pipette: 1 mL, 2 mL; Pear-shaped flask: 150 mL; Screw vial: 2.0 mL, with screw caps and PTFE septa; Reservoirs and adapters to fit SPE columns: 50 mL; pH meter: Capable of measuring $\pm$0.02 unit.

1.7.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath, warm at $\leq$60°C with occasional shaking until liquefied, mix thoroughly and promptly cool

to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label. The test samples should be stored at 20°C.

(2) Extraction

Weigh 5 g test sample (accurate to 0.01 g) into 150-mL Erlenmeyer flask. Add 25 mL phosphoric acid solution to each sample. Mix vigorously 1 min on vortex mixer, until honey is completely dissolved.

(3) Clean-up

Connect a reservoir with glass wool plug to cation exchange cartridge. The honey sample solution is decanted into the reservoir. Let solution pass through the cation exchange cartridge at reduced pressure, adjusting the flow rate to $\leq$3 mL/min. Wait till sample solution has thoroughly drained, then rinse reservoir and cartridge respectively with 5 mL phosphoric acid solution and 5 mL water. Discard all the effluents. Finally, elute sulfonamides with 40 mL phosphate buffer into the 100-mL flat flask. Add 1.5 mL 1-heptanesulfonic acid solution to the elute and adjust it to pH = 6 by drop-wise addition of phosphoric acid.

The sample solution is made to pass through the Oasis HLB cartridge in the same way as described previously, and the flow rate is adjusted to $\leq$3 mL/min. Wait till the sample solution has thoroughly drained, and then rinse the cartridge with 3 mL water and discard all the effluents. Dry the cartridge by drawing air through it for 5 min under 65 kPa vacuum. Finally, elute sulfonamides with 10 mL methanol into 150-mL pear-shaped flask. Evaporate to dryness on a rotary evaporator with vacuum in a water bath at 45°C. Add accurately 1 mL mobile phase to extract for LC-MS-MS analysis.

1.7.6 DETERMINATION

(1) Operating conditions

Chromatographic column: Lichrospher 100 PR-18 5 μm, 4.6 × 250 mm, or equivalent; Column temperature: 35°C; Mobile phase: acetonitrile-0.01 mol/L ammonium acetate solution (12 + 88); Flow rate: 0.8 mL/min; Injection volumes: 40 μm; Split ratio: 1:3.

Ion source: ESI source; Scan mode: Positive scan; Monitor mode: Multiple reaction monitor; Ionspray voltage: 5500 V; Nebulizer gas: 0.055 MPa; Curtain gas: 0.079 MPa; Turbo ionspray gas rate: 6 L/min; Source temperature: 400°C; Precursor/product ion combinations, declustering potential, collision energy: see Table 1.20.

(2) LC-MS/MS analysis

The standard working solutions of different concentrations for 16 sulfonamides are prepared with honey control sample extract on standard working solutions. Then 20 μL of the different concentration working standard solutions, respectively, are injected in duplication under LC and MS conditions. Draw the seven-point standard curves of each sulfonamide

TABLE 1.20 Precursor/Product Ion Combinations, Declustering Potential, Collision Energy of 16 Sulfonamides

Analytes	Qualifier Ion Pairs (*m*/*z*)	Target Ion Pairs (*m*/*z*)	Collision Energy (V)	Declustering Potential (V)
Sulfacetamide	215/156; 215/108	215/156	18; 28	40; 45
Sulfamethizole	271/156; 271/107	271/156	20; 32	50; 50
Sulfisoxazole	268/156; 268/113	268/156	20; 23	45; 45
Sulfachloropyridazine	285/156; 285/108	285/156	23; 35	50; 50
Sulfadiazine	251/156; 251/185	251/156	23; 27	55; 50
Sulfamethoxazole	254/156; 254/147	254/156	23; 22	50; 45
Sulfathiazole	256/156; 256/107	256/156	22; 32	55; 47
Sulfamonomethoxine	281/156; 281/215	281/156	25; 25	65; 50
Sulfamerazine	265/156; 265/172	265/156	25; 24	50; 60
Sulfadoxine	311/156; 311/108	311/156	31; 35	70; 55
Sulfapyridine	250/156; 250/184	250/156	25; 25	50; 60
Sulfameter	281/156; 281/215	281/156	25; 25	65; 50
Sulfamethoxypyridazine	281/156; 281/215	281/156	25; 25	65; 50
Sulfamethazine	279/156; 279/204	279/156	22; 20	55; 60
Sulfaphenazole	315/156; 315/160	315/156	32; 35	55; 55
Sulfadimethoxine	311/156; 311/218	311/156	31; 27	70; 70

(peak area vs. sulfonamide concentration). Calculate the concentrations of the corresponding content from the working curve. The responses of 16 sulfonamides in the standard working solution and sample solution should be in the linear range of the instrumental detection. Under LC and MS conditions, for the reference retention times of the 16 sulfonamides, see Table 1.21; the chromatogram of 16 sulfonamide standards is shown in Figs. 1.5 and 1.6.

TABLE 1.21 Retention Times of 16 Sulfonamides

Analytes	Retention Time (min)	Analytes	Retention Time (min)
Sulfacetamide	2.61	Sulfamerazine	9.93
Sulfamethizole	4.54	Sulfadoxine	11.29
Sulfisoxazole	4.91	Sulfapyridine	11.62
Sulfadiazine	5.20	Sulfameter	12.66
Sulfachloropyridazine	6.54	Sulfamethoxypyridazine	17.28
Sulfamethoxazole	8.41	Sulfamethazine	17.95
Sulfathiazole	9.13	Sulfaphenazole	22.29
Sulfamonomethoxine	9.48	Sulfadimethoxine	28.97

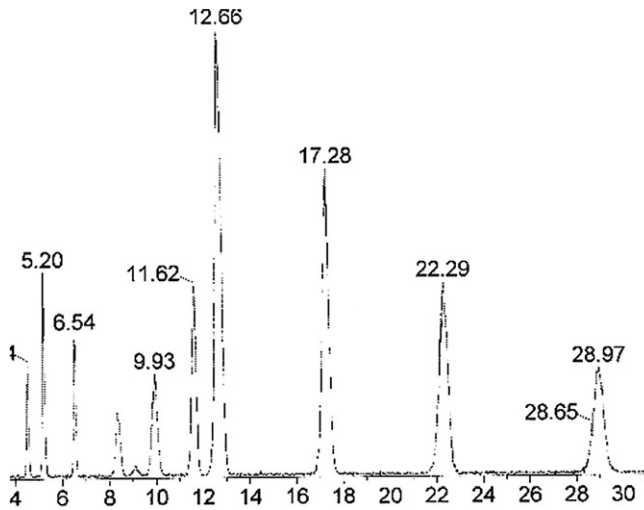

FIG. 1.5 Total ion chromatogram of 11 sulfonamide standards.

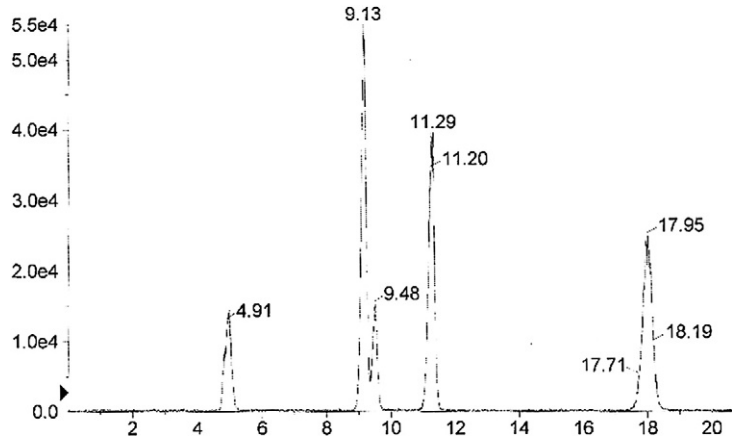

FIG. 1.6 Total ion chromatogram of 5 sulfonamide standards.

1.7.7 PRECISION

The precision data of the method for this standard have been determined in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for 16 sulfonamide drugs in honey are shown in Table 1.22.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations shall be reconducted, and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for 16 sulfonamide drugs in honey are shown in Table 1.22.

1.7.8 RECOVERY

Under optimized conditions, the recoveries of 16 sulfonamides in honey using this method are listed in Table 1.23.

TABLE 1.22 Analytical Range, Repeatability, and Reproducibility

Analytes	Analytical Range (μg/kg)	Repeatability (r)	Reproducibility (R)
Sulfacetamide	2.0–50.0	$\lg r = 0.9358 \lg m - 1.0259$	$\lg R = 0.1539 \lg m + 0.0561$
Sulfamethizole	2.0–50.0	$\lg r = 1.0841 \lg m - 1.0704$	$\lg R = 1.0744 \lg m - 0.6765$
Sulfisoxazole	2.0–50.0	$\lg r = 0.8428 \lg m - 0.8760$	$\lg R = 0.7392 \lg m - 0.5488$
Sulfachloropyridazine	2.0–50.0	$\lg r = 0.9705 \lg m - 1.0008$	$R = 0.1414m + 0.2000$
Sulfadiazine	2.0–50.0	$\lg r = 1.2712 \lg m - 1.2329$	$\lg R = 0.8401 \lg m - 0.5495$
Sulfamethoxazole	2.0–50.0	$\lg r = 1.0624 \lg m - 1.0368$	$\lg R = 0.9819 \lg m - 0.7392$
Sulfathiazole	4.0–100.0	$\lg r = 1.0918 \lg m - 1.0852$	$\lg R = 0.9107 \lg m - 0.6442$
Sulfamonomethoxine	2.0–50.0	$\lg r = 1.0931 \lg m - 1.1693$	$\lg R = 1.0980 \lg m - 0.8558$
Sulfamerazine	2.0–50.0	$\lg r = 1.1531 \lg m - 1.2676$	$\lg R = 0.7928 \lg m - 0.5102$
Sulfadoxine	2.0–50.0	$\lg r = 0.9147 \lg m - 0.8606$	$\lg R = 0.7911 \lg m - 0.4748$
Sulfapyridine	2.0–50.0	$\lg r = 0.8943 \lg m - 0.8190$	$\lg R = 0.8292 \lg m - 0.5530$
Sulfameter	8.0–200.0	$\lg r = 1.0755 \lg m - 1.0745$	$\lg R = 1.1364 \lg m - 0.8346$
Sulfamethoxypyridazine	4.0–100.0	$\lg r = 1.1270 \lg m - 1.1279$	$\lg R = 1.1294 \lg m - 0.8120$
Sulfamethazine	8.0–200.0	$\lg r = 1.0379 \lg m - 1.0642$	$\lg R = 1.0498 \lg m - 0.7143$
Sulfaphenazole	12.0–300.0	$\lg r = 1.1421 \lg m - 1.2258$	$\lg R = 1.0512 \lg m - 0.8053$
Sulfadimethoxine	4.0–100.0	$\lg r = 0.8598 \lg m - 0.8796$	$\lg R = 0.9760 \lg m - 0.6733$

TABLE 1.23 The Recoveries of 16 Sulfonamide Drugs in Honey

Analytes	Fortifying Concentration (μg/kg)	Average Recovery (%)
Sulfacetamide	2.0	77.1
	5.0	77.7
	10.0	77.3
	50.0	78.7
Sulfamethizole	1.0	78.7
	2.5	80.0
	5.0	79.3
	25.0	77.9
Sulfisoxazole	2.0	90.2
	5.0	75.5
	10.0	76.9
	50.0	87.5
Sulfachloropyridazine	2.0	82.1
	5.0	85.7
	10.0	77.2
	50.0	78.6
Sulfadiazine	2.0	84.7
	5.0	86.6
	10.0	86.4
	50.0	77.5
Sulfamethoxazole	2.0	83.0
	5.0	87.8
	10.0	97.8
	50.0	80.5
Sulfathiazole	4.0	85.3
	10.0	70.9
	20.0	76.5
	100.0	86.7
Sulfamonomethoxine	2.0	95.6
	5.0	88.9
	10.0	94.8
	50.0	102.5

TABLE 1.23 The Recoveries of 16 Sulfonamide Drugs in Honey—cont'd

Analytes	Fortifying Concentration (μg/kg)	Average Recovery (%)
Sulfamerazine	2.0	81.8
	5.0	83.3
	10.0	89.4
	50.0	81.6
Sulfadoxine	2.0	97.4
	5.0	86.5
	10.0	92.1
	50.0	95.4
Sulfapyridine	2.0	87.9
	5.0	85.0
	10.0	84.1
	50.0	77.9

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1.8

Determination of 18 Sulfonamide Residues in Royal Jelly—LC-MS-MS Method (GB/T 22947-2008)

1.8.1 SCOPE

This method is applicable to the determination of 18 sulfonamide residues in royal jelly.

The limit of determination of this method of 18 sulfonamides is 5.0 μg/kg.

1.8.2 PRINCIPLE

The sulfonamide drugs are extracted from the royal jelly with deionized water, precipitated protein with trichloroacetic acid. After being centrifuged, the supernatant is cleaned up by Oasis MCX solid extraction columns or equivalent solid extraction columns. The drugs are eluted with aqueous ammonia-methanol solution (1 + 19) and dried. The drugs are dissolved in acetonitrile-0.01 mol/L ammonium acetate solution. After passing through a 0.2-μm filter membrane, the solution is ready for determination by LC-MS-MS, using an internal standard method.

1.8.3 REAGENTS AND MATERIALS

Water: GB/T6682, First-level.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Ammonium acetate: G.R.
Trichloroacetic acid: G.R.
Formic acid: HPLC grade.
Aqueous ammonia. A.R.
0.01 mol/L Ammonium acetate solution: Weigh 0.77 g ammonium acetate dissolved in 1000 mL water.
2% formic acid solution: Dilute 2 mL formic acid to 100 mL with water.
50% trichloroacetic acid: Weigh 20 g trichloroacetic acid dissolved in 20 mL water.
Aqueous ammonia-methanol solution (1 + 19): Mix 5 mL aqueous ammonia and 95 mL methanol.
Acetonitrile-0.01 mol/L ammonium acetate solution (3 + 22): Mix 12 mL acetonitrile and 88 mL 0.01 mol/L ammonium acetate solution.

18 Sulfonamide standards: sulfadiazine(CAS 68-35-9), sulfathiazole (CAS 72-14-0), sulfapyridine (CAS 144-83-2), sulfamethoxypyridazine (CAS 127-79-7), sulfamonomethoxine (CAS 1220-83-3), sulfamethizole (CAS 144-82-1), sulfamethazine (CAS 57-68-1), sulfamethoxypyridazine (CAS 80-35-3), sulfameter (CAS 651-06-9), sulfachloropyridazine (CAS 80-32-0), sulfamethoxazole (CAS 723-46-6), sulfadoxine (CAS 2447-57-6), sulfisoxazole (CAS 127-69-5), sulfabenzamide (CAS 127-71-9), sulfachloropyrazine (CAS 102-65-8), sulfaphenazole (CAS 526-08-9), sulfadimethoxine (CAS 1037-50-9), sulfaquinoxaline (CAS 59-40-5); purity ≥99%.

Standard stock solutions of sulfonamide: 0.1 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volume of methanol, then dilute with methanol separately to prepare the standard stock solutions of 0.1 mg/mL in concentration. The standard stock solutions are stored at 4°C.

Mixed standard working solutions of 18 sulfonamides: 5.0 μg/mL. Pipette 0.5 mL each standard stock solutions into 10-mL volumetric flasks respectively. Dilute the standard solution to volume with methanol. The standard working solutions are stored at 4°C.

Sulfonamide internal standards: sulfamethizole-D4, sulfadiazine-D4, sulfathiazole-D4; purity ≥99%.

Internal standard stock solutions of sulfonamide: 0.1 mg/mL. Accurately weigh appropriate amount of each internal standard, dissolve in a small volume of methanol, then dilute with methanol separately to prepare the internal standard stock solutions of 0.1 mg/mL in concentration. The internal standard stock solutions are stored at 4°C.

Mixed internal standard working solutions of sulfonamides: 5.0 μg/mL. Pipette 0.5 mL each internal standard stock solutions into 10-mL volumetric flasks respectively. Dilute the internal standards solution to volume with methanol. The internal standard working solutions are stored at 4°C.

Base standard working solutions: Pipette different volume mixed standard working solutions of 18 sulfonamides and 20 μL mixed internal standard working solutions of sulfonamides. Prepare the base standard working solutions of 5.0, 10.0, 20.0, 50.0, 100.0 ng/mL different concentration for sulfonamides with control sample extract. Prepare fresh daily.

Oasis MCX columns or equivalent solid extraction columns: 150 mg, 6 mL. Condition column with 5 mL methanol followed by 10 mL water.

Filter membrane: 0.2 μm.

1.8.4 APPARATUS

LC-MS-MS: Equipped with ESI source.

Analytical balance: capable of weighing from 0.1 mg to 0.01 g.

Centrifuge tube: 50 mL.
Shaker.
Vortex mixer.
Solid phase extraction vacuum apparatus.
Reservoirs and adapters to fit SPE columns: 50 mL.
Nitrogen evaporator.
Conical tube: 10 mL.

1.8.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Regarding the frozen test sample, after defrosting, mix it thoroughly by stirring. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label.

The test samples should be stored at −18°C.

(2) Extraction

Weigh 2-g test sample (accurate to 0.01 g) into 50-mL centrifuge tube; add 20 μL internal standard working solutions and 20 mL water to each sample. Mix 1 min on vortex mixer and shake 10 min on shaker. Add 0.5 mL trichloroacetic acid into the sample solution. Mix vigorously for 30 s. Centrifuge the sample solution at 3000 rpm for 5 min. Transfer the supernatant into an Erlenmeyer flask, add 15 mL water into the dregs; repeat the operation. Combine the supernatant.

(3) Clean-up

Connect a reservoir with glass wool plug to Oasis MCX solid extraction columns. The supernatant is decanted into the reservoir. Let solution pass through the Oasis MCX columns at reduced pressure, adjusting the flow rate to $\leq$3 mL/min. Wait till sample solution has thoroughly drained, then rinse reservoir and cartridge respectively with 5 mL formic acid solution and 5 mL methanol. Discard all the effluents. Finally, elute the sulfonamides with 5 mL aqueous ammonia-methanol into the 10-mL conical tube. The elution solution is blown to dryness on a nitrogen evaporator at 50°C. Add accurately 1 mL acetonitrile −0.01 mol/L ammonium acetate solution to dregs; put through a 0.2-μm filter membrane, and it is then ready for determination by LC-MS-MS.

(4) Preparation of control sample extract

According to the above-mentioned extraction and cleanup steps to prepare the base standard working solutions of different concentrations for sulfonamides with royal jelly control sample extract.

1.8.6 DETERMINATION

(1) Operation conditions

Chromatographic column: Atlantis C18, 3 μm, 150 mm × 2.1 mm i.d. or equivalent;

Mobile phase: A: Acetonitrile, B: 0.1% formic acid solution, C: Methanol. Gradient elution condition: see Table 1.24;
Flow: 0.2 mL/min;
Column temperature: 35°C;
Injection volumes: 20 μL;
Ion source: ESI source;
Scan mode: Positive scan;
Monitor mode: Multiple reaction monitor;
Ionspray voltage: 5500 V;
Nebulizer gas: 0.069 MPa;
Curtain gas: 0.069 MPa;
Turbo ionspray gas rate: 6 L/min;
Source temperature: 350°C;
Precursor/product ion combinations, declustering potential, collision energy: see Table 1.25.

(2) Qualitative determination

Select one precursor and more than two daughters. In the same conditions, if the ratio error of the chromatographic retention time of the analyte and the standard corresponds is ±2.5% compared with the matrix standard solutions, the relative ion intensity of the sulfonamide in the sample and matrix standard solutions accords with Table 1.8; this sample has the sulfonamide.

(3) Quantitation determination

The base standard working solutions of different concentrations for 18 sulfonamides is prepared with fugu and eel control sample extract on base. Then inject 20 μL of the different concentration working standard solutions, respectively, in duplication under LC and MS conditions. Draw the five-point standard curves of each sulfonamide (peak area vs. sulfonamide concentration). Calculate the concentrations of the corresponding content from the working curve. The responses of 18 sulfonamides in

TABLE 1.24 Gradient Elution Condition

Time (min)	A (%)	B (%)	C (%)
0.00	10.0	80.0	10.0
3.00	20.0	70.0	10.0
8.00	35.0	55.0	10.0
12.00	70.0	20.0	10.0
12.01	10.0	80.0	10.0
17.00	10.0	80.0	10.0

TABLE 1.25 18 Sulfonamides Precursor/Product Ion Combinations, Declustering Potential, Collision Energy

Drug	Precursor Ion Combinations (*m*/*z*)	Production Ion Combinations (*m*/*z*)	Collision Energy (V)	Declustering Potential (V)
Sulfadiazine	251/156 251/185	251/156	23 27	55 50
Sulfathiazole	256/156 256/107	256/156	22 32	55 47
Sulfapyridine	250/156 250/184	250/156	25 25	50 60
Sulfamethoxypyridazine	265/156 265/172	265/156	25 24	50 60
Sulfamonomethoxine	281/156 281/215	281/156	25 25	65 50
Sulfamethizole	271/156 271/107	271/156	20 32	50 50
Sulfamethazine	279/156 279/204	279/156	22 20	55 60
Sulfamethoxypyridazine	281/156 281/215	281/156	25 25	65 50
Sulfameter	281/156 281/215	281/156	25 25	65 50

Sulfachloropyridazine	285/156 285/108	285/156	23 35	50 50
Sulfamethoxazole	254/156 254/147	254/156	23 22	50 45
Sulfadoxine	311/156 311/108	311/156	31 35	70 55
Sulfisoxazole	268/156 268/113	268/156	20 23	45 45
Sulfabenzamide	277/156 277/108	277/156	20 35	85 85
Sulfachloropyrazine	285/156 285/108	285/156	24 37	53 53
Sulfaphenazole	315/156 315/160	315/156	32 35	55 55
Sulfadimethoxine	311/156 311/218	311/156	31 27	70 70
Sulfaquinoxaline	301/156 301/208	301/156	25 28	42 42

the standard working solution and sample solution should be in the linear range of the instrumental detection. Under the LC and MS conditions, for the reference retention times of the 18 sulfonamides, see Table 1.26.

1.8.7 PRECISION

The precision data of the method for this standard have been determined in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for 18 sulfonamides in royal jelly are shown in Table 1.27.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations shall be reconducted, and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for 18 sulfonamides in fugu and eel are shown in Table 1.27.

TABLE 1.26 Retention Times of 18 Sulfonamides

Drug	Retention Time (min)	Drug	Retention Time (min)
Sulfadiazine	4.45	Sulfachloropyridazine	8.91
Sulfathiazole	4.77	Sulfamethoxazole	9.29
Sulfapyridine	5.39	Sulfadoxine	9.29
Sulfamethoxypyridazine	6.20	Sulfisoxazole	9.72
Sulfamonomethoxine	7.75	Sulfabenzamide	10.50
Sulfamethizole	7.76	Sulfachloropyrazine	10.85
Sulfamethazine	7.83	Sulfaphenazole	11.11
Sulfamethoxypyridazine	8.01	Sulfadimethoxine	11.12
Sulfameter	8.75	Sulfaquinoxaline	11.22

TABLE 1.27 Fortifying Concentration Range and Repeatability and Reproducibility (Unit μg/kg)

Drug	Fortifying concentration range	Repeatability (r)	Reproducibility (R)
Sulfadiazine	5.0–50.0	$\lg r = 1.3195 \lg m - 1.5661$	$\lg R = 1.2613 \lg m - 1.2872$
Sulfathiazole	5.0–50.0	$\lg r = 1.4281 \lg m - 1.7573$	$\lg R = 1.3766 \lg m - 1.3357$
Sulfapyridine	5.0–50.0	$\lg r = 0.9337 \lg m - 1.0499$	$\lg R = 1.1830 \lg m - 0.9735$
Sulfamethoxypyridazine	5.0–50.0	$\lg r = 1.2787 \lg m - 1.4852$	$\lg R = 1.2050 \lg m - 1.1419$
Sulfamonomethoxine	5.0–50.0	$\lg r = 1.3061 \lg m - 1.6252$	$\lg R = 1.1186 \lg m - 1.0905$
Sulfamethizole	5.0–50.0	$\lg r = 1.0851 \lg m - 1.4269$	$\lg R = 1.1774 \lg m - 1.1627$
Sulfamethazine	5.0–50.0	$\lg r = 0.9582 \lg m - 1.1850$	$\lg R = 1.0745 \lg m - 1.0287$
Sulfamethoxypyridazine	5.0–50.0	$\lg r = 1.3423 \lg m - 1.2995$	$\lg R = 1.3141 \lg m - 0.9663$
Sulfameter	5.0–50.0	$\lg r = 0.8920 \lg m - 1.0160$	$\lg R = 0.9875 \lg m - 0.9051$
Sulfachloropyridazine	5.0–50.0	$\lg r = 1.4154 \lg m - 1.4363$	$\lg R = 1.3900 \lg m - 1.1803$
Sulfamethoxazole	5.0–50.0	$\lg r = 0.8369 \lg m - 1.0303$	$\lg R = 1.0966 \lg m - 1.1560$
Sulfadoxine	5.0–50.0	$\lg r = 1.0991 \lg m - 1.1818$	$\lg R = 1.0857 \lg m - 0.9053$
Sulfisoxazole	5.0–50.0	$\lg r = 1.1470 \lg m - 1.2670$	$\lg R = 1.4686 \lg m - 1.3792$
Sulfabenzamide	5.0–50.0	$\lg r = 1.0569 \lg m - 1.2776$	$\lg R = 1.4528 \lg m - 1.4866$
Sulfachloropyrazine	5.0–50.0	$\lg r = 0.8449 \lg m - 1.1208$	$\lg R = 0.9769 \lg m - 0.8218$
Sulfaphenazole	5.0–50.0	$\lg r = 1.1813 \lg m - 1.4811$	$\lg R = 1.3630 \lg m - 1.3707$
Sulfadimethoxine	5.0–50.0	$\lg r = 1.0477 \lg m - 1.3232$	$\lg R = 1.3350 \lg m - 1.3173$
Sulfaquinoxaline	5.0–50.0	$\lg r = 0.9185 \lg m - 1.0654$	$\lg R = 1.3522 \lg m - 1.1577$

Note: *The* m *is average value of parallel test results.*

1.8.8 RECOVERY

Under optimized condition, the recoveries of 18 sulfonamides in royal jelly using this method are listed in Table 1.28.

TABLE 1.28 The Data of 18 Sulfonamide Fortifying Concentrations and Recovery

Drugs	Fortifying Concentration (μg/kg)	Average Recovery (%)
Sulfadiazine	5.0	104.9
	10.0	109.6
	20.0	99.0
	50.0	107.0
Sulfathiazole	5.0	99.5
	10.0	98.5
	20.0	95.8
	50.0	108.0
Sulfapyridine	5.0	92.8
	10.0	89.0
	20.0	94.4
	50.0	91.3
Sulfamethoxypyridazine	5.0	82.9
	10.0	86.8
	20.0	84.8
	50.0	82.5
Sulfamonomethoxine	5.0	92.2
	10.0	91.5
	20.0	93.0
	50.0	98.2
Sulfamethizole	5.0	95.4
	10.0	97.9
	20.0	96.1
	50.0	100.2

TABLE 1.28 The Data of 18 Sulfonamide Fortifying Concentrations and Recovery—cont'd

Drugs	Fortifying Concentration (μg/kg)	Average Recovery (%)
Sulfamethazine	5.0	97.4
	10.0	94.5
	20.0	94.1
	50.0	97.4
Sulfamethoxypyridazine	5.0	95.3
	10.0	94.7
	20.0	92.2
	50.0	92.9
Sulfameter	5.0	94.4
	10.0	97.5
	20.0	98.1
	50.0	94.9
Sulfachloropyridazine	5.0	96.5
	10.0	97.3
	20.0	95.9
	50.0	110.5
Sulfamethoxazole	5.0	105.8
	10.0	109.1
	20.0	97.9
	50.0	112.6
Sulfadoxine	5.0	86.5
	10.0	93.4
	20.0	91.6
	50.0	96.9
Sulfisoxazole	5.0	92.1
	10.0	88.9
	20.0	93.0
	50.0	93.2

Continued

TABLE 1.28 The Data of 18 Sulfonamide Fortifying Concentrations and Recovery—cont'd

Drugs	Fortifying Concentration (μg/kg)	Average Recovery (%)
Sulfabenzamide	5.0	93.4
	10.0	91.6
	20.0	94.8
	50.0	102.5
Sulfachloropyrazine	5.0	91.0
	10.0	90.5
	20.0	89.3
	50.0	96.1
Sulfaphenazole	5.0	98.6
	10.0	86.7
	20.0	80.2
	50.0	84.4
Sulfadimethoxine	5.0	105.5
	10.0	102.3
	20.0	87.4
	50.0	82.6
Sulfaquinoxaline	5.0	86.4
	10.0	83.4
	20.0	84.2
	50.0	85.8

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Chapter 2

β-Adrenoceptor Agonists

2.1

Determination of 12 β-Agonist Residues in Milk and Milk Powder—LC-MS-MS Method (GB/T 22965-2008)

2.1.1 SCOPE

The method is applicable to milk and milk powder. Twelve β-agonists detectable by this method are brombuterol, cimaterol, clenbuterol, clenpenterol, hydroxymethylclenbuterol, isoxsuprine, mabuterol, ractopamin, ritodrine, salbutamol, terbutaline, and tulobuterol.

The limit of determination of the method in milk of ractopamin, salbutamol, cimaterol, clenpenterol, and clenbuterol is 0.05 μg/kg.

The limit of determination of the method in milk of brombuterol, tulobuterol, mabuterol, terbutaline, ritodrine, isoxsuprine, and hydroxymethylclenbuterol is 0.25 μg/kg.

The limit of determination of the method in milk powder of ractopamin, salbutamol, cimaterol, clenpenterol, and clenbuterol is 0.4 μg/kg.

The limit of determination of the method in milk powder of brombuterol, tulobuterol, mabuterol, terbutaline, ritodrine, isoxsuprine, and hydroxymethylclenbuterol is 2.0 μg/kg.

2.1.2 PRINCIPLE

The samples are hydrolyzed by hydrochloric acid solution. The β-agonist residues are extracted by acetic ether-isopropol and cleaned up by a mixed cation-exchange solid phase extraction column (MCX). Residues are determined by LC-MS-MS and quantified by an internal standard.

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2.1.3 REAGENTS AND MATERIALS

All reagents shall be of recognized analytical quality unless specified, and water for analytical shall be grade first specified in GB/T 6682.

Methanol: HPLC grade
Isopropanol
Ethyl acetate
Acetonitrile: HPLC grade
Formic acid: HPLC grade
Perchloric acid: 70%–72%.
Hydrochloric acid
Sodium hydroxide
Ammonium hydroxide: HPLC grade
Ammonia
Sodium chloride
Sodium hydroxide solution (10 mol/L): Weigh 40 g sodium hydroxide in 100 mL water.
Perchloric acid (0.1 mol/L): Dilute 0.4 mL perchloric acid to final volume of 100 mL with water.
Hydrochloric acid (0.1 mol/L). Dilute 0.85 mL hydrochloric acid to final volume of 100 mL with water.
Ammonium acetate buffer solution: 5 mmol/L. Weigh 0.385 g ammonium acetate in 800 mL water, dilute 2 mL acetic acid, and add water to final volume of 1 L.
Methanol-formic acid solution: 0.1%. Pipette 0.1 mL formic acid into 50 mL water and mix well with 50 mL methanol.
12 β-agonist standards: brombuterol, cimaterol, clenbuterol, clenpenterol, hydroxymethylclenbuterol, isoxsuprine, mabuterol, ractopamin, ritodrine, salbutamol, terbutaline, and tulobuterol, purity $\geq$98%.
Internal standards: Clenbuterol_D_9, Salbutamol_D_3, Ractopamin_D_5, purity $\geq$98.0%.
Stock solutions of β-agonists (100 mg/L): weigh 10.0 mg β-agonist standard materials, dissolve with methanol to a volume of 100 mL respectively, and store at approximately −20°C.
Working standard solutions (1 mg/L): As needed, freshly dilute the standard solutions with methanol.
Internal standard stock solutions of β-agonists (100 mg/L): Weigh 10.0 mg β-agonist internal standard materials, dissolve with methanol to a volume of 100 mL respectively, and store at approximately −20°C.
Working internal standard solutions (1 mg/L): As needed, freshly dilute the standard solutions with methanol.
Matrix standard solutions: dilute appropriate volume of stock solutions and internal standard solutions to the intended concentration with blank extraction solution and mix well. These solutions should be prepared before use.

Cation-exchange solid phase extraction (SPE) cartridge: 60 mg/3 mL; the extraction cartridge is conditioned using 3 mL methanol, 3 mL water before use; prevent the columns from running dry.
0.22 μm hydrophilic membrane filter.

2.1.4 APPARATUS

High Performance Liquid Chromatography-Mass Spectrometer equipment: equipped with electrospray (ESI) LC interface.

Analytical balance: sensibility reciprocal is 0.1 mg and 0.01 g respectively.
Tissues blender
High-speed Refrigerated Centrifuge: 15,000 rpm, temperature: 4°C.
Centrifuge: rotate speed ≥4000 rpm.
Vortex mixer
DK Electro-Thermostatic Water Cabinet.
PH meter.
Rotary vacuum evaporator.
Solid phase extraction equipment.
Nitrogen evaporator.

2.1.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Weigh to the nearest 500 g of sample into the beaker. Divide into two groups after mixing well. Seal and label.

Milk may be stored in a refrigerated cabinet, and milk powder may be stored in an air desiccator at room temperature.

(2) Extraction

(a) Milk

Accurately weigh 10.0 mg (0.01 g) sample into a 50 mL polypropylene centrifuge tube; add 20 mL hydrochloric acid (0.1 mol/L) and 50 μL working internal standard (10 ng/mL) to sample matrix and vibrate for 5 min. Shake for 16 h (overnight) at 37°C in a shaking water bath. Cool samples to room temperature and then add 5 mL perchloric acid. Homogenize for 30 s at 10,000 rpm and then centrifuge 10 min at 15,000 rpm. Transfer the 10 mL supernatant into a 50-mL colorimetric tube. Adjust pH to 9.7 ±0.3 with 10 mol/L sodium hydroxide. Add 2.0 g sodium chloride to the sample solution. Add 25 mL isopropanol-ethyl acetate (6+4, v/v) and homogenize for 2 min at 10,000 rpm. Then centrifuge 5 min at 4500 rpm. Transfer the supernatant into another 50-mL colorimetric tube and add another 15 mL isopropanol-ethyl acetate (3+7, v/v) and extract once more. Combine the supernatants and evaporate to dryness at 35°C under a stream of nitrogen with pressured gas blowing concetrator. The residues are redissolved in 5 mL hydrochloric acid. The dissolution is achieved using the vortex for 30 s.

(b) Milk powder

Weigh to the nearest 12.5 g of sample into a beaker. Using appropriate amount 35°C to 50°C water, dissolve and mix well, until a homogeneous mixture is obtained. Allow the solution of milk powder to cool to room temperature and then quantitatively transfer it to a 100-g volumetric flask using small amounts of water. Accurately weigh 10.0 mg (0.01 g) sample into a 50-mL polypropylene centrifuge tube. And then the subsequent procedures were identical to the extraction from milk sample.

(3) Clean-up

Pass extracts through strong cation exchange (MCX) solid phase extraction (SPE) cartridges at a flow rate ca. 1 mL/min. Wash loaded SPE column with 3 mL water, 3 mL 2% formic acid solution and 3 mL methanol. Afterwards, purge the cartridges by air and then elute with 5 mL methanol/ammonium hydroxide (95+5). Concentrate the eluate to near dryness using a gentle nitrogen stream and add 0.5 mL methanol–formic acid solution to resolve the residual. Filter using a 0.22-μm membrane filter prior to LC-MS-MS analysis.

(4) Sample blank solution preparation

Accurately weigh 5.0 g of the blank samples, and use the same procedure as the above-mentioned extraction and cleanup steps to prepare the blank sample solution.

2.1.6 DETERMINATION

(1) Operation conditions

Column: Acquity UPLC BEH C_{18}column (55 mm × 2.1 mm, 1.7 μm) or equivalent columns;

Mobile phase: A: 5 mM/L Ammonium acetate solution+0.2% formic acid.

B: Acetonitrile+0.1% formic acid

Gradient elution procedure: Program pump for the following gradient (Table 2.1):

Flow rate: 250 μL/min.

Column temperature: 30°C

Injection volume: 10 μL

Ion source: ESI, positive ionization mode.

Scan mode: multiple reaction monitoring (MRM) mode.

Capillary (kV): 1.5

Source Temperature (°C): 120

Desolvation Temperature (°C): 450

Cone Gas Flow (L/Hr): 45

Desolvation Gas Flow (L/Hr): 700

Collision Cell Pressure: $2.20 \times 10-6$ Pa

Dwell time: 0.05 s

TABLE 2.1 LC Gradient Elution Procedure

Time (min)	A (%)	B (%)
0.00	85	15
1.0	85	15
2.0	30	70
2.5	30	70
3.0	85	15
5.0	85	15

Calibrate the mass spectrometer and electrospray interface according to the manufacturer's specifications. Optimum parameters for LC-MS-MS.

(2) Qualification determination

The qualification ions for every compound must be found, and at least include one precursor ion and two daughter ions. Under the same determination conditions, the variation range of the retention time for the peak of analyte in the unknown sample and in the standard working solution cannot be out of range more than ±2.5%. For the same analysis batch and the same compound, the variation range of the ion ratio between the two daughter ions for the unknown sample and the standard working solution at the similar concentration cannot be out of range given in Table 2.2. Then the corresponding analyte must be present in the sample.

(3) Quantitation determination

Interstandard quantitative method**:** After standard working resolution mixture injection, the regression equation is obtained with the concentrations ratio as abscissa and the peak area ratio as ordinate. According to the approximate concentration of analyte in the sample solution, select the standard working solution with similar responses to that of the sample solution. The responses of the analytes in the standard working solutions and the sample solutions should be within the linear range of the instrument detection. The mixed standard working solutions and the

TABLE 2.2 Maximum Permitted Tolerances for Relative Ion Intensities During Confirmation (%)

Relative intensity	$k > 50$	$20 < k < 50$	$10 < k < 20$	$k \leqq 10$
Permitted tolerances	±20	±25	±30	±50

sample solutions should be injected with equal volume alternatively. Under the preceding LC-MS-MS operating conditions, the retention times of 12 β-agonists are listed in Table 2.3; for the multireaction monitor chromatograms of 12 β-agonists, see Fig. 2.1.

TABLE 2.3 Conditions for the MS-MS Detection of 12 β-Agonists

Analyte	RT/min	Transition (*m*/*z*)	Ions for Quantity (*m*/*z*)	Cone (V)	Collision energy (eV)
Ractopamin	2.00	302 > 164	302 > 164	25	*15*
		302 > 284			*12*
Salbutamol	0.75	240 > 148	240 > 148	20	*18*
		240 > 166			*13*
Cimaterol	0.84	220 > 160	220 > 160	18	*15*
		220 > 202			*10*
Clenpenterol	2.25	291 > 203	291 > 203	20	*15*
		291 > 273			*10*
Clenbuterol	2.15	277 > 203	277 > 203	26	*15*
		277 > 259			*11*
Brombuterol	2.20	367 > 293	367 > 293	18	*18*
		367 > 349			*13*
Tulobuterol	2.13	228 > 119	228 > 154	21	*25*
		228 > 154			*16*
Mabuterol	2.25	311 > 237	311 > 237	20	*16*
		311 > 293			*11*
Terbutaline	0.78	226 > 125	226 > 152	20	*22*
		226 > 152			*16*
Ritodrine	1.25	288 > 121	288 > 270	20	*20*
		288 > 270			*12*
Isoxsuprine	2.22	302 > 150	302 > 150	20	*22*
		302 > 284			*14*
Hydroxymethylclenbuterol	1.95	293 > 203	293 > 203	18	*15*
		293 > 275			*12*
Clenbuterol_D_9	2.13	286 > 204	286 > 204	26	*15*

TABLE 2.3 Conditions for the MS-MS Detection of 12 β-Agonists—cont'd

Analyte	RT/ min	Transition (*m/z*)	Ions for Quantity (*m/z*)	Cone (V)	Collision energy (eV)
Salbutamol_D_3	0.75	243 > 151	243 > 151	20	*18*
Ractopamin_D_5	*2.00*	*308 > 168*	*308 > 168*	*25*	*15*

Annotation: The compounds quantified by clenbuterol_D9: clenpenterol, clenbuterol, brombuterol, tulobuterol, mabuterol, isoxsuprine. The compounds quantified by salbutamol_D3: salbutamol, cimaterol, terbutaline. The compounds quantified by ractopamin_D5: ractopamin, ritodrine, hydroxymethylclenbuterol.

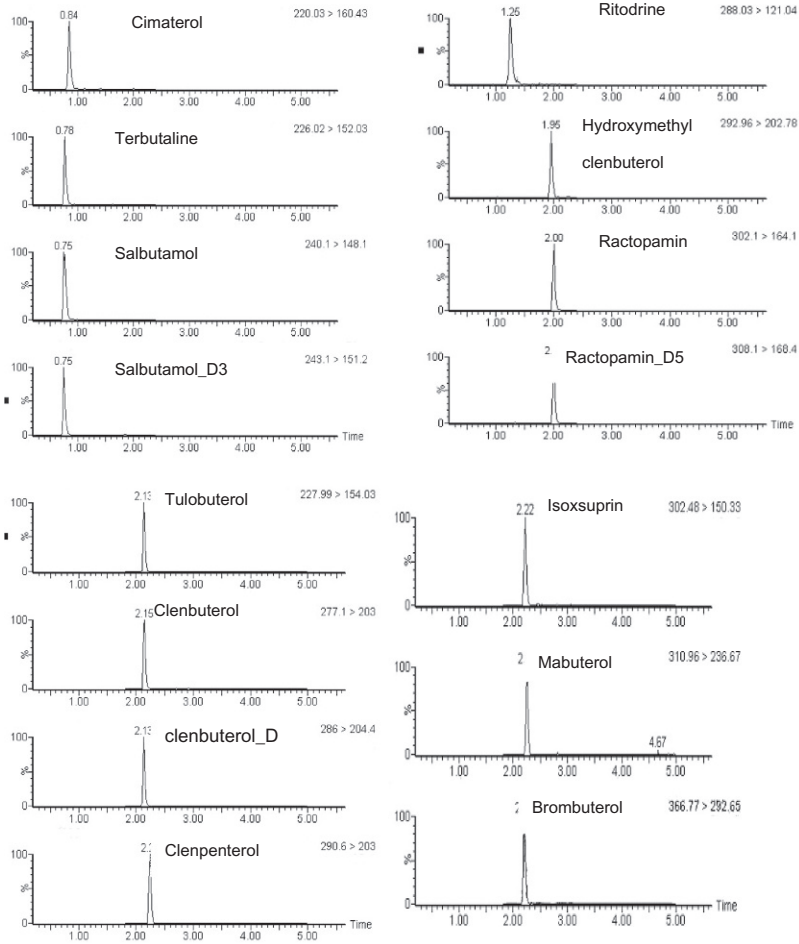

FIG. 2.1 MRM chromatograms of 12 β-agonists.

TABLE 2.4 The Repeatability and Reproducibility Equations of 12 β-Agonists

Analyte	Sample Matrix	Concentration Range (μg/kg)	Repeatability Limit	Reproducibility
Ractopamin	Milk	0.05–0.5	$\lg r = 0.9423 \lg m - 0.7681$	$\lg R = 0.9808 \lg m - 0.5091$
	Milk powder	0.4–4.0	$\lg r = 1.0250 \lg m - 10.706$	$\lg R = 1.0194 \lg m - 0.5622$
Salbutamol	Milk	0.05–0.5	$\lg r = 0.9634 \lg m - 0.4150$	$\lg R = 0.9812 \lg m - 0.4887$
	Milk powder	0.4–4.0	$\lg r = 1.0291 \lg m - 0.7085$	$\lg R = 0.9754 \lg m - 0.5549$
Cimaterol	Milk	0.05–0.5	$\lg r = 1.0971 \lg m - 0.6762$	$\lg R = 0.9191 \lg m - 0.4941$
	Milk powder	0.4–4.0	$\lg r = 1.1077 \lg m - 0.6913$	$\lg R = 0.9225 \lg m - 0.4898$
Clenpenterol	Milk	0.05–0.5	$\lg r = 0.9467 \lg m - 0.6084$	$\lg R = 1.0066 \lg m - 0.4705$
	Milk powder	0.4–4.0	$\lg r = 1.1021 \lg m - 0.6751$	$\lg R = 1.0096 \lg m - 0.4702$
Clenbuterol	Milk	0.05–0.5	$\lg r = 0.9884 \lg m - 0.7787$	$\lg R = 1.0564 \lg m - 0.5136$
	Milk powder	0.4–4.0	$\lg r = 1.0816 \lg m - 0.6544$	$\lg R = 1.0757 \lg m - 0.5569$
Brombuterol	Milk	0.25–2.5	$\lg r = 0.8930 \lg m - 0.7134$	$\lg R = 1.0330 \lg m - 0.4531$
	Milk powder	2.0–20.0	$\lg r = 0.9310 \lg m - 0.6180$	$\lg R = 1.0842 \lg m - 0.5120$
Tulobuterol	Milk	0.25–2.5	$\lg r = 0.9054 \lg m - 0.4936$	$\lg R = 0.9751 \lg m - 0.5611$
	Milk powder	2.0–20.0	$\lg r = 1.1843 \lg m - 0.6298$	$\lg R = 0.9575 \lg m - 0.4913$
Mabuterol	Milk	0.25–2.5	$\lg r = 0.9825 \lg m - 0.6372$	$\lg R = 0.9335 \lg m - 0.4684$
	Milk powder	2.0–20.0	$\lg r = 0.9124 \lg m - 0.6297$	$\lg R = 0.9722 \lg m - 0.4504$

Terbutaline	Milk	0.25–2.5	$\lg r = 0.9366 \lg m - 0.7328$	$\lg R = 0.9715 \lg m - 0.4991$
	Milk powder	2.0–20.0	$\lg r = 0.9934 \lg m - 0.7355$	$\lg R = 0.9570 \lg m - 0.5358$
Ritodrine	Milk	0.25–2.5	$\lg r = 0.9585 \lg m - 0.7316$	$\lg R = 0.9326 \lg m - 0.4887$
	Milk powder	2.0–20.0	$\lg r = 1.0999 \lg m - 0.7742$	$\lg R = 1.0122 \lg m - 0.6059$
Isoxsuprine	Milk	0.25–2.5	$\lg r = 0.9632 \lg m - 0.7558$	$\lg R = 0.9840 \lg m - 0.5208$
	Milk powder	2.0–20.0	$\lg r = 0.9789 \lg m - 0.6768$	$\lg R = 0.9506 \lg m - 0.5540$
Hydroxymethyl clenbuterol	Milk	0.25–2.5	$\lg r = 0.9891 \lg m - 0.7396$	$\lg R = 1.0012 \lg m - 0.4684$
	Milk powder	2.0–20.0	$\lg r = 0.9186 \lg m - 0.4970$	$\lg R = 0.9677 \lg m - 0.4824$

2.1.7 PRECISION

The precision data is determined according to GB/T6379.1 and GB/T6379.2. The repeatability and reproducibility limits given are obtained at the 95% probability level.

(1) Repeatability

Under repeatability conditions, the value of the absolute difference between two independent test results should be less than or equal to the repeatability limit (r). The concentration range of the analytes and the type of relationship are listed in Table 2.4.

If the difference exceeds the repeatability limit, the test results should be discarded and the tests redone.

(2) Reproducibility

Under reproducibility conditions, the value of the absolute difference between two independent test results should be less than or equal to the reproducibility limit (R). The concentration range of the analytes and the type of relationship are listed in Table 2.4.

2.1.8 RECOVERY

Under optimized condition, the recoveries of 12 β-Agonists in milk and milk powder using this method are listed in Table 2.5.

TABLE 2.5 The Spiked Concentration and Corresponding Recoveries of 12 β-Agonists

Analyte	Spiked Concentration (μg/kg)	Recoveries Range (%)	
		Milk	Milk Powder
Ractopamin	0.10	91.8	92.2
	0.20	89.3	91.8
	0.50	92.7	91.4
	1.0	91.9	94.6
Salbutamol	0.10	89.7	93.2
	0.20	90.5	91.9
	0.50	90.3	92.3
	1.0	91.6	93.0

TABLE 2.5 The Spiked Concentration and Corresponding Recoveries of 12 β-Agonists—cont'd

Analyte	Spiked Concentration (μg/kg)	Recoveries Range (%)	
		Milk	Milk Powder
Cimaterol	0.10	86.4	86.7
	0.20	86.3	87.9
	0.50	86.8	87.6
	1.0	87.5	86.3
Clenpenterol	0.10	86.7	88.5
	0.20	91.7	87.5
	0.50	91.0	88.5
	1.0	89.9	88.1
Clenbuterol	0.10	93.0	94.1
	0.20	94.7	91.9
	0.50	93.2	91.8
	1.0	93.6	91.3
Brombuterol	0.10	87.7	86.6
	0.20	88.9	84.9
	0.50	88.5	88.2
	1.0	87.1	86.2
Tulobuterol	0.50	88.1	88.9
	1.0	89.9	85.1
	2.5	87.8	90.4
	5.0	91.2	90.7
Mabuterol	0.50	87.6	82.2
	1.0	89.7	82.8
	2.5	83.7	86.2
	5.0	86.7	88.0
Terbutaline	0.50	85.5	87.2
	1.0	88.7	84.6
	2.5	87.0	88.5
	5.0	86.4	86.7

Continued

TABLE 2.5 The Spiked Concentration and Corresponding Recoveries of 12 β-Agonists—cont'd

Analyte	Spiked Concentration (μg/kg)	Recoveries Range (%)	
		Milk	Milk Powder
Ritodrine	0.50	86.0	88.6
	1.0	87.6	86.1
	2.5	82.5	85.5
	5.0	89.0	83.8
Isoxsuprine	0.50	87.9	87.9
	1.0	88.8	87.3
	2.5	88.1	87.2
	5.0	89.8	87.1
Hydroxymethylclenbuterol	0.50	85.1	86.0
	1.0	87.7	87.9
	2.5	87.4	88.9
	5.0	86.6	86.1

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2.2

Determination of 12 β-Agonist Residues in Fugu, Eel, and Baked Eel—LC-MS-MS Method (GB/T 22950-2008)

2.2.1 SCOPE

The method is applicable to fugu, eel, and roasted eel products. 12 β-agonists detectable by this method are brombuterol, cimaterol, clenbuterol, clenpenterol, hydroxymethylclenbuterol, isoxsuprine, mabuterol, ractopamin, ritodrine, salbutamol, terbutaline and tulobuterol.

The limit of determination of the method for ractopamin, salbutamol, cimaterol, clenpenterol, and clenbuterol is 0.1 μg/kg.

The limit of determination of the method for brombuterol, tulobuterol, mabuterol, terbutaline, ritodrine, isoxsuprine, and hydroxymethylclenbuterol is 0.5 μg/kg.

2.2.2 PRINCIPLE

The samples are hydrolyzed by a hydrochloric acid solution. The β-agonist residues are extracted by acetic ether-isopropol and cleaned up by a mixed cation-exchange solid phase extraction column (MCX). Residues are determined by LC-MS-MS and quantified by an internal standard.

2.2.3 REAGENTS AND MATERIALS

All reagents shall be of recognized analytical quality unless specified, and water for analytical shall be grade first specified in GB/T 6682.

Methanol: HPLC grade
Isopropanol
Ethyl acetate
Acetonitrile: HPLC grade
Formic acid: HPLC grade
Perchloric acid: 70%–72%.
Hydrochloric acid
Sodium hydroxide
Ammonium hydroxide: HPLC grade
Ammonia

Sodium chloride
Sodium hydroxide solution (10 mol/L): Weigh 40 g sodium hydroxide in 100 mL water.
Perchloric acid (0.1 mol/L): Dilute 0.4 mL perchloric acid to final volume of 100 mL with water.
Hydrochloric acid (0.1 mol/L). Dilute 0.85 mL hydrochloric acid to final volume of 100 mL with water.
Ammonium acetate buffer solution: 5 mmol/L. Weigh 0.385 g ammonium acetate in 800 mL water, dilute 2 mL acetic acid and add water to final volume of 1 L.
Methanol-formic acid solution: 0.1%. Pipette 0.1 mL formic acid into 50 mL water and mix well with 50 mL methanol.
12 β-Agonist standards: brombuterol, cimaterol, clenbuterol, clenpenterol, hydroxymethylclenbuterol, isoxsuprine, mabuterol, ractopamin, ritodrine, salbutamol, terbutaline, and tulobuterol, purity ≥98%.
Internal standards: Clenbuterol_D_9, Salbutamol_D_3, Ractopamin_D_5, purity ≥98.0%.
Stock solutions of β-agonists (100 mg/L): Weigh 10.0 mg β-agonist standard materials, dissolve with methanol to a volume of 100 mL respectively, and store at approximately −20°C.
Working standard solutions (1 mg/L): As required for use, freshly dilute the standard solutions with methanol.
Internal standard stock solutions of β-agonists (100 mg/L): Weigh 10.0 mg β-agonist internal standard materials, dissolve with methanol to a volume of 100 mL respectively, and store at approximately −20°C.
Working internal standard solutions (1 mg/L): As required for use, freshly dilute the standard solutions with methanol.
Matrix standard solutions: Dilute appropriate volume of stock solutions and internal standard solutions to the intended concentration with blank extraction solution and mix well. These solutions should be prepared just before use.
Cation-exchange solid phase extraction (SPE) cartridge: y/3 mL, the extraction cartridge is conditioned using 3 mL methanol, 3 mL water before use; prevent the columns from running dry.
0.22 μm hydrophilic membrane filter.

2.2.4 APPARATUS

High Performance Liquid Chromatography-Mass Spectrometry equipment: equipped with electrospray (ESI) LC interface
Analytical Balance: Sensibility Reciprocal is 0.1 mg and 0.01 g Respectively
Tissues Blender
High-Speed Refrigerated Centrifuge: 15,000 rpm; Temperature: 4°C

Centrifuge: Rotate Speed ≥4000 rpm
Vortex Mixer
DK Electro-Thermostatic Water Cabinet
PH Meter
Rotary Vacuum Evaporator
Solid Phase Extraction Equipment
Nitrogen Evaporator

2.2.5 SAMPLE PRETREATMENT

(1) Sample Preparation

For fugu and eel samples, combine the muscle tissues and skins heated in microwave oven and mix well using homogenizer; for eel products, combine all the edible portions and mix well using homogenizer. Seal them in a clean vessel and label.

Precaution measures should be taken to avoid contamination or any other factors that may cause the change of residue concentrations in samples.

Samples may be stored at −18°C; fresh or frozen tissues may be stored at 2–6°C for 72 h.

(2) Extraction

Accurately weigh 5.0 (0.01 g) sample into a 50-mL polypropylene centrifuge tube; add 20 mL hydrochloric acid (0.1 mol/L) and 50 μL working internal standard (10 ng/mL) to sample matrix and vibrate for 5 min. Shake for 16 h (overnight) at 37°C in a shaking water bath. Cool samples to room temperature and then add 5 mL perchloric acid. Homogenize for 30 s at 10,000 rpm and then centrifuge 10 min at 15,000 rpm. Transfer the 10 mL supernatant into a 50-mL colorimetric tube. Adjust pH to 9.7 ± 0.3 with 10 mol/L sodium hydroxide. Add 2.0 g sodium chloride to the sample solution. Add 25 mL isopropanol-ethyl acetate (6+4, v/v) and homogenize for 2 min at 10,000 rpm; then centrifuge 5 min at 4500 rpm. Transfer the supernatant into another 50-mL colorimetric tube and add another 15 mL isopropanol-ethyl acetate (3+7, v/v) and extract once more. Combine the supernatants and evaporate to dryness at 35°C under a stream of nitrogen with pressured gas blowing concetrator. Redissolve residues in 5 mL hydrochloric acid. The dissolution is achieved using the vortex for 30 s.

(3) Clean-up

Pass extracts through strong cation exchange (MCX) solid phase extraction (SPE) cartridges at a flow rate ca. 1 mL/min. Wash loaded SPE column with 3 mL water, 3 mL 2% formic acid solution and 3 mL methanol. Afterwards purge the cartridges by air, then elute with 5 mL methanol/ammonium hydroxide (95+5). Concentrate the eluate to near

dryness by gentle stream of nitrogen and add 0.5 mL methanol-formic acid solution to resolve the residual, and filter using a 0.22-μm membrane filter prior to LC-MS-MS analysis.

(4) Sample blank solution preparation

Accurately weigh 5.0 g of the blank samples, and use the same procedure as the above-mentioned extraction and cleanup steps to prepare the blank sample solution.

2.2.6 DETERMINATION

(1) Operation conditions:

Column: Acquity UPLC BEH C_{18}column (55 mm × 2.1 mm, 1.7 μm) or equivalent columns;

Mobile phase: A: 5 mM/L Ammonium acetate solution + 0.2% formic acid.

B: Acetonitrile + 0.1% formic acid

Gradient elution procedure: Program pump for the following gradient (Table 2.1):

Flow rate: 250 μL/min.

Column temperature: 30°C.

Injection volume: 10 μL.

Ion source: ESI, positive ionization mode.

Scan mode: multiple reaction monitoring (MRM) mode.

Capillary (kV): 1.5

Source Temperature (°C): 120

Desolvation Temperature (°C): 450

Cone Gas Flow (L/h): 45

Desolvation Gas Flow (L/h): 700

Collision Cell Pressure: 2.20×10^{-6} Pa

Dwell time: 0.05 s

Calibrate the mass spectrometer and electrospray interface according to the manufacturer's specifications. Optimum parameters for LC-MS-MS.

(2) Qualification determination

The qualification ions for every compound must be found and must at least include one precursor ion and two daughter ions. Under the same determination conditions, the variation range of the retention time for the peak of analyte in the unknown sample and in the standard working solution cannot be out of range more than ±2.5%. For the same analysis batch and the same compound, the variation range of the ion ratio between the two daughter ions for the unknown sample and the standard working solution at the similar concentration cannot be out of range given in Table 2.2. Then the corresponding analyte must be present in the sample.

(3) Quantitation determination

Interstandard quantitative method: After mixture standard working resolution injection, the regression equation is obtained with the concentrations

ratio as abscissa and the peak area ratio as ordinate. According to the approximate concentration of analyte in the sample solution, select the standard working solution with similar responses to that of the sample solution. The responses of the analytes in the standard working solutions and the sample solutions should be within the linear range of the instrument detection. The mixed standard working solutions and the sample solutions should be injected with equal volume alternatively. Under the preceding LC-MS-MS operating conditions, the retention times of 12 β-agonists are listed in Table 2.3; for the multireaction monitor chromatograms of 12 β-agonists, see Fig. 2.1.

2.2.7 PRECISION

The precision data are determined according to GB/T6379.1 and GB/T6379.2. The repeatability and reproducibility limits given are obtained at a 95% probability level.

(1) Repeatability

Under repeatability conditions, the value of the absolute difference between two independent test results should be less than or equal to the repeatability limit (r). The concentration range of the analytes and the type of relationship are listed in Table 2.6.

If the difference exceeds the repeatability limit, the test results should be discarded and the tests redone.

(2) Reproducibility

Under reproducibility conditions, the value of the absolute difference between two independent test results should be less than or equal to the reproducibility limit (R). The concentration range of the analytes and the type of relationship are listed in Table 2.6.

2.2.8 RECOVERY

Under optimized condition, the recoveries of 12 β-Agonists in fugu, eel, and baked eel using this method are listed in Table 2.7.

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TABLE 2.6 The Repeatability and Reproducibility Equations of 12 β-Agonists

Analyte	Concentration Range (μg/kg)	Repeatability Limit	Reproducibility
Ractopamin	0.1–1.0	$\lg r = 1.2554 \lg m - 0.8080$	$\lg R = 1.0324 \lg m - 0.4984$
Salbutamol	0.1–1.0	$\lg r = 1.2767 \lg m - 0.4946$	$\lg R = 1.0380 \lg m - 0.5013$
Cimaterol	0.1–1.0	$\lg r = 1.1148 \lg m - 0.7804$	$\lg R = 1.0153 \lg m - 0.5039$
Clenpenterol	0.1–1.0	$\lg r = 1.0797 \lg m - 0.6752$	$\lg R = 0.8926 \lg m - 0.5742$
Clenbuterol	0.1–1.0	$\lg r = 1.0062 \lg m - 0.7697$	$\lg R = 0.9598 \lg m - 0.4974$
Brombuterol	0.5–5.0	$\lg r = 1.0360 \lg m - 0.5762$	$\lg R = 1.0858 \lg m - 0.5339$
Tulobuterol	0.5–5.0	$\lg r = 1.1378 \lg m - 0.4915$	$\lg R = 0.9408 \lg m - 0.5826$
Mabuterol	0.5–5.0	$\lg r = 0.9981 \lg m - 0.6873$	$\lg R = 1.0205 \lg m - 0.5182$
Terbutaline	0.5–5.0	$\lg r = 0.9798 \lg m - 0.7052$	$\lg R = 1.0387 \lg m - 0.4810$
Ritodrine	0.5–5.0	$\lg r = 0.9654 \lg m - 0.7129$	$\lg R = 0.9735 \lg m - 0.4816$
Isoxsuprine	0.5–5.0	$\lg r = 1.1228 \lg m - 0.8142$	$\lg R = 0.9505 \lg m - 0.5014$
Hydroxymethylclenbuterol	0.5–5.0	$\lg r = 1.0170 \lg m - 0.7182$	q

TABLE 2.7 The Spiked Concentration and Corresponding Recoveries of 12 β-Agonists

Analyte	Spiked Concentration (μg/kg)	Recoveries Range (%)		
		Fugu	Eel	Eel product
Ractopamin	0.10	90.4	81.0	84.8
	0.20	88.9	81.5	84.1
	0.50	89.9	82.5	86.0
	1.0	94.7	84.3	87.2
Salbutamol	0.10	89.7	84.8	88.0
	0.20	90.7	84.4	87.0
	0.50	90.9	88.7	85.0
	1.0	90.8	87.1	88.7
Cimaterol	0.10	86.6	85.2	87.4
	0.20	90.6	93.9	83.2
	0.50	92.4	87.2	85.0
	1.0	91.8	89.5	88.9
Clenpenterol	0.10	87.7	86.8	84.2
	0.20	87.3	89.5	84.0
	0.50	90.4	86.9	87.6
	1.0	89.9	87.3	88.0
Clenbuterol	0.10	85.6	88.5	86.4
	0.20	87.1	90.4	84.9
	0.50	91.7	89.6	86.7
	1.0	82.0	93.3	87.1
Brombuterol	0.10	89.4	86.7	83.7
	0.20	92.5	93.5	84.7
	0.50	90.2	90.5	87.7
	1.0	92.7	90.9	86.6
Tulobuterol	0.50	90.5	89.5	86.0
	1.0	92.8	91.8	85.0
	2.5	95.7	93.2	89.9
	5.0	92.9	92.0	90.0

Continued

TABLE 2.7 The Spiked Concentration and Corresponding Recoveries of 12 β-Agonists—cont'd

Analyte	Spiked Concentration (μg/kg)	Recoveries Range (%)		
		Fugu	Eel	Eel product
Mabuterol	0.50	90.5	82.8	85.6
	1.0	91.4	89.2	85.5
	2.5	90.6	91.2	86.4
	5.0	90.8	96.9	86.9
Terbutaline	0.50	88.8	85.9	91.4
	1.0	87.4	92.1	89.8
	2.5	91.6	94.6	93.5
	5.0	89.2	91.9	90.3
Ritodrine	0.50	88.8	83.9	87.6
	1.0	94.2	83.8	87.4
	2.5	93.5	84.2	87.6
	5.0	91.1	90.0	90.7
Isoxsuprine	0.50	88.0	82.4	90.8
	1.0	90.0	86.3	85.5
	2.5	90.0	87.3	86.9
	5.0	93.7	91.9	87.5
Hydroxymethyl clenbuterol	0.50	90.8	87.3	90.2
	1.0	90.7	94.6	90.1
	2.5	91.5	97.3	88.8
	5.0	91.8	95.1	91.3

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Chapter 3

Aminoglycoside

3.1

Curative Effects and Side Effects of Streptomycin

Streptomycin (STR) and dihydrostreptomycin (DHSTR) are aminoglycoside antibiotics. STR is produced by *Streptomyces griseus*. All aminoglycosides contain amino-sugars bonded to an aminocyclitol ring by glycosidic links. They have a narrow-spectrum activity mainly against Gram-negative bacteria. They act primarily by impairing bacterial protein synthesis through binding to prokaryotic ribosomes. Susceptible strains include *Actinomyces bovis*, *Pasturella* spp., *Escherichia coli*, *Salmonella* spp., *Campylobacter* fetus, *Leptospira* spp., and *Brucella* spp. Mycobacterium tuberculosis is also sensitive. Both drugs have been widely used for the treatment of infectious diseases in food-producing animals. However, they are potentially toxic by causing damage to vestibular and auditory functions. The EU has a limited number of veterinary drugs permitted for use in foods of animal origin because the use of antibiotics may increase the resistance of target pathogens and must, therefore, be subject to strict control. In the EU maximum residue limits (MRLs) for STR and DHSTR have been established in Regulation 2377/90/EEC (1990) for food-producing animals at $500\,\mu g\,kg^{-1}$ in muscle, skin, fat, and liver, $1000\,\mu g\,kg^{-1}$ in kidney, and $200\,\mu g\,kg^{-1}$ in milk. In honey, contamination by STR was found after the direct treatment of infected bees with this antibiotic. However, there are no MRLs established for bee products in the EU. Because the antibiotic is also used for the control of bacterial infections caused by *Erwina amylovora* that affect fruit trees during flowering, this practice could potentially lead to STR contamination from pollen to nectar, to bees and honey.

The presence of STR residues in foods of animal origin can lead to allergic reaction following the ingestion of contaminated milk or meat. There is also concern about increasing bacterial resistance to antibiotics, which has been observed in livestock. The transmission of these resistance factors to man would be catastrophic and drastically reduce the effectiveness of antibiotics on human diseases.

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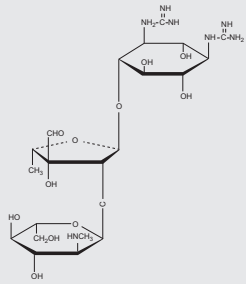
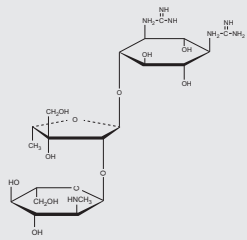
3.2

Chemical Structures and Maximum Residue Limits for Streptomycin and Dihydrostreptomycin (Table 3.1)

TABLE 3.1 Chemical Structures and Maximum Residue Limits for Streptomycin and Dihydrostreptomycin

Compound Names	Structures	M.W	Cas. No	MRL (μg/kg)
Streptomycin		581.58	3810-74-0	America: 2000 (kidney), 500 (other tissue) China: 200 (milk), 500 (muscle) EU: 200 (milk), 500 (muscle)
Dihydrostreptomycin		583.58	5490-27-7	America: 125 (milk) China: 200 (milk), 500 (muscle) EU: 200 (milk), 500 (muscle) Japan: 300 (fat, liver)

3.3

Determination of Streptomycin Residues in Honey—LC-FLD (GB/T 18932.3-2002)

3.3.1 SCOPE

This method is applicable to the determination of streptomycin residues in honey.

The limit of determination of this method: 0.010 mg/kg.

3.3.2 PRINCIPLE

Streptomycin residue in honey sample is extracted with phosphoric acid solution. The extract is filtered, cleaned up on an action exchange column and a C_{18} solid phase extraction cartridge. The retained analyses on the cartridge are eluted with methanol and the eluate evaporated to dryness in a rotary evaporator. The residue is dissolved in 1-heptanesulfonic acid sodium solution. The extract is analyzed by HPLC with postcolumn derivatization and fluorescence detection.

3.3.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be of analytical grade; water is deionized water.

Methanol: HPLC grade;
Acetonitrile: HPLC grade;
n-Hexane;
Acetic acid;
Phosphoric acid: G.R.;
Di-potassium hydrogen phosphate: G.R.;
Potassium dihydrogen phosphate: G.R.;
Sodium hydroxide: G.R.;
1-Heptanesulfonic acid sodium salt: $C_7H_{15}NaO_3S.H_2O$, HPLC grade;
1,2-Naphthoquinone-4-sulfonic acid sodium salt;
Tertiary butyl methyl ether;
Glass wool: Treated with phosphoric acid.
Cation exchange column: aromatic sulfonic solid phase extraction (SPE) cartridge: 500 mg, 3 mL. Condition aromatic sulfonic SPE cartridge with

5 mL methanol and 10 mL water before using. The cartridge is kept wet. C_{18} Solid phase extraction (SPE) cartridge: 500 mg, 3 mL. Condition C_{18} SPE cartridge with 5 mL methanol and 10 mL water before using.
Phosphoric acid solution: pH = 2. Add 1 mL phosphoric acid in 1000 mL water and adjust to pH = 2 by dropwise addition of phosphoric acid.
Phosphate buffer: 0.2 M, pH = 8. Weigh 33.46 g di-potassium hydrogen phosphate and 1.05 g potassium dihydrogen phosphate into 2000-mL beaker, add 900 mL water, and stir with a magnetic bar to dissolve. Transfer quantitatively into a 1000-mL volumetric flask and dilute to volume with water. Adjust to pH = 8 with phosphoric acid.
Sodium hydroxide: 0.2 M. Weigh 8 g sodium hydroxide and dissolve in 1000 mL water.
1-Heptanesulfonic acid sodium solution: 0.5 mol/L. Weigh 11 g 1-heptanesulfonic acid sodium salt, dissolve in 80 mL water and dilute to volume in a 100-mL volumetric flask.
1-Heptanesulfonic acid sodium solution: 0.01 mol/L, pH = 3.3. Weigh 2.2 g 1-heptanesulfonic acid sodium salt into 2000-mL beaker and add 900 mL water. Transfer quantitatively into a 1000-mL volumetric flask and dilute to volume with water; adjust to pH = 3.3 with acetic acid.
Tertiary butyl methyl ether-n-hexane mixed solution: 4 + 1. Measure 80 mL tertiary butyl methyl ether and 20 mL n-hexane in a 100-mL volumetric flask.
Streptomycin standard stock solution: Accurately weigh an appropriate amount of streptomycin standard and dissolve with water to prepare a standard stock solution of 100 μg/mL. This standard stock solution should be stored at 4°C.
Streptomycin standard working solution: According to the requirement, pipette adequate amount of standard stock solution and dilute with 0.01 mol/L 1-heptanesulfonic acid sodium solution to prepare standard working solution of suitable concentrations.

3.3.4 APPARATUS

High-performance liquid chromatograph, equipped with a fluorescence detector, and postcolumn derivatization apparatus;

Rotary evaporator;
Vacuum air pump;
pH meter: Capable of measuring ±0.02 unit;
Vortex mixer;
Solid phase extraction vacuum apparatus;
Reservoir: 50 mL;
Evaporation flask: 150 mL;
Flat-bottomed flask: 200 mL;
Microsyringe: 100 μL.

3.3.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath, warm at $\leq$60°C with occasional shaking until liquefied, mix thoroughly, and promptly cool to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label. The test samples should be stored at room temperature.

(2) Extraction

Weigh 10 g honey sample, accurate to 0.01 g, into a 150-mL Erlenmeyer flask. Add 25 mL phosphoric acid solution. Agitate on a vortex mixer at high speed for 5 min. Dissolve the honey sample completely.

(3) Clean-up

(a) Clean-up by cation exchange column

Insert a small amount of glass wool into a reservoir and wash with adequate amount of water. Insert it into a preconditioned aromatic sulfonic SPE cartridge. Pour the sample solution obtained into the reservoir. Use solid phase extraction vacuum apparatus so as to make the sample solution flow at a speed of 1.5 mL/min through the aromatic sulfonic SPE cartridge. Wash the SPE cartridge with 5 mL phosphoric acid solution and 10 mL water. Discard all of these effluents. Elute streptomycin from the SPE cartridge with 30 mL phosphate buffer into a 200-mL flat-bottomed flask at 1.5 mL/min. Add 3 mL 1-heptanesulfonic acid sodium solution to the eluate and shake well. Then adjust to pH = 2 by drop-wise addition of phosphoric acid.

(b) Clean-up by C_{18} SPE cartridge

Insert a reservoir into a preconditioned C_{18} SPE cartridge. Transfer the eluate obtained into the reservoir. Use solid phase extraction vacuum apparatus so as to make the eluate pass through C_{18} SPE at a flow rate of 1.5 mL/min. Rinse the C_{18} SPE cartridge with 5 mL phosphoric acid solution. Dry the C_{18} SPE cartridge for 5 min at 65 kPa by vacuum pump. Rinse the C_{18} SPE cartridge with 4 mL tertiary butyl methyl ether-n-hexane mixed solution and continue to suck dry for 5 min. Elute the streptomycin residue with 10 mL methanol into a 150-mL evaporation flask. Evaporate the eluate to dryness at 45°C using a rotary evaporator. The residue is dissolved in 1.0 mL 1-heptanesulfonic acid solution, and the solution is used for LC analysis.

3.3.6 DETERMINATION

(1) Operating conditions

Chromatographic column: Hypersil C_{18} 5 μm, 150 × 4.6 mm (i.d.), or equivalent;

Mobile phase: Weigh 1.10 g 1-heptanesulfonic acid sodium salt and 0.052 g 1,2-naphthoquinone-4-sulfonic acid sodium salt. Dissolve in 500 mL acetonitrile-water mixed solution (27 + 73) and adjust the solution to pH = 3.3 with acetic acid. Adjust the retention time of streptomycin to about 9 min with acetonitrile share. Produce fresh daily.

Mobile phase flow rate: 1.0 mL/min;

Detection wavelength: excitation 263 nm, emitting 435 nm;

Oven temperature: 50°C;

Derivatization column: 10 m × 0.25 mm (i.d.), stainless steel chamber;

Derivatization reagent: sodium hydroxide solution;

Derivatization temperature: 50°C;

Derivatization reagent flow rate: 0.4 mL/min;

Injection volume: 80 μL.

(2) HPLC determination

According to the approximate concentration of streptomycin in the sample solution, select the standard working solution with similar peak high to that of the sample solution. The responses of the streptomycin in the standard working solution and sample solution should be within the linear range of instrumental detection. The standard solution should be randomly injected in between the injections of sample solution of equal volume. Under these operating conditions, the retention time of streptomycin is about 9 min. The chromatogram of the streptomycin standard is shown in Fig. 3.1.

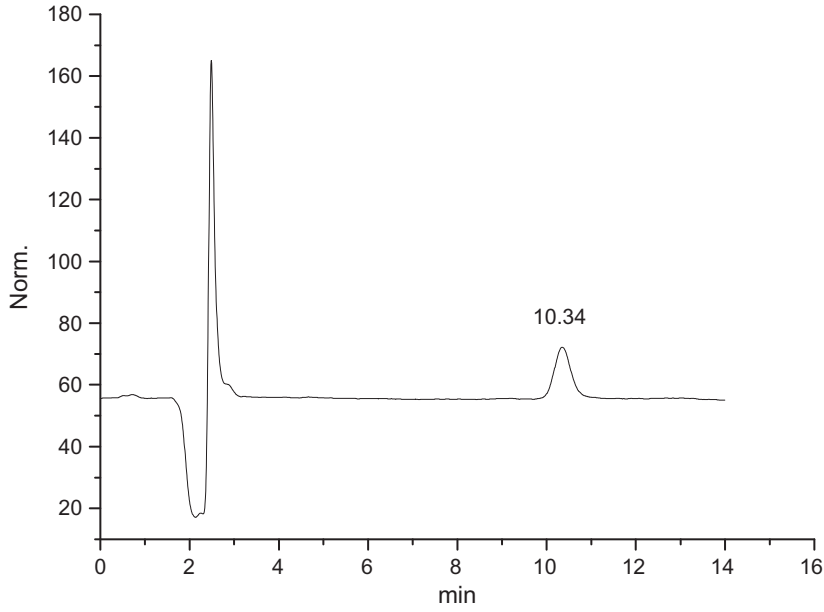


FIG. 3.1 Typical LC chromatogram of streptomycin standard.

3.3.7 PRECISION

The precision data of the method for this standard have been determined from the four fortification samples tested by 15 laboratories in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r). Repeatability of this method shall be calculated by:

The analytical range and repeatability equations for streptomycin in 0.010–0.10 mg/kg are:

$\lg r = 0.8504 \lg m - 0.9735$, where m is the average value obtained from two independent determinations.

If the difference in value exceeds the limit of repeatability, the test results should be discarded and two individual determinations repeated.

(2) Reproducibility

Under reproducibility conditions, the difference of the absolute values obtained from two independent determinations shall not exceed the limit of reproducibility (R). Reproducibility of this method shall be calculated by: $\lg R = 0.8416 \lg m - 0.8795$, where m is the average value obtained from the two independent determinations. The content range and repeatability equations of streptomycin in 0.010–0.10 mg/kg are: $\lg R = 0.8416 \lg m - 0.8795$, where m is the average value obtained from two independent determinations.

3.3.8 RECOVERY

Under optimized conditions, the recoveries of streptomycin from honey using this method are listed as follows:

Fortifying Concentration	Average Recovery
0.010 mg/kg	86.5%
0.020 mg/kg	82.4%
0.050 mg/kg	82.7%
0.10 mg/kg	82.1%

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3.4

Determination of Streptomycin, Dihydrostreptomycin, and Kanamycin Residues in Milk and Milk Powder—LC-MS-MS Method (GB/T 22969-2008)

3.4.1 SCOPE

This method is applicable to the determination of streptomycin, dihydrostreptomycin, and kanamycin residues in milk and milk powder.

The limit of determination of this method for streptomycin, dihydrostreptomycin, and kanamycin is 10 μg/kg in milk; the limit of determination of this method for streptomycin, dihydrostreptomycin, and kanamycin is 80 μg/kg in milk powder.

3.4.2 PRINCIPLE

Streptomycin, dihydrostreptomycin, and kanamycin residues are extracted with phosphoric acid solutions, and the proteins were precipitated with trichloroacetic acid. The solution obtained is cleaned up with aromatic sulfonic acid and carboxylic acid SPE cartridges. Residues are determined by LC-MS-MS.

3.4.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be guaranteed pure; "water" is deionized water.

Water: GB/T 6682, the first degree.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Formic acid.
Phosphoric acid.
Di-potassium hydrogen phosphate, K_2HPO_4.
Trichloroacetic acid, $C_2HCl_3O_2$.
Heptanes sulfonate sodium salt $C_7H_{15}NaO_3S{\cdot}H_2O$.
5% phosphoric acid solution (1 + 19): Dilute 50 mL concentrated phosphoric acid into 1000-mL volumetric flask. Dilute solution to volume with water.
0.2 mol/L Phosphate salt buffer solution: pH = 8.5. Dissolve 34.8 g di-potassium hydrogen phosphate into 1000-mL volumetric flask. Dilute

solution to volume with water. Then adjust to pH = 8.5 with sodium hydroxide solution.
Trichloroacetic acid solution: 50%. Dissolve 20 g trichloroacetic acid into 20-mL volumetric flask. Dilute solution to volume with water.
0.01 mol/L heptanes sulfonate sodium salt solution: Place 2.20 g heptanes sulfonate sodium salt into 1000-mL volumetric flask and dissolve in water. Dilute solution to volume with water.
SPE elute solution: Combine 4 mL formic acid into 100-mL volumetric flask. Dilute solution in 100-mL volumetric flask with 0.01 mol/L heptanes sulfonate sodium salt solution.
25% methanol solution (1 + 3): Combine 25 mL methanol into 100-mL volumetric flask. Dilute solution to volume with water.
Standard: streptomycin (CAS: 3810-74-0), dihydrostreptomycin (CAS: 128-46-1), and kanamycin (CAS: 25389-94-0); purity ≥98%.
1.0 mg/mL standard stock solutions: Accurately weigh an adequate amount of streptomycin, dihydrostreptomycin, and kanamycin standard, dissolve in 0.3% acetic acid–water to prepare a solution of 1.0 mg/mL in concentration as stock standard solutions. Stock standard solutions are stored at −18°C away from exposure to any light.
0.1 μg/mL Working standard mix solutions in sample matrix: According to the requirement, pipette adequate amount of streptomycin, dihydrostreptomycin, and kanamycin standard stock standard solution, and dilute with 0.3% acetic acid–water to prepare working standard mix solutions. Stock standard solutions are stored at −18°C away from exposure to any light.
Oasis HLB SPE (500 mg, 3 mL) or equivalent: The SPEs are conditioned using 5 mL methanol, 10 mL water before use; keep wet.
Carboxylic acid SPE (500 mg, 3 mL) or equivalent: The SPEs are conditioned by 5 mL methanol, 10 mL water before use; keep wet.
Filter: 0.2 μm.

3.4.4 APPARATUS

LC-MS-MS: Equipped with ESI source
Analytical balance: Capable of weighing to 0.1 mg, 0.01 g.
Homogenizer.
Solid phase extraction vacuum apparatus.
Sharker.
Vacuum pump: Vacuum to 80 kPa.
Microsyringes: 25 μL, 100 μL.
pH Meter: capable of measuring ±0.02 unit.
Centrifuge tubes with stopper: 100 mL.
Sample calibration tube: 5 mL.
Reservoir: 50 mL.
Centrifugal machine: rotate speed >4000 rpm.

3.4.5 PREPARATION AND STORAGE OF TEST SAMPLE

(1) Preparation of test sample

Take representative portions from the whole primary sample. Grind about 1 kg in a blender. Keep the prepared sample in two sample bottles, sealed and labeled. In the course of sample preparation, precautions must be taken to avoid contamination or any factors that may cause a change in residue content.

The test samples should be stored at −18°C.

(2) Mixed Matrix Experiment Sample Solution

Extraction and first purification of milk sample

Weigh 8 g milk sample (accurate to 0.01 g) into 100-mL centrifuge tube. Add 30 mL 5% phosphoric acid solution and add 3 mL trichloroacetic acid solution; stir mix and centrifuge at 4000 rpm for 10 min. Supernatant is decanted into preconditioned aromatic sulfonic acid SPE cartridge with reservoir, and the flow rate is adjusted to $\leq$2 mL/min. Let full supernatant pass through aromatic sulfonic acid SPE cartridge. Wait until solution has thoroughly drained and then rinse aromatic sulfonic acid SPE cartridge followed by 10 mL 5% phosphoric acid solution and 10 mL water. Discard all the effluents. Then with 20 mL di-potassium hydrogen phosphate solution elute aromatic sulfonic acid SPE cartridge into 50-mL centrifuge tube.

Extraction and first purification of milk powder sample

Weigh 1 g milk powder sample (accurate to 0.01 g) into 100-mL centrifuge tube and add 8 mL water; add 30 mL 5% phosphoric acid solution and add 3 mL trichloroacetic acid solution; stir mix and centrifuge at 4000 rpm for 10 min. Supernatant is decanted into preconditioned aromatic sulfonic acid SPE cartridge with reservoir, and the flow rate is adjusted to $\leq$2 mL/min. Let full supernatant pass through aromatic sulfonic acid SPE cartridge. Wait until solution has thoroughly drained and then rinse aromatic sulfonic acid SPE cartridge followed by 10 mL 5% phosphoric acid solution and 10 mL water. Discard all the effluents. Then with 20 mL di-potassium hydrogen phosphate solution elute aromatic sulfonic acid SPE cartridge into 50-mL centrifuge tube.

Sample solution further purification

The preceding elute solution is decanted into a preconditioned carboxylic acid SPE cartridge with reservoir, and the flow rate is adjusted to $\leq$2 mL/min. Let solution pass through the carboxylic acid SPE cartridge. Wait until the solution has thoroughly drained and then rinse the carboxylic acid SPE cartridge followed with 10 mL water and 10 mL 25% methanol solution. Discard all the effluents. Dry carboxylic acid SPE cartridge by drawing air through it for 10 min under 65 kPa vacuum. Finally, elute with 2 mL SPE elute solution into 5-mL scale centrifuge tube. Filter with 0.2 μm syringe filter and it is ready for determination with LC-MS-MS.

(3) Mixed Matrix Calibration Standard Solution

Mixed matrix calibration standard solution of milk

Weigh 5 × 10 g negative milk samples (accurate to 0.01 g) into 100-mL centrifuge tubes. Add standard solutions of the four metabolites of nitrofuran to form 5.0 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, and 100 ng/mL solutions of the four metabolites of nitrofuran. Add internal standard as 2 ng/mL of the four metabolites of nitrofuran, and then the subsequent procedures were identical to the extraction and clean-up from milk sample.

Mixed matrix calibration standard solution of milk powder

Weigh 5 × 1 g negative milk powder samples (accurate to 0.01 g) and add 8 mL water into 100-mL centrifuge tubes. Add standard solutions of the four metabolites of nitrofuran to form 40 ng/mL, 80 ng/mL, 160 ng/mL, 800 ng/mL, and 1600 ng/mL solutions of the four metabolites of nitrofuran. Add internal standard as 2 ng/mL of the four metabolites of nitrofuran, and then the subsequent procedures were identical to the extraction and clean-up from milk powder sample.

3.4.6 DETERMINATION

(1) Operating conditions

LC column: Atlantis-C_{18}, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent;
Column temperature: 40°C;
Injection volumes: 30 μL.
Mobile phase: mobile phase A is 0.1% formic acid solution, mobile phase B is acetonitrile + 0.1% formic acid, mobile phase C is methanol, as seen in Table 3.2.
Ion source: ESI source;
Scan mode: Positive scan;
Monitor mode: Multiple reaction monitor;

TABLE 3.2 Mobile Phase and Flow Rate

Time (min)	Flow Rate (μL/min)	Mobile Phase A (%)	Mobile Phase B (%)	Mobile phase C (%)
0	200	85	10	5
3.01	200	60	35	5
6.00	200	60	35	5
6.01	200	85	10	5
16.00	200	85	10	5

Ion spray voltage: 5000 V;
Turbo ion spray gas rate: 7 L/min;
Source temperature: 550°C;
Focusing Potential: 150 V;
LC-MS-MS parameters of streptomycin, dihydrostreptomycin, and kanamycin: see Table 3.3;

(2) Identification

Under the same experimental conditions, if the ratio between the retention times of analytes and internal standards, called relative retention times, of the sample chromatogram peaks are consistent with the standards and the differences are within ±2.5%; and if after background compensation all the diagnostic ions are present, and the relative ion intensities correspond to those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 3.4, then the corresponding analyte must be present in the sample.

(3) Quantification

Internal standard method: Use the software of the instrument. External standard method: Inject the different concentrations of mixed matrix

TABLE 3.3 LC-MS-MS Parameters of Streptomycin, Dihydrostreptomycin, and Kanamycin

Name	Qualitative Ions Pairs (m/z)	Quantitative Ions Pairs (m/z)	Declustering Potential (V)	Collection Time (ms)	Collision Energy (V)
Streptomycin	582/263 582/246	582/263	110	100	45 55
Dihydrostreptomycin	584/263 584/246	584/263	100	100	43 55
Kanamycin	485/163 485/324	485/163	50	100	34 23

TABLE 3.4 Maximum Permitted Tolerances for Relative Ion Intensities

Relative ion intensities	$K > 50\%$	$20\% < K < 50\%$	$10\% < K < 20\%$	$K \leq 10\%$
Maximum permitted tolerances	±20%	±25%	±30%	±50%

calibration standard solution of streptomycin, dihydrostreptomycin, and kanamycin, respectively, in duplication under LC and MS conditions. Draw the standard curves for streptomycin, dihydrostreptomycin, and kanamycin (concentration vs. peak area). Calculate the concentrations of the corresponding content from the working curve. The responses of streptomycin, dihydrostreptomycin, and kanamycin in the sample solutions should be in the linear range of the instrumental detection.

Total ion chromatograms of the analyte standard and internal standards are shown in Fig. 3.2.

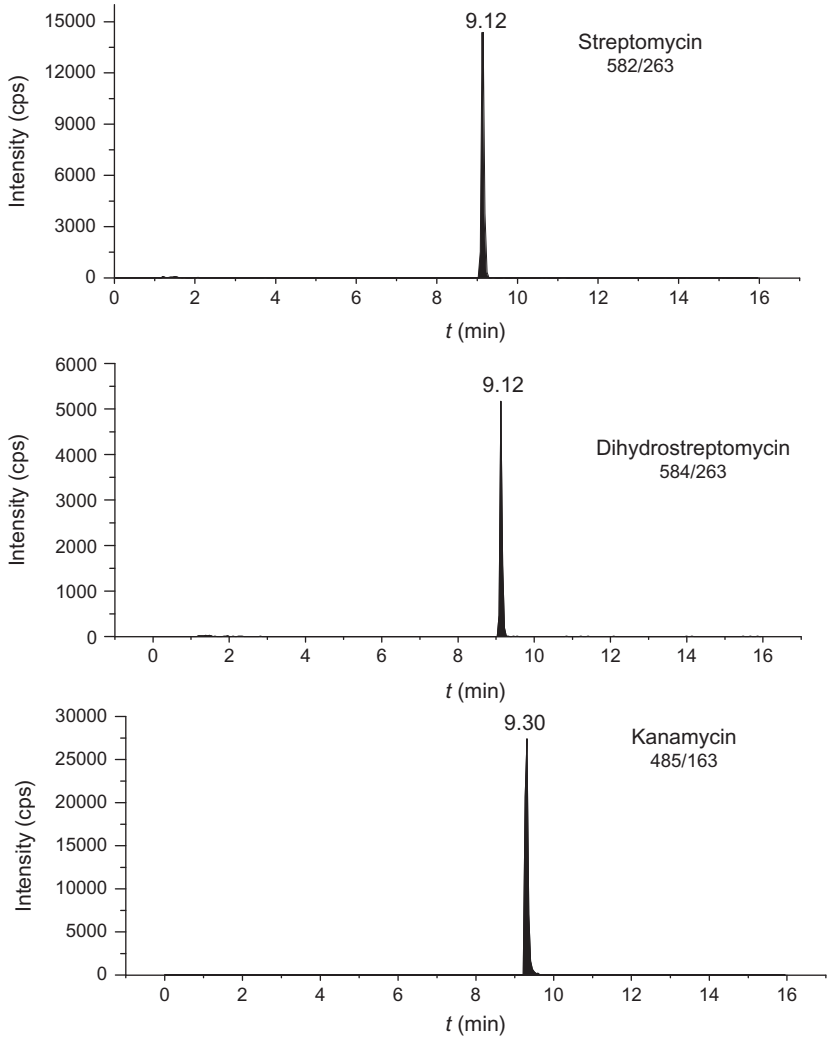

FIG. 3.2 MRM chromatogram of the analyte standard and internal standards.

3.4.7 PRECISION

The precision data of this part are confirmed according to GB/T 6379.1 and GB/T6379.2. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r), and repeatability and content range of this method are listed in Table 3.5.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R). The content range and reproducibility equations for streptomycin, dihydrostreptomycin, and kanamycin are shown in Table 3.5.

3.4.8 RECOVERY

Under optimized condition, the recoveries of streptomycin, dihydrostreptomycin, and kanamycin in milk and milk powder using this method are listed in Table 3.6.

TABLE 3.5 Content Range and Limits of Repeatability and Reproducibility

Name	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Streptomycin	5.0–200.0	lg $r = 0.8448$ lg $m - 0.8428$	lg $R = 0.9102$ lg $m - 0.8145$
Dihydrostreptomycin	5.0–200.0	lg $r = 0.8990$ lg $m - 0.9508$	lg $R = 0.9449$ lg $m - 0.9205$
Kanamycin	5.0–200.0	lg $r = 0.8896$ lg $m - 0.9208$	lg $R = 0.7263$ lg $m - 0.6916$

Note: m *equals to the average of two results.*

TABLE 3.6 Test Data of Fortification Concentration and Average Recovery for Streptomycin, Dihydrostreptomycin, and Kanamycin ($n = 11$)

Fortifying Concentration (μg/kg)	Streptomycin	Dihydrostreptomycin	Kanamycin
The recovery of streptomycin, dihydrostreptomycin, and kanamycin in milk (%)			
5.0	97.31	96.35	95.15
10.0	95.88	93.75	98.66
20.0	96.36	94.86	95.91
50.0	96.96	94.80	95.56
The recovery of streptomycin, dihydrostreptomycin, and kanamycin in milk powder (%)			
40.0	91.78	93.17	89.32
80.0	92.58	94.19	91.57
160.0	92.79	92.78	93.45
400.0	90.43	93.61	89.13

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3.5

Determination of Streptomycin, Dihydrostreptomycin, and Kanamycin Residues in Fugu and Eel—LC-MS-MS Method (GB/T 22954-2008)

3.5.1 SCOPE

This method is applicable to the determination of streptomycin, dihydrostreptomycin, and kanamycin residues in fugu and eel.

The limit of determination of this method for streptomycin, dihydrostreptomycin, and kanamycin is 10.0 μg/kg in fugu and eel.

3.5.2 PRINCIPLE

Streptomycin, dihydrostreptomycin, and kanamycin residues are extracted with phosphoric acid solutions, and the proteins were precipitated with trichloroacetic acid. The solution obtained is cleaned up with aromatic sulfonic acid and carboxylic acid SPE cartridges. Residues are determined by LC-MS-MS.

3.5.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be guaranteed pure; "water" is deionized water.

Water: GB/T 6682, the first degree.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Formic acid.
Phosphoric acid.
Di-potassium hydrogen phosphate, K_2HPO_4.
Trichloroacetic acid, $C_2HCl_3O_2$.
Heptanes sulfonate sodium salt $C_7H_{15}NaO_3S{\cdot}H_2O$
5% phosphoric acid solution (1 + 19): Dilute 50 mL concentrated phosphoric acid into 1000-mL volumetric flask. Dilute solution to volume with water.
0.2 mol/L Phosphate salt buffer solution: pH = 8.5. Dissolve 34.8 g di-potassium hydrogen phosphate into 1000-mL volumetric flask. Dilute solution to volume with water. Then adjust to pH = 8.5 with sodium hydroxide solution.
Trichloroacetic acid solution: 50%. Dissolve 20 g trichloroacetic acid into 20-mL volumetric flask. Dilute solution to volume with water.
0.01 mol/L heptanes sulfonate sodium salt solution: Place 2.20 g heptanes sulfonate sodium salt into 1000-mL volumetric flask and dissolve in water. Dilute solution to volume with water.
SPE elute solution: Combine 4 mL formic acid into 100-mL volumetric flask. Dilute solution into 100-mL volumetric flask with 0.01 mol/L heptanes sulfonate sodium salt solution.
25% methanol solution (1 + 3): Combine 25 mL methanol into 100-mL volumetric flask. Dilute solution to volume with water.
Standard: streptomycin (CAS: 3810-74-0), dihydrostreptomycin (CAS: 128-46-1), and kanamycin (CAS: 25389-94-0); purity $\geq$98%.
1.0 mg/mL standard stock solutions: Accurately weigh an adequate amount of streptomycin, dihydrostreptomycin, and kanamycin standard, dissolve in 0.3% acetic acid–water to prepare a solution of 1.0 mg/mL in concentration as stock standard solutions. Stock standard solutions are stored at −18°C.
0.1 μg/mL working standard mix solutions in sample matrix: According to the requirement, pipette adequate amount of streptomycin, dihydrostreptomycin, and kanamycin standard stock standard solution, and dilute with

0.3% acetic acid–water to prepare working standard mix solutions. Stock standard solutions are stored at −18°C away from exposure to any light.
Oasis HLB SPE (500 mg, 3 mL) or equivalent: The SPE are conditioned by 5 mL methanol, 10 mL water, before use; keep wet.
Carboxylic Acid SPE (500 mg, 3 mL) or equivalent: The SPE are conditioned by 5 mL methanol, 10 mL water before use; keep wet.
Filter: 0.2 μm.

3.5.4 APPARATUS

LC-MS-MS: Equipped with ESI source.
Analytical balance: Capable of weighing to 0.1 mg, 0.01 g.
Sharker.
Solid phase extraction vacuum apparatus.
Homogenizer.
Vacuum pump: Vacuum to 80 kPa.
Microsyringes: 25 μL, 100 μL.
pH meter: capable of measuring ±0.02 unit.
Centrifuge tubes with stopper: 100 mL.
Sample calibration tube: 5 mL.
Reservoir: 50 mL.
Centrifugal machine: rotate speed >4000 rpm.

3.5.5 SAMPLE PRETREATMENT

(1) Preparation of Test Sample

Take representative portion from the whole primary sample, about 1 kg, and grind in a blender. Keep the prepared sample in two sample bottles, sealed and labeled. In the course of sample preparation, precautions must be taken to avoid contamination or any factors that may cause a change of residue content.

The test samples should be stored at −18°C.

(2) Extraction and first purification of fugu and eel sample

Weigh 10 g fugu or eel samples (accurate to 0.01 g) into 100-mL centrifuge tubes. Add 30 mL 5% phosphoric acid solution and homogenize for 3 min, then rinse the homogenizer with additional phosphoric acid solutions and incorporate into preceding solution. Add 3 mL trichloroacetic acid solution (4.11); stir mix and centrifuge at 4000 rpm for 10 min. Supernatant is decanted into preconditioned aromatic sulfonic acid SPE cartridge with reservoir, and the flow rate is adjusted to ≤2 mL/min. Let full supernatant pass through aromatic sulfonic acid SPE cartridges. Wait until solution has thoroughly drained, then rinse SPE cartridge with 10 mL 5% phosphoric acid solution and 10 mL water. Discard all the effluents. Then elute aromatic sulfonic acid SPE cartridge with 20 mL di-potassium hydrogen phosphate solution into 50-mL centrifuge tube.

(3) Sample solution further purification

The elute solution just described is decanted into a preconditioned carboxylic acid SPE cartridge with reservoir, and the flow rate is adjusted to $\leq$2 mL/min. Let solution pass through carboxylic acid SPE cartridge. Wait till solution has thoroughly drained and then rinse carboxylic acid SPE cartridge with 10 mL water and 10 mL 25% methanol solution. Discard all the effluents. Dry carboxylic acid SPE cartridge by drawing air through for 10 min under 65 kPa vacuum. Finally, elute with 2 mL SPE elute solution into 5-mL scale centrifuge tube. Filter with 0.2 μm syringe filter and proceed to determination with LC-MS-MS.

(4) Mixed matrix calibration standard solution

Weigh 5 × 10 g negative fugu or eel samples (accurate to 0.01 g) into 100-mL centrifuge tubes. Add standard solutions of the four metabolites of nitrofuran to form 5.0 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, and 100 ng/mL solutions of the four metabolites of nitrofuran. Add internal standard as 2 ng/mL of the four metabolites of nitrofuran. And then the subsequent procedures were identical to the above-mentioned extraction and cleanup steps to prepare the mixed matrix calibration standard solution.

3.5.6 DETERMINATION

(1) Operating conditions:

LC column: Atlantis-C_{18}, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent;
Column temperature: 40°C;
Injection volumes: 30 μL.
Mobile phase: mobile phase A is 0.1% formic acid solution, mobile phase B is acetonitrile + 0.1% formic acid, mobile phase C is methanol, as seen in Table 3.2:
Ion source: ESI source;
Scan mode: Positive scan;
Monitor mode: Multiple reaction monitor;
Ion spray voltage: 5000 V;
Turbo ion spray gas rate: 7 L/min;
Source temperature: 550°C;
Focusing Potential: 150 V;
LC-MS-MS parameters of streptomycin, dihydrostreptomycin, and kanamycin: see Table 3.3.

(2) Identification

Under the same experimental conditions, if the ratio between the retention times of analytes and internal standards, called relative retention times, of the sample chromatogram peaks are consistent with the standards and the differences are within ±2.5%; and if after background compensation, all the diagnostic ions are present, the relative ion intensities correspond to

those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 3.4, then the corresponding analyte must be present in the sample.

(3) Quantification

Internal standard method: Use the software of the instrument. External standard method: Inject the different concentrations of mixed matrix calibration standard solution of streptomycin, dihydrostreptomycin, and kanamycin, respectively, in duplication under LC and MS conditions. Draw the standard curves of streptomycin, dihydrostreptomycin, and kanamycin (concentration vs. peak area). Calculate the concentrations of the corresponding content from the working curve. The responses of streptomycin, dihydrostreptomycin, and kanamycin in the sample solutions should be in the linear range of the instrumental detection.

3.5.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379.1 and GB/T6379.2 The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r). Repeatability and content range of this method are shown in Table 3.7.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

TABLE 3.7 Content Range and Limits of Repeatability and Reproducibility

Name	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Streptomycin	5.0–200.0	$\lg r = 1.1141$ $\lg m - 1.0366$	$\lg R = 0.9080$ $\lg m - 0.8333$
Dihydrostreptomycin	5.0–200.0	$\lg r = 1.2478$ $\lg m - 1.0861$	$\lg R = 1.0584$ $\lg m - 0.9876$
Kanamycin	5.0–200.0	$\lg r = 0.8670$ $\lg m - 0.8214$	$\lg R = 0.7085$ $\lg m - 0.6047$

Note: m *equals to the average of two results.*

TABLE 3.8 The Test Data of Fortification Concentration and Average Recovery for Streptomycin, Dihydrostreptomycin, and Kanamycin in Fugu and Eel ($n = 11$)

Fortifying Concentration (μg/kg)	Streptomycin	Dihydrostreptomycin	Kanamycin
The recovery of streptomycin, dihydrostreptomycin, and kanamycin in fugu (%)			
5.0	90.48	98.17	93.08
10.0	91.21	93.97	92.69
20.0	95.67	94.63	96.50
50.0	94.35	96.12	94.35
The recovery of streptomycin, dihydrostreptomycin, and kanamycin in eel (%)			
5.0	89.61	91.39	90.45
10.0	91.27	93.71	92.45
20.0	90.45	90.45	90.15
50.0	92.48	89.46	90.46

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R). The content range and reproducibility equations for streptomycin, dihydrostreptomycin, and kanamycin are shown in Table 3.7.

3.5.8 RECOVERY

Under optimized condition, the recoveries of streptomycin, dihydrostreptomycin, and kanamycin in fugu and eel using this method are listed in Table 3.8.

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3.6

Determination of Streptomycin, Dihydrostreptomycin, and Kanamycin Residues in Honey—LC-MS-MS Method (GB/T 22995-2008)

3.6.1 SCOPE

This method is applicable to the determination of streptomycin, dihydrostreptomycin, and kanamycin residues in honey.

The limit of determination of this method for streptomycin, dihydrostreptomycin, and kanamycin is 5.0 μg/kg in honey.

3.6.2 PRINCIPLE

Streptomycin, dihydrostreptomycin, and kanamycin residues are extracted from honey samples with phosphate buffer solution. The solution obtained is cleaned up with Oasis HLB carboxylic acid SPE cartridges, followed by LC-MS-MS analysis.

3.6.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be guaranteed pure; "water" is deionized water.

Water: GB/T 6682, the first degree.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Formic acid.
Di-potassium hydrogen phosphate, K_2HPO_4.
heptanes sulfonate sodium salt $C_7H_{15}NaO_3S{\cdot}H_2O$.
Phosphate buffer solution: 0.1mol/L pH = 8.0. Place 34.8 g di-potassium hydrogen phosphate into 1000-mL volumetric flask and dissolve in water. Dilute solution to volume with water, then adjust to pH = 8.5 with phosphoric acid.
0.01 mol/L heptanes sulfonate sodium salt solution: Place 2.20 g heptanes sulfonate sodium salt into 1000-mL volumetric flask and dissolve in water. Dilute solution to volume with water.

SPE elute solution: Combine 4 mL formic acid into 100-mL volumetric flask. Dilute solution to volume with 0.01 mol/L heptanes sulfonate sodium salt solution.
20% methanol solution (1+3): Combine 25 mL methanol into 100-mL volumetric flask. Dilute solution to volume with water.
Standard: streptomycin (CAS: 3810-74-0), dihydrostreptomycin (CAS: 128-46-1), and kanamycin (CAS: 25389-94-0); purity $\geq$98%.
1.0 mg/mL standard stock solutions: Accurately weigh an adequate amount of streptomycin, dihydrostreptomycin, and kanamycin standard, dissolve in 0.3% acetic acid–water to prepare a solution of 1.0 mg/mL in concentration as stock standard solutions. Stock standard solutions are stored at -18°C.
0.1 μg/mL working standard mix solutions in sample matrix: According to the requirement, pipette adequate amount of streptomycin, dihydrostreptomycin, and kanamycin standard stock standard solution, and dilute with 0.3% acetic acid–water to prepare working standard mix solutions. Stock standard solutions are stored at -18°C.
Oasis HLB SPE (60 mg, 3 mL) or equivalent: The SPEs are conditioned by 5 mL methanol, 10 mL water, before use; keep wet.
Carboxylic acid SPE (500 mg, 3 mL) or equivalent: The SPEs are conditioned by 5 mL methanol, 10 mL water before use; keep wet.
Filter: 0.2 μm.

3.6.4 APPARATUS

LC-MS-MS: Equipped with ESI source
Analytical balance: Capable of weighing to 0.1 mg, 0.01 g
Sharker.
Solid phase extraction vacuum apparatus.
Homogenizer.
Vacuum pump: Vacuum to 80 kPa.
Microsyringes: 25 μL, 100 μL.
pH meter: capable of measuring $\pm$0.02 unit.
Erlenmeyer flask: 200 mL.
Reservoir: 50 mL.

3.6.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath, warm at $\leq$60 C with occasional shaking until liquefied, mix thoroughly, and promptly cool to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label.

The test samples should be stored at ambient temperature.

(2) Extraction

Weigh 10 g honey sample (accurate to 0.01 g) into a 200-mL conical flask, add 20 mL 0.2 mol/L di-potassium hydrogen phosphate buffer solution, and mix for 5 min, until sample is completely dissolved.

(3) Purification

The previously described sample solution is decanted into preconditioned Oasis HLB solid extraction columns with reservoir. The underside of these Oasis HLB solid extraction columns connect carboxylic acid SPE cartridges with an adapter and the flow rate is adjusted to $\leq$2 mL/min. Let the solution successively pass through the Oasis HLB solid extraction columns and the carboxylic acid SPE cartridges. Wait till the solution has thoroughly drained and then rinse the Oasis HLB solid extraction columns and carboxylic acid SPE cartridges with 10 mL water and 10 mL 25% methanol solution. Discard all the effluents and the Oasis HLB solid extraction columns. Dry the darboxylic acid SPE cartridges by drawing air through for 10 minutes under a 65 kPa vacuum. Finally, elute with 2 mL SPE elute solution into a 5-mL scale centrifuge tube. Filter with a 0.2-μm syringe filter and proceed to determination using LC-MS-MS.

(4) Mixed matrix calibration standard solution

Weigh 10×10 g negative honey samples (accurate to 0.01 g) into 100-mL centrifuge tubes. Add standard solutions of the four metabolites of nitrofuran to form 2.5 ng/mL, 5.0 ng/mL, 10 ng/mL, 25 ng/mL, and 50 ng/mL solutions of the four metabolites of nitrofuran. Add an internal standard as 2 ng/mL of the four metabolites of nitrofuran, and then the subsequent procedures were identical to the above-mentioned extraction and cleanup steps to prepare the mixed matrix calibration standard solution.

3.6.6 DETERMINATION

(1) Operating conditions

LC column: Atlantis-C_{18}, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent;
Column temperature: 40°C;
Injection volumes: 30 μL.
Mobile phase: mobile phase A is 0.1% formic acid solution, mobile phase B is acetonitrile + 0.1% formic acid, mobile phase C is methanol; see Table 3.2.
Ion source: ESI source;
Scan mode: Positive scan;
Monitor mode: Multiple reaction monitor;
Ion spray voltage: 5000 V;
Turbo ion spray gas rate: 7 L/min;

Source temperature: 550°C;
Focusing potential: 150 V;
LC-MS-MS parameters of streptomycin, dihydrostreptomycin, and kanamycin: see Table 3.3.

(2) Identification

Under the same experimental conditions, if the ratios between the retention times of analytes and internal standards, called relative retention times, of the sample chromatogram peaks are consistent with the standards and the differences are within ±2.5%; and if after background compensation, all the diagnostic ions are present, and the relative ion intensities correspond to those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 3.4, then the corresponding analyte must be present in the sample.

(3) Quantification

Internal standard method: Use the software of the instrument. External standard method: Inject the different concentrations mixed matrix calibration standard solutions of streptomycin, dihydrostreptomycin, and kanamycin, respectively, in duplication under LC and MS conditions. Draw the standard curves of streptomycin, dihydrostreptomycin, and kanamycin (concentration vs. peak area). Calculate the concentrations of the corresponding content from the working curve. The responses of the streptomycin, dihydrostreptomycin, and kanamycin in the sample solutions should be in the linear range of the instrumental detection.

3.6.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379.1 and GB/T6379.2 The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r). Repeatability and content range of this method are shown in Table 3.9.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R). The content range and reproducibility equations for streptomycin, dihydrostreptomycin, and kanamycin are shown in Table 3.9.

TABLE 3.9 Content Range and Limits of Repeatability and Reproducibility

Name	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Streptomycin	2.5–100.0	lg $r = 1.3130$ lg $m - 1.0831$	lg $R = 1.1699$ lg $m - 1.0069$
Dihydrostreptomycin	2.5–100.0	lg $r = 1.0309$ lg $m - 0.9123$	lg $R = 1.1031$ lg $m - 1.0132$
Kanamycin	2.5–100.0	lg $r = 0.9354$ lg $m - 0.9475$	lg $R = 1.0934$ lg $m - 1.0202$

Note: m *equals the average of two results.*

TABLE 3.10 The Test Data of Fortification Concentration and Average Recovery for Streptomycin, Dihydrostreptomycin, and Kanamycin in Honey ($n = 11$)

Fortifying Concentration (μg/kg)	The Recovery of Streptomycin, Dihydrostreptomycin, and Kanamycin in Honey (%)		
	Streptomycin	Dihydrostreptomycin	Kanamycin
2.5	95.56	97.54	95.11
5.0	96.38	92.22	96.67
10.0	94.12	91.35	94.46
25.0	96.10	94.35	96.00

3.6.8 RECOVERY

Under optimized condition, the recoveries of streptomycin, dihydrostreptomycin, and kanamycin in honey using this method are listed in Table 3.10.

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3.7

Determination of Streptomycin, Dihydrostreptomycin, and Kanamycin Residues in Royal Jelly—LC-MS-MS Method (GB/T 22945-2008)

3.7.1 SCOPE

This method is applicable to the determination of streptomycin, dihydrostreptomycin, and kanamycin residues in royal jelly.

The limit of determination of this method for streptomycin, dihydrostreptomycin, and kanamycin is 10.0 μg/kg in royal jelly.

3.7.2 PRINCIPLE

Streptomycin, dihydrostreptomycin, and kanamycin residues are extracted with phosphoric acid solutions, and the proteins were precipitated with trichloroacetic acid. The solution obtained is cleaned up with aromatic sulfonic acid and carboxylic acid SPE cartridges. Residues are determined by LC-MS-MS.

3.7.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be guaranteed pure; "water" is deionized water.

Water: GB/T 6682, the first degree.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Formic acid.
Phosphoric acid.
Di-potassium hydrogen phosphate, K_2HPO_4.
Trichloroacetic acid, $C_2HCl_3O_2$.
Heptanes sulfonate sodium salt $C_7H_{15}NaO_3S{\cdot}H_2O$
5% phosphoric acid solution (1 + 19): Dilute 50 mL concentrated phosphoric acid into 1000-mL volumetric flask. Dilute solution to volume with water.
0.2 mol/L Phosphate salt buffer solution: pH = 8.5. Dissolve 34.8 g di-potassium hydrogen phosphate into 1000-mL volumetric flask. Dilute solution to volume with water. Then adjust to pH = 8.5 with sodium hydroxide solution.

Trichloroacetic acid solution: 50%. Dissolve 20 g trichloroacetic acid into 20-mL volumetric flask. Dilute solution to volume with water.
0.01 mol/L heptanes sulfonate sodium salt solution: Place 2.20 g heptanes sulfonate sodium salt into 1000-mL volumetric flask and dissolve in water. Dilute solution to volume with water.
SPE elute solution: Combine 4 mL formic acid into 100-mL volumetric flask. Dilute solution in 100-mL volumetric flask with 0.01 mol/L heptanes sulfonate sodium salt solution.
25% methanol solution (1+3): Combine 25 mL methanol into 100-mL volumetric flask. Dilute solution to volume with water.
Standard: streptomycin (CAS: 3810-74-0), dihydrostreptomycin (CAS: 128-46-1), and kanamycin (CAS: 25389-94-0); purity $\geq$98%.
1.0 mg/mL standard stock solutions: Accurately weigh an adequate amount of streptomycin, dihydrostreptomycin, and kanamycin standard, dissolve in 0.3% acetic acid–water to prepare a solution of 1.0 mg/mL in concentration as stock standard solutions. Stock standard solutions are stored at -18°C.
0.1 μg/mL working standard mix solutions in sample matrix: According to the requirement, pipette adequate amount of streptomycin, dihydrostreptomycin, and kanamycin standard stock standard solution, and dilute with 0.3% acetic acid–water to prepare working standard mix solutions. Stock standard solutions are stored at -18°C away from exposure to any light.
Aromatic sulfonic acid SPE (500 mg, 3 mL) or equivalent: The SPE is conditioned by 5 mL methanol, 10 mL water, before use; keep wet.
Carboxylic acid SPE (500 mg, 3 mL) or equivalent: The SPE is conditioned by 5 mL methanol, 10 mL water before use; keep wet.
Filter: 0.2 μm.

3.7.4 APPARATUS

LC-MS-MS: Equipped with ESI source
Analytical balance: Capable of weighing to 0.1 mg, 0.01 g
Sharker.
Solid phase extraction vacuum apparatus.
Homogenizer.
Vacuum pump: Vacuum to 80 kPa.
Microsyringes: 25 μL, 100 μL.
pH meter: capable of measuring $\pm$0.02 unit.
Centrifuge tubes with stopper: 100 mL.
Sample calibration tube: 5 mL
Reservoir: 50 mL.
Centrifugal machine: rotate speed >4000 rpm.

3.7.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Take representative portions from the whole primary sample of about 1 kg and grind in a blender. Keep the prepared sample in two sample bottles, sealed and labeled. In the course of sample preparation, precautions must be taken to avoid contamination or any factors that may cause a change of residue content.

The test samples should be stored at −18°C.

(2) Extraction and first purification of milk sample

Weigh 10 g of the royal jelly sample (accurate to 0.01 g) into a 100-mL centrifuge tube. Add 30 mL 5% phosphoric acid solution and homogenize for 3 min, then rinse the homogenizer with additional phosphoric acid solution and incorporate into the previous solution. Add 3 mL trichloroacetic acid solution; revolve mix and centrifuge at 4000 rpm for 10 min. Supernatant is decanted into a preconditioned aromatic sulfonic acid SPE cartridge with reservoir, and the flow rate is adjusted to $\leq$2 mL/min. Let full supernatant pass through the aromatic sulfonic acid SPE cartridge. Wait till solution has thoroughly drained, then rinse aromatic sulfonic acid SPE cartridges followed by 10 mL 5% phosphoric acid solutions and 10 mL water. Discard all the effluents. Then with 20 mL di-potassium hydrogen phosphate solution, elute aromatic sulfonic acid SPE cartridges into 50-mL centrifuge tube.

(3) Sample solution further purification

The previous elute solution is decanted into a preconditioned carboxylic acid SPE cartridge with reservoir, and the flow rate is adjusted to $\leq$2 mL/min. Let solution pass through carboxylic acid SPE cartridge. Wait till solution has thoroughly drained and then rinse carboxylic acid SPE cartridge followed by 10 mL water and 10 mL 25% methanol solution. Discard all the effluents. Dry the carboxylic acid SPE cartridge by drawing air through it for 10 min under 65 kPa vacuum (5.7). Finally, elute with 2 mL SPE elute solution into 5-mL scale centrifuge tube. Filter with 0.2-μm syringe filter and proceed to determination with LC-MS-MS.

(4) Mixed matrix calibration standard solution

Weigh 5 × 10 g negative royal jelly sample (accurate to 0.01 g) into a 100-mL centrifuge tube. Add standard solutions of the four metabolites of nitrofuran to form 5.0, 10, 20, 50, and 100 ng/mL solutions of the four metabolites of nitrofuran. Add internal standard as 2 ng/mL of the four metabolites of nitrofuran, and then the subsequent procedures were identical to the above-mentioned extraction and cleanup steps to prepare the mixed matrix calibration standard solution.

3.7.6 DETERMINATION

(1) Operating conditions

LC column: Atlantis-C_{18}, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent;
Column temperature: 40°C;

Injection volumes: 30 μL.
Mobile phase: mobile phase A is 0.1% formic acid solution, mobile phase B is acetonitrile + 0.1% formic acid, mobile phase C is methanol, as seen in Table 3.2.
Ion source: ESI source;
Scan mode: Positive scan;
Monitor mode: Multiple reaction monitor;
Ion spray voltage: 5000 V;
Turbo ion spray gas rate: 7 L/min;
Source temperature: 550°C;
Focusing potential: 150 V;
LC-MS-MS parameters of streptomycin, dihydrostreptomycin, and kanamycin: see Table 3.3.

(2) Identification

Under the same experimental conditions, if the ratios between the retention times of analytes and internal standards, called relative retention times, of the sample chromatogram peaks are consistent with the standards and the differences are within ±2.5%; and if after background conpensation, all the diagnostic ions are present, and the relative ion intensities correspond to those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 3.4, then the corresponding analyte must be present in the sample.

(3) Quantification

Internal standard method: Use the software of the instrument. External standard method: Inject the different concentrations mixed matrix calibration standard solutions of streptomycin, dihydrostreptomycin, and kanamycin, respectively, in duplication under LC and MS conditions. Draw the standard curves of streptomycin, dihydrostreptomycin, and kanamycin (concentration vs. peak area). Calculate the concentrations of the corresponding content from the working curve. The responses of streptomycin, dihydrostreptomycin, and kanamycin in the sample solutions should be in the linear range of the instrumental detection.

3.7.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379.1 and GB/T6379.2 The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r), and repeatability and content range of this method are listed in Table 3.11.

TABLE 3.11 Content Range and Limits of Repeatability and Reproducibility

Name	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Streptomycin	5.0–200.0	lg $r = 1.1244$ lg $m - 1.0316$	lg $R = 1.1401$ lg $m - 1.0213$
Dihydrostreptomycin	5.0–200.0	lg $r = 1.1797$ lg $m - 1.0162$	lg $R = 1.0232$ lg $m - 0.9838$
Kanamycin	5.0–200.0	lg $r = 0.9810$ lg $m - 1.0029$	lg $R = 0.9953$ lg $m - 0.9844$

Note: m *equals to the average of two results.*

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for streptomycin, dihydrostreptomycin, and kanamycin are shown in Table 3.11.

3.7.8 RECOVERY

Under optimized condition, the recoveries of streptomycin, dihydrostreptomycin, and kanamycin in royal jelly using this method are listed in Table 3.12.

TABLE 3.12 The Test Data of Fortification Concentration and Average Recovery for Streptomycin, Dihydrostreptomycin, and Kanamycin ($n = 11$)

Fortifying Concentration (μg/kg)	The Recovery of Streptomycin, Dihydrostreptomycin, and Kanamycin in royal jelly (%)		
	Streptomycin	Dihydrostreptomycin	Kanamycin
5.0	94.40	96.66	95.80
10.0	92.74	94.74	93.55
20.0	94.81	94.49	94.73
50.0	93.40	94.47	95.44

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Chapter 4

Chloramphenicol

4.1

Curative Effect and Side Effects of Chloramphenicol

Chloramphenicol (CAP) is a broad-spectrum antibiotic, exhibiting activity against a variety of aerobic and anaerobic microorganisms. It is widely used as an antimicrobial agent, because it interferes with protein synthesis of many Gram-negative and Gram-positive bacteria.

CAP is, in certain susceptible individuals, associated with serious toxic effects in humans in the form of bone marrow depression, particularly severe in the form of fatal aplastic anemia. Since this condition is dose independent, CAP has been banned for use in food-producing animals in many countries, including the EU. As a consequence, CAP is included in Annex IV of Council Decision 2077/90, which lists the drugs with an established zero-tolerance level in edible tissues.

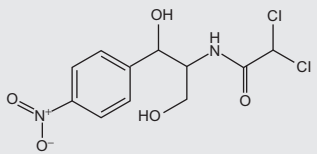
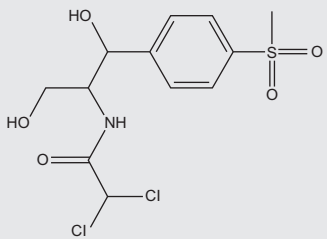
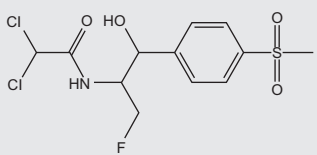
4.2

Chemical Structures and Maximum Residue Limits for Chloramphenicol, Thiamphenicol, and Florfenicol

See Table 4.1.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00004-X

TABLE 4.1 Chemical Structures and Maximum Residue Limits for Chloramphenicol, Thiamphenicol, and Florfenicol

Compounds	Structure	Molecular Weight	Cas. No.	MRL (μg/kg)	
Chloramphenicol		322.01	56-75-7	USA CN EU JP	Banned
Thiamphenicol		355.00	15318-45-3	EU: JP:	50 20
Florfenicol		357.00	73231-34-2	US China Canada Australia EU JP:	200 300 100 30

4.3

Determination of Chloramphenicol, Thiamphenicol, and Florfenicol Residues in Edible Animal Muscles, Liver, and Aquatic Products—LC-MS-MS Method (GB/T 20756-2006)

4.3.1 SCOPE

This method is applicable to the determination of chloramphenicol, thiamphenicol, and florfenicol residues in edible animal muscle, liver, and aquatic products.

The limits of quantitation of the method are 0.1 for chloramphenicol and 1.0 μg/kg for thiamphenicol and florfenicol in edible animal muscle, liver, and aquatic products.

4.3.2 PRINCIPLE

Chloramphenicol, thiamphenicol, and florfenicol residues in sample are extracted with ethyl acetate under alkali conditions. The ethyl acetate is evaporated to dryness and the residue is reconstituted in water. The water is partitioned with n-hexane to remove the fat and determined by LC-MS-MS using an internal standard.

4.3.3 REAGENTS AND MATERIALS

Methanol, ethyl acetate, n-hexane are HPLC grade;
Ammonia solution: 25%–28% (g/g);
Anhydrous sodium sulfate: Ignite at 650°C for 4 h, and store in a desiccator.
Standards: Chloramphenicol, thiamphenicol, and florfenicol, purity ≥99.5%; Chloramphenicol-D5 (100 μg/ mL), purity ≥98%.
Stock standard solutions (100 μg/mL): Into separate volumetric flasks accurately weigh an adequate amount of chloramphenicol, thiamphenicol, and florfenicol standards, and dissolve in methanol to prepare a solution of 100 μg/mL as the stock standard solutions. Solutions are stable for a year at –18°C. Mixed stock standard solution (1 μg/mL): into separate 100-mL volumetric flasks pipette 1.00 mL of chloramphenicol, thiamphenicol, and florfenicol stock solutions and dilute to volume with methanol. Solution

is stable for 6 months at −18°C. Mixed stock standard solution (20 ng/mL): into separate 50-mL volumetric flasks pipette 1.00 mL of mixed stock standard solution and dilute to volume with water to prepare a 20 ng/mL standard solution. Solution is stable for 3 months at 4°C.

Internal stock standard solution (1 μg/mL): Accurately pipette 100 μL of chloramphenicol -D_5 standard (100 μg/mL) into a 10-mL volumetric flask and dilute to volume with methanol. The concentration of solution is 1 μg/mL. The solution is stable for 6 months at −18°C. Internal stock standard solution (20 ng/mL): Accurately pipette 1.00 mL of internal stock standard solution into a 50-mL volumetric flask and dilute to volume with water. The concentration of the solution is 20 ng/mL. The solution is stable for 3 months at 4°C.

Matrix extracted mix standard working solutions: According to the sensitivity of each standard and the linear range of the instrument detector, separately pipette an adequate volume of mix stock standard solution and internal stock standard solution and dilute with matrix extracted solution to prepare appropriate standard working solutions containing the matrix of interest and the internal standard at a concentration of 0.3 ng/mL.

4.3.4 APPARATUS

Liquid chromatography—electrospray ionization tandem mass spectrometer; Balance: 0.1 mg and 0.01 g sensitivity; Centrifuge: 4000 rpm; High speed centrifuge: 13,000 rpm; Food blender; Homogenizer; Rotary vacuum evaporator; Ultrasonic bath; Vortex mixer; Polypropylene centrifuge tube; Heart-shaped flask, 25 mL; Colorimetric tube, 50 mL with stopper. Membrane filter: 0.22 μm.

4.3.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Sample, about 500 g, is thoroughly blended and homogenized in a blender, placed in clean containers as the test sample, sealed, and labeled. The test sample should be stored below –18°C.

(2) Extraction

Weigh 5 g of test sample (accurate to 0.01 g) into a 50-mL polypropylene centrifuge tube and add 75.0 μL of internal standard working solution, 15 mL of ethyl acetate, 0.45 mL of ammonia solution, and 5 g of anhydrous sodium sulfate. Homogenize for 30 s and centrifuge for 5 min at 4000 rpm. Transfer the supernatant to a 50-mL colorimetric tube. Rinse the probe with 15 mL of ethyl acetate and 0.45 mL of ammonia solution in another centrifuge tube. Pour the rinse solution into the first centrifuge tube, stir the residue with a glass rod, vortex mix for 1 min, and sonicate for 5 min. After centrifugation for 5 min at 4000 rpm, the supernatant is combined in the colorimetric tube. Add another 15 mL of ethyl acetate and 0.45 mL of

ammonia solution to the residue, vortex mix for 1 min, and centrifuge for 5 min at 4000 rpm. Combine the supernatant in the colorimetric tube and make up to the mark with ethyl acetate. Mix well and pipette 10.0 mL of ethyl acetate into a 25-mL heart-shaped flask and evaporate to dryness with rotary evaporator at 45°C.

(3) Clean-up

The residue is reconstituted in 3.00 mL of water, vortex mixed, and sonicated for 5 min. Add 3 mL of n-hexane and vortex mix for 30 s. Let stand to separate and discard the n-hexane. Add another y of n-hexane and vortex to mix. Transfer a portion of aqueous layer to a 1.5-mL polypropylene centrifuge tube and centrifuge for 5 min at 13,000 rpm. Filter through a 0.20-μm aqueous filter for LC-MS-MS determination.

4.3.6 DETERMINATION

(1) Operating conditions

Column: C_{18}: 150 mm × 2.1 mm (i.d.), 5-μm particle size; Mobile phase: Methanol-H2O (40 + 60); Flow rate: 0.3 mL/min; Column temperature: 40°C; Injection volume: 20 μL. Ion source: ESI, Scan mode: negative mode; Monitor mode: Multiple reaction monitoring; Ionspray voltage: −1750 V; Nebulizer gas, curtain gas, heater gas, nitrogen and collision gas are high purity nitrogen gases or equivalent; optimize the flow rate of each gas to reach the requirement of the sensitivity of mass spectrometry. Temp: 500°C; Qualification ions, quantification ions, declustering potential (DP), dwell time, and collision energy (CE) are listed in Table 4.2.

(2) Qualitation determination

The qualitative ions for each analyst include one precursor ion and two product ions at least. Under the same determination conditions, the ratio of the chromatographic retention time of the analyte to that of the internal standard, i.e., the relative retention time of the analyte, shall correspond to that of the calibration solution at a tolerance of ±2.5%. The relative intensities of the detected ions of each analyst shall correspond to those of the calibration standard at comparable concentrations within the tolerances shown in Table 4.3 for the analyte to be considered present in the sample.

(3) Quantitative analysis

Under optimum working conditions of LC-MS-MS, inject mixed standard working solutions and generate a linear regression curve based on the ratio of the chromatographic peak area of the analyte to that of the internal standard vs. the ratio of the concentration of the analyte to that of the internal standard. Quantify the concentration of the analyte in test sample solution from the regression curve. The responses of the analyte in the standard working solution and the sample solution should be within the linear range

TABLE 4.2 Mass Spectrometric Acquisition Parameters for Chloramphenicol, Thiamphenicol, Florfenicol, and Chloramphenicol-D5

Analytes	Qualification Ions (*m*/*z*)	Quantification Ions (*m*/*z*)	Dwell Time (ms)	CE (V)	DP (V)
Choramphenicol	320.9 → 257.0	320.9/152.0	200	−16	−55
	320.9 → 152.0			−26	−55
Thiamphenicol	354.0 → 290.0	354.0/185.0	200	−18	−55
	354.0 → 185.0			−27	−55
Florfenicol	356.0 → 336.0	356.0/336.0	200	−14	−55
	356.0 → 185.0			−27	−55
Choramphenicol-D5	326.0 → 157.0	326.0/157.0	200	−26	−55

TABLE 4.3 Maximum Permitted Tolerances for Relative Ion Intensities While Confirmation

Relative intensity (*k*)	$k > 50$	$20 < k \leq 50$	$10 < k \leq 20$	$k \leq 10$
Permitted tolerances	±20	±25	±30	±50

of the instrument detection (Fig. 4.1). Typical retention times are as follows: chloramphenicol: 4.06 min; thiamphenicol: 2.15 min; florfenicol: 2.63 min; chloramphenicol-D5: 4.00 min.

4.3.7 PRECISION

The method for the determination of precision of this standard is carried out according to GB/T6379. Repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

For two independent test results carried out under repeatability conditions, the absolute difference value would be $\leq r$ (repeatability limit). The analytical range and repeatability of the method are shown in Tables 4.4 and 4.5.

If the absolute difference value exceeds r (repeatability limit), the two independent test results would be rejected and the experiment repeated.

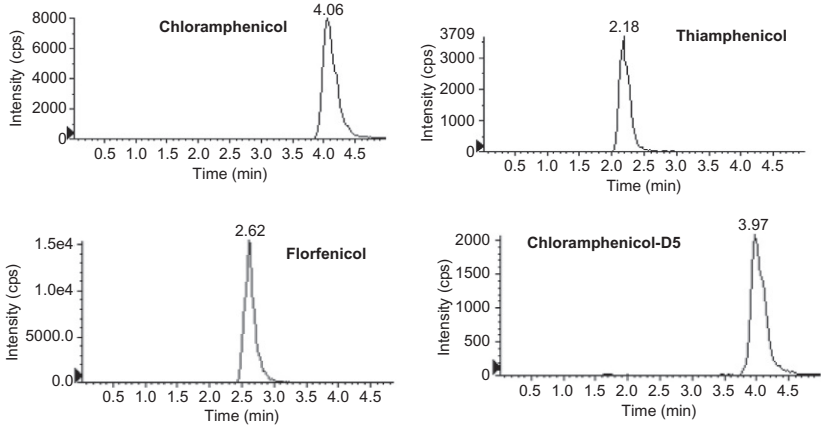


FIG. 4.1 MRM chromatogram of chloramphenicol, thiamphenicol, florfenicol, and chloramphenicol-D5.

TABLE 4.4 The Analytical Range, Repeatability, and Reproducibility Equations (Muscle)

Analytes	Fortified Levels Range	Repeatability Limit (r)	Reproducibility Limit (R)
Chloramphenicol	0.0500–1.00	$r=0.075m+0.0186$	$R=0.1142m+0.0174$
Thiamphenicol	0.500–4.00	$r=0.252m+0.103$	$R=0.264+0.102$
Florfenicol	0.500–4.00	$\lg r=0.9116\lg m-0.618$	$\lg R=0.916\lg m-0.617$

Note: m *is the average of two test results.*

TABLE 4.5 The Analytical Range, Repeatability, and Reproducibility Equations (Liver)

Analytes	Fortified Levels Range	Repeatability Limit (r)	Reproducibility Limit (R)
Chloramphenicol	0.0500–1.00	$r=0.143m+0.0034$	$R=0.196m+0.0049$
Thiamphenicol	0.500–4.00	$r=0.228m+0.187$	$R=0.292m+0.0622$
Florfenicol	0.500–4.00	$r=0.164m+0.0519$	$R=0.205m+0.036$

Note: m *is the average of two test results.*

(2) Reproducibility

Two independent test results carried out under reproducibility conditions, the absolute difference value would be $\leq R$ (reproducibility limit). The analytical range and reproducibility equations of analystrs are shown in Tables 4.4 and 4.5.

4.3.8 RECOVERY

Under optimized conditions, the recoveries of chloramphenicol, thiamphenicol, and florfenicol over the analytical range of the method are listed in Table 4.6.

TABLE 4.6 The Recoveries for Chloramphenicol, Thiamphenicol, and Florfenicol

Matrix	Analytes	Fortified Level (μg/kg)	Average Recovery (%)
Muscle	Chloramphenicol	0.100	97.8
		0.500	97.9
		1.00	113
	Thiamphenicol	1.00	101
		2.00	104
		4.00	99.8
	Florfenicol	1.00	99.0
		2.00	100
		4.00	108
Liver	Chloramphenicol	0.100	100
		0.500	98
		1.00	105
	Thiamphenicol	1.00	104
		2.00	97.1
		4.00	77.3
	Florfenicol	1.00	103
		2.00	106
		4.00	99.1
Fish	Chloramphenicol	0.100	118
		0.500	112
		1.00	108

TABLE 4.6 The Recoveries for Chloramphenicol, Thiamphenicol, and Florfenicol—cont'd

Matrix	Analytes	Fortified Level (μg/kg)	Average Recovery (%)
	Thiamphenicol	1.00	97.0
		2.00	97.3
		4.00	91.7
	Florfenicol	1.00	90.0
		2.00	92.9
		4.00	86.2
Shrimp	Chloramphenicol	0.100	113
		0.500	107
		1.00	109
	Thiamphenicol	1.00	96.4
		2.00	98.9
		4.00	96.1
	Florfenicol	1.00	93.0
		2.00	83.9
		4.00	86.4

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4.4

Determination of Chloramphenicol, Thiamphenicol, and Florfenicol Residues in Fugu, Eel, and Baked Eel—LC-MS-MS Method (GB/T 22959-2008)

4.4.1 SCOPE

This method is applicable to the determination of chloramphenicol, thiamphenicol, florfenicol, and florfenicol amine in balloonfish, eel, and roasted eel.

The limits of quantitation of the standard are 0.1 for chloramphenicol and 1.0 μg/kg for thiamphenicol, florfenicol, and florfenicol amine in fugu, eel, and roasted eel.

4.4.2 PRINCIPLE

Chloramphenicol, thiamphenicol, florfenicol, and florfenicol amine residues in sample are extracted with alkali ethyl acetate. The ethyl acetate is evaporated to dryness and the residue is reconstituted in water. The water is partitioned with n-hexane to remove the fat, analyzed by LC-MS-MS, and quantified by peak area, applying an internal standard method for chloramphenicol, thiamphenicol, and florfenicol, and applying an external standard method for florfenicol amine.

4.4.3 REAGENTS

Unless otherwise specified, all reagents used are A.R. and "water" is gradeIspecified by GB/T 6682.

Methanol: HPLC grade.
Ethyl acetate.
n-Hexane.
Ammonia solution: 25%–28% (g/g).
Anhydrous sodium sulfate: Ignite at 650°C for 4 h.
Standards: Chloramphenicol (CAS 56-75-7), thiamphenicol (CAS 15318-45-3), and florfenicol (CAS 76639-94-6), purity ≥99.5%; florfenicol amine, purity ≥98%.
Internal stock standard solution: Chloramphenicol-D5, 100 μg/mL, solvent is acetonitrile, deuterated ≥98%.

100 μg/mL stock standard solutions: Separately, accurately weigh an adequate amount of chloramphenicol, thiamphenicol, florfenicol, and florfenicol amine standards, dissolve in methanol, and prepare a solution of 100 μg/mL as the stock standard solutions. Solutions are stable for a year at −18°C.
1 μg/mL mixed stock standard solution of chloramphenicol, thiamphenicol, and florfenicol: Separately pipette 1.00 mL of chloramphenicol, thiamphenicol, and florfenicol stock standard solutions into a 100-mL volumetric flask and dilute to volume with methanol. Solutions are stable for 6 months at −18°C.
1 μg/mL stock standard solution of florfenicol amine: Pipette 1.00 mL of florfenicol amine stock standard solutions into a 100-mL volumetric flask and dilute to volume with methanol. Solution is stable for 6 months at −18°C.
20 ng/mL mixed middle standard solution of chloramphenicol, thiamphenicol, and florfenicol: Separately pipette 1.00 mL of mixed stock standard solution into a 50-mL volumetric flask and dilute to volume with water. The concentration of this solution is 20 ng/mL. Solution is stable for 3 months at 4°C.
20 ng/mL middle standard solution of florfenicol amine: Separately pipette 1.00 mL of mixed stock standard solution of florfenicol amine into a 50-mL volumetric flask and dilute to volume with water. The concentration of this solution is 20 ng/mL. Solution is stable for 3 months at 4°C.
1 μg/mL internal stock standard solution: Accurately pipette 100 μL of internal stock standard solution into a 10-mL volumetric flask and dilute to volume with methanol. Solution is stable for 6 months at −18°C.
20 ng/mL internal middle standard solution: Accurately pipette 1.00 mL of internal stock standard solution into a 50-mL volumetric flask and dilute to volume with water. The concentration of this solution is 20 ng/mL. Solution is stable for 3 months at 4°C.
Mix matrix working standard solutions of chloramphenicol, thiamphenicol, and florfenicol: According to the sensitivity of each standard and the linear range of the instrument detector, separately pipette an adequate volume of mixed middle standard solution and internal middle standard solution and dilute with water to prepare the proper concentration standard working solution, containing 0.15 ng/mL chloramphenicol-D5.
Matrix working standard solutions of florfenicol amine: According to the sensitivity of each standard and the linear range of the instrument detector, separately pipette an adequate volume of middle standard solution, dilute with water to prepare proper concentration standard working solution.
Membrane filter: 0.2 μm, aqueous filter.

4.4.4 APPARATUS

Liquid chromatography: electrospray ionization tandem mass spectrometry.
Electronic balance: sensitivity is 0.1 mg, 0.01 g.

Centrifuge: ≥4000 rpm.
High speed centrifuge: ≥13,000 rpm.
Food blender.
Homogenizer.
Rotary vacuum evaporator.
Ultrasonic bath.
Vortex mixer.
Polypropylene centrifuge tube, 50 mL, 1.5 mL.
Heart-shaped flask, 25 mL.
Colorimetric tube with cap: 50 mL.

4.4.5 SAMPLE PRETREATMENT

(1) Sample preparation

Sample, about 500 g, is thoroughly blended and homogenized in a blender and placed in clean containers as the test sample, which is sealed and labeled.

Measures should be taken to prevent samples from contamination and from residue content changes during sample preparation.

The test sample should be stored below –18°C.

(2) Extraction

Weigh 5 g of test sample (accurate to 0.01 g) into a 50-mL polypropylene centrifuge tube. Add 75.0 μL of internal middle standard solution, 25 mL of ethyl acetate, 0.75 mL of 25% ammonia solution, and 3 g of anhydrous sodium sulfate. Homogenize for 30 s and centrifuge for 5 min at 4000 rpm. Transfer the supernatant to a 50-mL colorimetric tube. Rinse the probe with 20 mL of ethyl acetate and 0.60 mL of 25% ammonia solution in another centrifuge tube. Pour the rinse solution into the first centrifuge tube, stir the residue with a glass stick, vortex to mix for 1 min, and sonicate for 5 min. After centrifugation for 5 min at *y*, the supernatant is combined into the colorimetric tube and made up to the mark with ethyl acetate. Mix well and pipette 10.0 mL of ethyl acetate into a 25-mL heat-shaped flask and evaporate to dryness at 45°C.

(3) Clean-up

The residue is reconstituted in 2.00 mL of water. Vortex to mix and sonicate for 5 min. Add 3 mL of n-hexane and vortex to mix for 30 s; let stand for separating and then decant the n-hexane. Add another 3 mL of n-hexane and vortex to mix. Transfer a portion of the aqueous layer to a 1.5-mL polypropylene centrifuge tube and centrifuge for 5 min at 13,000 rpm. Filter through a 0.20-μm aqueous filter and it is ready for LC-MS-MS determination of chloramphenicol, thiamphenicol, and florfenicol. Pipette another 100 μL filter solution, add 400 μL of water, and mix well for LC-MS-MS determination of florfenicol amine.

(4) Preparation for blank matrix solution

Weigh 5 g of test sample (accurate to 0.01 g) and follow steps that described above.

4.4.6 DETERMINATION

(1) Operation conditions

Determination of chloramphenicol, thiamphenicol, and florfenicol

SUPELCO Discovery column: C_{18}, 150 mm × 2.1 mm (i.d.), 5-μm particle size;
Column temperature: 40°C;
Mobile phase: Methanol-H2O (40+60);
Flow rate: 0.3 mL/min;
Injection volume: 20 μL.

Determination of florfenicol amine

Intersil C_8-3 column, 150 mm × 4.6 mm (i.d.), 5-μm particle size;
Column temperature: 40°C;
Mobile phase: Methanol-H2O (80+20);
Flow rate: 0.3 mL/min;
Injection volume: 10 μL.

MS operation conditions

Ion source: ESI;
Scan mode: negative mode for chloramphenicol, thiamphenicol, and florfenicol detection; positive mode for florfenicol amine detection;
Monitor mode: multiple reaction monitoring;
Ionspray voltage: −4200 V for chloramphenicol, thiamphenicol, and florfenicol detection; 4500 V for florfenicol amine detection;
Nebulizer gas, curtain gas, heater gas and collision gas are high purity nitrogen gases or equivalent; optimize the flow rate of each gas to reach the requirement of the sensitivity of mass spectrometry.
Source Temp: 600°C;
Quality ions, quantity ions, declustering potential, dwell time, and collision energy are listed in Table 4.7.

(2) Confirmation

One precursor and at least two daughters should be measured for each analyte. Under the same determination conditions, the ratio of the chromatographic retention time of the analyte to that of the internal standard, i.e., the relative retention time of the analyte, shall correspond to that of the calibration solution at a tolerance of ±2.5%. The relative intensities of the quality ions of the analyst shall correspond to those of the calibration standard at

TABLE 4.7 Mass Parameters of Chloramphenicol, Thiamphenicol, Florfenicol, Chloramphenicol-D5 and Florfenicol Amine

Analyte	Quality Ions (*m*/*z*)	Quantity Ions (*m*/*z*)	Dwell Time (ms)	DP/V	CE/V
Choramphenicol	320.9/257.0	320.9/152.0	200	−55	−16
	320.9/152.0		200		−26
Thiamphenicol	353.9/290.3	353.9/185.2	200	−55	−17
	353.9/185.2		200		−27
Florfenicol	356.0/336.0	356.0/336.0	200	−55	−14
	356.0/185.0		200		−27
Choramphenicol-D5	326.0/157.0	326.0/157.0	200	−55	−26
Florfenicol amine	248.3/230.2	248.3/130.2	200	45	18
	248.3/130.2		200		33

comparable concentrations, within the tolerances shown in Table 4.3. Then the corresponding analyte must be present in the sample.

(3) Quantification

Internal standard method for determination of chloramphenicol, thiamphenicol, and florfenicol: Under the best working conditions of the instrument, inject the matrix working standard solution. Plot the calibration curve using the ratio of the chromatographic peak area of the analyte to that of choramphenicol-D5 as the *y*-value and the ratio of the concentration of the analyte to that of choramphenicol-D5 as the *x*-value. Use the calibration curve to quantify the sample. The responses of the analyte in the working standard solution and the sample solution should be within the linear range of the instrument detection.

External standard method for determination of florfenicol amine: Under the best working conditions of the instrument, inject the matrix working standard solution. Plot the calibration curve using the chromatographic peak area of the analyte as the *y*-value and the concentration of the analyte as the *x*-value. Using the calibration curve to quantify the sample. The responses of the analyte in the standard working solution and the sample solution should be within the linear range of the instrument detection.

Multiple reaction monitoring chromatograms of the standards of chloramphenicol, thiamphenicol, florfenicol, and florfenicol amine are shown in Figs. 4.2–4.4.

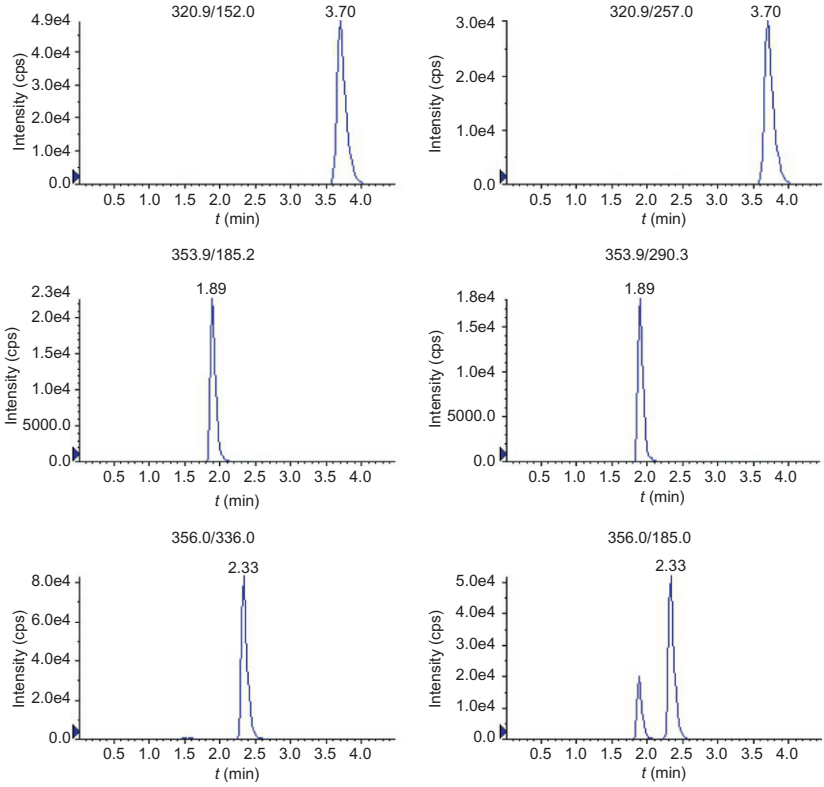

FIG. 4.2 Multiple reaction monitoring chromatogram of the standards of chloramphenicol, thiamphenicol, and florfenicol.

4.4.7 PRECISION

The precision of the part is in accordance with the prescribes of GB/T6379.1 and GB/T6379.2. Calculate the repeatability and reproducibility at a 95% confidence level.

(1) Repeatability

Under repeatability conditions, the absolute difference value of two independent test results shall not exceed the repeatability limit *r*. The content range and repeatability equations are shown in Table 4.8.

If the value exceeds the repeatability limit *r*, the two independent test results should be negated and retested.

(2) Reproducibility

Under reproducibility conditions, the absolute difference value of two independent test results shall not exceed the reproducibility limit *R*. The content range and reproducibility equations are shown in Table 4.8.

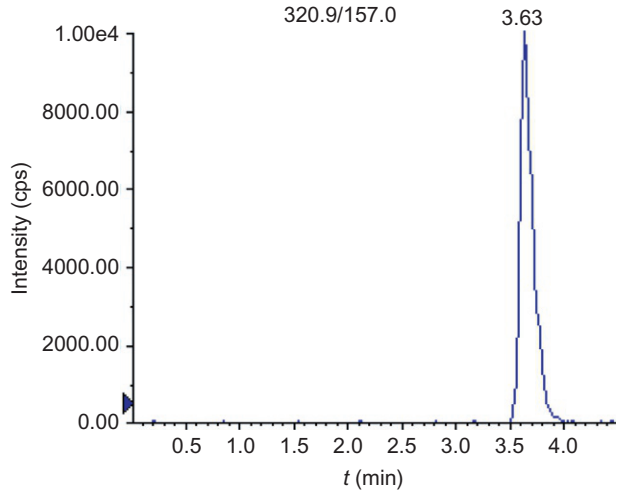

FIG. 4.3 Multiple reaction monitoring chromatogram of the standard of deuterium chloramphenicol.

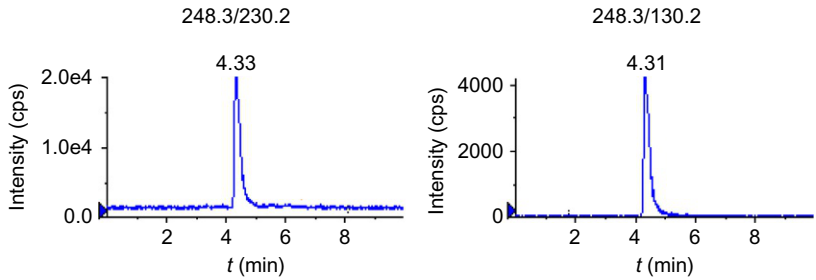

FIG. 4.4 Multiple reaction monitoring chromatogram of the standard of florfenicol amine.

4.4.8 RECOVERY

Under optimized condition, the recoveries of streptomycin, dihydrostreptomycin, and kanamycin in fugu, eel and baked eel using this method are listed in Table 4.9.

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TABLE 4.8 Spiked Concentration Range and Repeatability and Reproducibility Equations (μg/kg)

Analyte	Spiked Concentration	Sample Matrix	Repeatability r	Reproducibility R
Chloramphenicol	0.100–1.00	Balloonfish	$\lg r = 1.13\lg m + 0.691$	$\lg R = 1.07\lg m + 0.677$
		Eel	$\lg r = 0.979\lg m + 0.762$	$\lg R = 1.00\lg m + 0.762$
		Roasted eel	$\lg r = 1.11\lg m + 0.635$	$\lg R = 1.01\lg m + 0.632$
Tthiamphenicol	1.00–10.0	Balloonfish	$\lg r = 0.753\lg m - 0.0331$	$\lg R = 1.08\lg m - 0.0622$
		Eel	$\lg r = 0.991\lg m - 0.170$	$\lg R = 0.996\lg m - 0.150$
		Roasted eel	$\lg r = 0.883\lg m - 0.0969$	$\lg R = 104\lg m - 0.111$
Florfenicol	1.00–10.0	Balloonfish	$\lg r = 0.985\lg m - 0.146$	$\lg R = 0.997\lg m - 0.127$
		Eel	$\lg r = 0.956\lg m - 0.159$	$\lg R = 0.981\lg m - 0.130$
		Roasted eel	$\lg r = 0.973\lg m - 0.115$	$\lg R = 1.01\lg m - 0.148$
Florfenicol amine	1.00–8.00	Balloonfish	$\lg r = 1.09\lg m - 0.179$	$\lg R = 0.974\lg m - 0.143$
		Eel	$\lg r = 1.12\lg m - 0.190$	$\lg R = 0.983\lg m - 0.170$
		Roasted eel	$\lg r = 1.17\lg m - 0.200$	$\lg R = 0.969\lg m - 0.177$

Note: m *is the arithmetic average of two test results.*

TABLE 4.9 The Corresponding Average Recoveries of Spiked Levels of Chloramphenicol, Thiamphenicol, Florfenicol, and Florfenicol Amine

Sample Matrix	Analyte	Spiked Levels (μg/kg)	Average Recoveries (%)
Eel	Chloramphenicol	0.100	92.0
		0.200	96.2
		0.500	99.0
		1.00	92.0
	Thiamphenicol	1.00	102
		2.00	92.6
		4.00	96.4
		10.0	94.3
	Florfenicol	1.00	95.0
		2.00	95.2
		4.00	98.0
		10.0	98.0
	Florfenicol amine	1.00	97.7
		2.00	95.7
		4.00	95.4
		8.00	102
Balloonfish	Chloramphenicol	0.100	93.2
		0.200	96.7
		0.500	95.6
		1.00	95.1
	Thiamphenicol	1.00	91.6
		2.00	91.3
		4.00	88.9
		10.0	88.2
	Florfenicol	1.00	92.6
		2.00	102
		4.00	92.5
		10.0	89.4

TABLE 4.9 The Corresponding Average Recoveries of Spiked Levels of Chloramphenicol, Thiamphenicol, Florfenicol, and Florfenicol Amine—cont'd

Sample Matrix	Analyte	Spiked Levels (μg/kg)	Average Recoveries (%)
	Florfenicol amine	1.00	96.1
		2.00	91.3
		4.00	94.4
		8.00	93.5
Roasted Eel	Chloramphenicol	0.100	96.7
		0.200	97.9
		0.500	97.6
		1.00	95.9
	Thiamphenicol	1.00	85.7
		2.00	91.2
		4.00	92.1
		10.0	87.8
	Florfenicol	1.00	97.0
		2.00	97.1
		4.00	95.9
		10.0	91.8
	Florfenicol amine	1.00	93.7
		2.00	93.4
		4.00	95.7
		8.00	96.3

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Chapter 5

β-Lactams

5.1

Curative Effects and Side Effects of Penicillins

Penicillin, the archetype of antibiotics, is a derivative of the mold *Penicillium notatum*. Penicillin was found to be effective in laboratory cultures against many disease-producing bacteria in 1928. Today, penicillins are widely used to prevent and combat diseases like mastitis in food-producing animals.

The extensive use of these antibiotics may explain the presence of their residues in food animal products. They are also known to have side effects on consumers and their widespread use in human medicine seems to be associated with the emergence of bacterial resistance to these drugs in humans. Authorities worldwide, such as the European Union (EU), the American Food and Drug Administration (FDA), and the World Health Organization (WHO) for example, are actively involved in controlling the legitimate use and levels of these antibiotic residues in foods of animal origin.

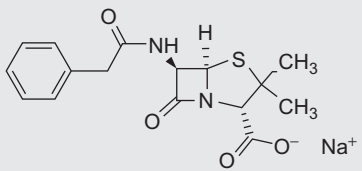
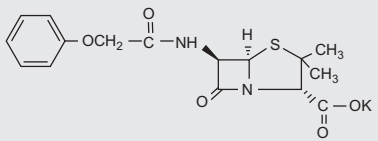
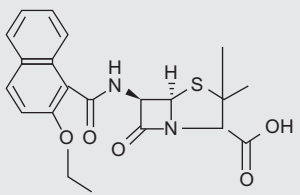
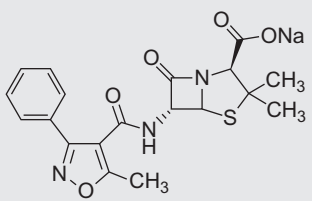
5.2

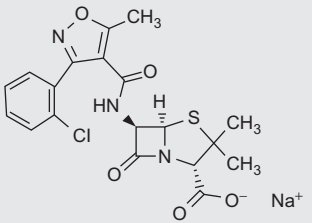
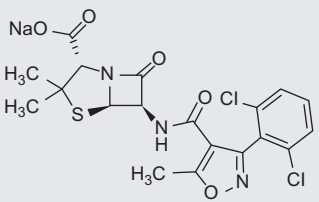
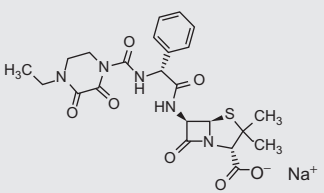
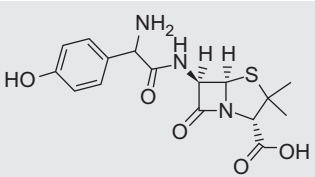
Chemical Structures and Maximum Residue Limits for Penicillins

Chemical structures and maximum residue limits (MRLs) for penicillin G, penicillin V, nafcillin, oxacillin, cloxacillin, dicloxacillin, piperacillin, amoxicillin, and ampicillin are found in Table 5.1.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00005-1

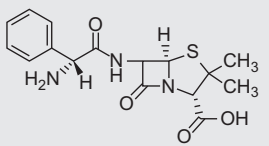
TABLE 5.1 Chemical Structures and MRLs for Penicillins

Compound Names	Molecular Structure	Molecular Weight	Cas. No	MRLs (μg/kg)
Penicillin G		356.37	69-57-8	CA:10 (edible of turkey) 50 (edible of pork and cattle), 10 (milk)
Penicillin V		388.48	87-08-1	–
Nafcillin		203.24	7177-50-6	EU: 300 (liver, muscle, kidney, fat of cattle), 30 (milk)
Oxacillin		245.28	66-79-5	CN: 300 (liver, muscle, kidney, fat) EU:300 (liver, muscle, kidney, fat), 30 (milk)

Cloxacillin		457.86	642-78-4	CN: 300 (liver, muscle, kidney, fat), 30 (milk) EU: 300 (liver, muscle, kidney, fat), 30 (milk) US: 10 (edible of cattle) AU: 10 (milk)
Dicloxacillin		474.30	3116-76-5	EU: 300 (milk, muscle, kidney, fat), 30 (liver)
Piperacillin		539.54	59703-84-3	–
Amoxicillin		419.45	61336-70-7	CN: 50 (liver, muscle, kidney, fat), 10 (milk) EU: 50 (liver, muscle, kidney, fat), 4 (milk) US: 10 (edible of cattle) AU: 10

Continued

TABLE 5.1 Chemical Structures and MRLs for Penicillins—cont'd

Compound Names	Molecular Structure	Molecular Weight	Cas. No	MRLs (μg/kg)
Ampicillin		349.45	7177-48-2	CN: 50 (liver, muscle, kidney, fat), 10 (milk) EU: 50 (liver, muscle, kidney, fat), 4 (milk) US: 10 (edible of pork and cattle) CA: 10 (milk and edible of pork and cattle)

5.3

Determination of Nine Penicillin Residues in Livestock and Poultry Muscles — LC-MS-MS Method (GB/T 20755-2006)

5.3.1 SCOPE

This method is applicable to the determination of nine penicillin residues in beef, mutton, pork, and chicken.

The limit of determination of this method: nafcillin is 0.25 μg/kg; penicillin G is 0.5 μg/kg; piperacillin, penicillin V and oxacillin is 1.0 μg/kg; amoxicillin, ampicillin, cloxacillin, and dicloxacillin is 2.0 μg/kg.

5.3.2 PRINCIPLE

The penicillin residues in test samples are extracted with 0.15 mol/L phosphate buffer (pH = 8.5), and then centrifuged. The supernatant is further cleaned up by solid phase extraction (SPE) cartridge. The residues are determined by the LC-MS/MS method and quantified by an external standard method.

5.3.3 REAGENTS AND MATERIALS

Methanol and acetonitrile are of LC grade. Sodium dihydrogen phosphate, sodium hydroxide, and acetic acid are of GR grade. Acetonitrile-water (1+1): Mix 50 mL acetonitrile and 50 mL water. 5 M sodium hydroxide solution; Phosphate buffer: 0.15 M, pH = 8.5; Bond Elut C_{18} SPE cartridge or equivalent: 500 mg, 6 mL; condition each cartridge with 5 mL methanol followed by 10 mL phosphate buffer before use. LC sample filter: 0.2 μm.

Penicillin standards: Purity ≥99%.

Stock standard solutions: 1.0 mg/mL. Accurately weigh the desired amount of each penicillin standard and dissolve in water to prepare a solution of 1.0 mg/mL in concentration as stock standard solutions. Stock standard solutions are stored at −18°C.

Matrix standard calibration solution: pipette adequate amount of penicillin standard solutions and dilute with the extraction solution for blank sample to prepare working standard solutions in sample matrix.

5.3.4 APPARATUS

LC-MS-MS: Equipped with ESI source. Analytical balance: capable of weighing to 0.1 mg and 0.01 g. Mechanical shaker. SPE vacuum apparatus. Reservoirs: 50 mL. Microsyringe: 25 μL, 100 μL. Sample tube: 5 mL, accurate to 0.1 mL. Centrifuge tubes: 50 mL. pH meter: Capable of measuring ±0.02 unit.

5.3.5 SAMPLE PRETREATMENT

(1) Extraction

Weigh 3 g of test sample (accurate to 0.01 g) into centrifuge tubes, add 25 mL phosphate buffer to each sample, and shake on mechanical shaker for 10 min; sample solutions are centrifuged at 4000 rpm.

(2) Clean-up

Connect a reservoir to a C_{18} SPE cartridge. The sample solution is decanted into the reservoir, and let it run through the cartridge at up to 3 mL/min. Rinse reservoir and cartridge with 2 mL deionized water; discard all eluates from this point. Finally, elute penicillin residues with 3 mL acetonitrile-water (1 + 1) into 5-mL sample tube. Dilute to 3 mL with acetonitrile-water (1 + 1) and shake well. Use this for the further determination by LC-MS-MS.

5.3.6 DETERMINATION

(1) Operation conditions

LC operating conditions: LC column: SunFire C_{18} 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent; Mobile phase and the elution gradient are listed in Table 5.2; Column temperature: 30°C. Injection volume: 20 μL.

TABLE 5.2 Mobile Phase Composition and Elution Gradient

Run Time (min)	Flow (μL/min)	0.3% Acetic Acid in Water (%)	0.3% Acetic Acid in Acetonitrile (%)
0.00	200	95.0	5.0
3.00	200	95.0	5.0
3.01	200	50.0	50.0
13.00	200	50.0	50.0
13.01	200	25.0	75.0
18.00	200	25.0	75.0
18.01	200	95.0	5.0
25.00	200	95.0	5.0

MS operating conditions: Ion source mode: ESI. Scan mode: Positive ion scan. Monitor mode: Multiple reaction monitor. Ionspray voltage: 5500 V; Nebulizer gas: 0.055 MPa; Curtain gas: 0.079 MPa; Turbo ionspray gas rate: 6 L/min; Ion source temperature: 400°C; MRM transitions, declustering potential, focusing potential, collision energy, and collision cell exit potential: see Table 5.3.

(2) Qualitative analysis

The qualification ions for every compound must be found, and at least must include one precursor ion and two daughter ions. Under the same determination conditions, the variation range of the retention time for the peak of the analyte in an unknown sample and in the standard working solution cannot be out of the range of ±0.25%. For the same analysis batch and the same compound, the variation range of the ion ratio between the two product ions for the unknown sample and the standard working solution at equivalent concentration cannot be out of the range listed in Table 5.4. When these conditions are met, it can be concluded that the corresponding analyte is present in the sample.

(3) Quantitative analysis

Under the best analytical conditions, the mixed standard working solutions are separately injected. Plot a standard curve of peak area as dependent variable versus concentration of analyte as independent variable. Measure the peak area of the analyte of interest in the test sample and use that to calculate the concentrations from the standard curve. A typical LC-MS chromatogram from an extract containing the nine penicillins is shown in Fig. 5.1. The retention times for the nine penicillins are listed in Table 5.5.

5.3.7 PRECISION

The precision data of the method are determined in accordance with the stipulations of GB/T6379. Repeatability and reproducibility are calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determinative results shall not exceed the limit of repeatability (r). Repeatability of this method is shown in Table 5.6, where m is the average value in micrograms per kilogram obtained from two independent determinations.

If the difference of the values exceeds the limit of repeatibility, the testing results should be discarded and two individual testing determinations made again.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not

TABLE 5.3 LC-MS-MS Parameters for the Determination of Nine Penicillins

Analytes	Qualitative Ion Pairs (*m*/*z*)	Quantitative Ion Pairs (*m*/*z*)	Collision Energy (V)	Declustering Potential (V)	Focusing Potential (V)	Collision Cell Exit Potential (V)
Amoxicillin	366/114 366/208	336/208	30 19	21	90	10
Ampicillin	350/192 350/160	350/160	23 20	20	90	10
Piperacillin	518/160 518/143	518/143	35 35	27 25	90	10
Penicillin G	335/160 335/176	335/160	20 20	23	90	10
Penicillin V	351/160 351/192	351/160	20 15	40	90	10
Oxacillin	402/160 402/243	402/160	20 20	23	90	10
Cloxacillin	436/160 436/277	436/160	21 22	20	90	10
Nafcillin	415/199 415/171	415/199	23 52	23	90	10
Dicloxacillin	470/160 470/311	470/160	20 22	20	90	10

TABLE 5.4 Maximum Permitted Tolerances for Relative Ion Intensities Using a Range of Qualitative Confirmatory Ions

Relative ion intensities	>50%	>20% to 50%	>10% to 20%	≤10%
Maximum permitted tolerances	±20%	±25%	±30%	±50%

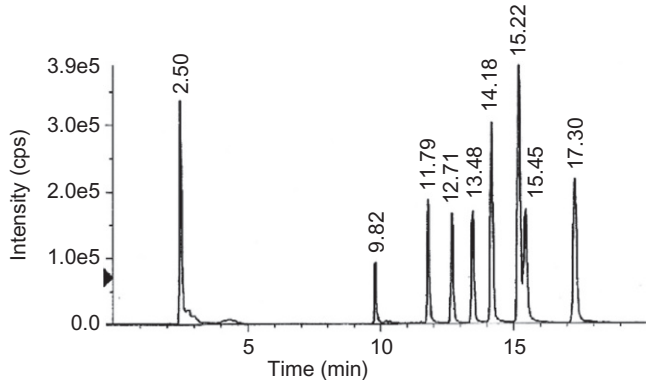


FIG. 5.1 The total ion current chromatogram of nine penicillin standards.

TABLE 5.5 The Retention Times of Nine Penicillins

Analytes	Retention Times (min)
Amoxicillin	2.50
Ampicillin	9.82
Piperacillin	11.79
Penicillin G	12.71
Penicillin V	13.48
Oxacillin	14.18
Cloxacillin	15.22
Nafcillin	15.45
Dicloxacillin	17.30

TABLE 5.6 Repeatability and Reproducibility Calculations

Analytes	Range (μg/kg)	Repeatability	Reproducibility
Amoxicillin	2.0–20	$\lg r = 0.9248 \lg m - 1.3073$	$\lg R = 0.9881 \lg m - 0.9461$
Ampicillin	2.0–20	$\lg r = 0.9511 \lg m - 1.3831$	$\lg R = 0.9857 \lg m - 0.9464$
Piperacillin	1–10	$\lg r = 0.0182m + 0.0362$	$\lg R = 0.9930 \lg m - 0.9616$
Penicillin G	0.5–5	$\lg r = 0.3252 \lg m - 1.3260$	$\lg R = 0.9683 \lg m - 0.9527$
Penicillin V	1–10	$\lg r = 1.4963 \lg m - 1.7513$	$\lg R = 1.0201 \lg m - 0.9754$
Oxacillin	1–10	$\lg r = 0.8719 \lg m - 1.4323$	$\lg R = 0.9921 \lg m - 0.9616$
Cloxacillin	2–20	$\lg r = 0.8313 \lg m - 1.3317$	$\lg R = 0.9973 \lg m - 0.9582$
Nafcillin	0.25–2.5	$\lg r = 0.9980 \lg m - 1.4927$	$R = 0.1081m + 0.0004$
Dicloxcillin	2–20	$\lg r = 1.2800 \lg m - 1.6791$	$\lg R = 1.0343 \lg m - 0.9823$

Note: m *is average value of parallel test results.*

exceed the limit of reproducibility (R). Repeatability of this method shall be calculated as shown in Table 5.6, where m is the average value obtained from two independent determinations, in micrograms per kilogram.

5.3.8 RECOVERY

Under optimized conditions, the recoveries of the nine penicillins in tissue matrix at different concentrations are listed in Table 5.7.

TABLE 5.7 The Recoveries of Penicillins from Matrix at Different Concentrations

Analytes	Fortification Concentration (μg/kg)	Recovery (%)
Amoxicillin	2.0	73.1
	4.0	75.6
	8.0	73.6
	20.0	78.6
Ampicillin	2.0	82.6
	4.0	85.7
	8.0	89.7
	20.0	97.2

TABLE 5.7 The Recoveries of Penicillins from Matrix at Different Concentrations—cont'd

Analytes	Fortification Concentration (μg/kg)	Recovery (%)
Piperacillin	1.0	81.7
	2.0	90.9
	4.0	98.2
	10.0	95.7
Penicillin G	0.5	85.9
	1.0	92.5
	2.0	103.4
	5.0	89.8
Penicillin V	1.0	86.4
	2.0	89.6
	4.0	96.5
	10.0	93.4
Nafcillin	0.25	82.5
	0.5	105.6
	1.0	95.9
	2.5	94.3
Oxacillin	1.0	90.7
	2.0	97.3
	4.0	85.6
	10.0	90.5
Cloxacillin	2.0	89.2
	4.0	84.7
	8.0	97.2
	20.0	87.6
Dicloxacillin	2.0	87.3
	4.0	91.7
	8.0	82.4
	20.0	97.1

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5.4

Determination of Nine Penicillin Residues in Milk and Milk Powder—LC-MS-MS Method (GB/T 22975-2008)

5.4.1 SCOPE

This method is applicable for the determination and identification of amoxicillin, ampicillin, piperacillin, penicillin G, penicillin V, oxacillin, cloxacillin, nafcillin, dicloxacillin residues in milk and milk powder.

The limit of determination of this method: ampicillin and nafcillin is 1 μg/kg; amoxicillin, piperacillin, penicillin G, penicillin V, cloxacillin is 2 μg/kg; oxacillin, dicloxacillin is 4 μg/kg in milk. Ampicillin and nafcillin is 8 μg/kg; amoxicillin, piperacillin, penicillin G, penicillin V, cloxacillin is 16 μg/kg; ampicillin and nafcillin is 32 μg/kg.

5.4.2 PRINCIPLE

After the residues of the analyte are extracted by acetonitrile-water solution from the sample, clean up by solid-phase extraction (SPE), detected by LC-MS-MS, with quantitative analysis using an external standard method.

5.4.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents are analytically pure; "water" is distilled water.

Acetonitrile: HPLC grade.
Acetic acid: HPLC grade.
Dibasic sodium phosphate.
Sodium hydroxide.
Sodium hydroxide solution of 5 mol/L: 100 g sodium hydroxide dissolved in 450 mL water; make up to 500 mL with water.
Phosphate buffered solution of 0.5 mol/L: 6 g dibasic sodium phosphate dissolved in 450 mL water; adjust pH to 8 with sodium hydroxide solution. Make up to 500 mL with water.
Acetonitrile-water solution (3+1, *v*/*v*): 300 mL acetonitrile mixed 100 mL water.
Acetonitrile-water solution (1+1, *v*/*v*): 100 mL acetonitrile mixed 100 mL water.
Amoxicillin (CAS: 26787-78-0), ampicillin (CAS: 69-53-4), piperacillin (CAS: 61477-96-1), penicillin G (CAS: 69-57-8), penicillin V (CAS: 132-98-9), oxacillin (CAS: 7240-38-2), cloxacillin (CAS: 642-78-4), nafcillin (CAS: 7177-50-6), dicloxacillin (CAS: 3116-76-5) nine standard substance, purity $\geq$98%.
Standard stock solutions: weigh an adequate amount of standard (accurate to 0.0001 g), dissolve in acetonitrile-water solution to make up standard stock solutions of 100 μg/mL.
Standard medium working solutions: accurately measure 1 mL of stock standard solution to 100-mL volumetric flask; make up to 100 mL with acetonitrile-water solution; concentration is 1 μg/mL.
Standard working solutions: according to the content of analyzed matter, accurately measure an adequate volume of stock standard medium working solution, diluting with blank sample extraction.
Column of HLB, 500 mg, 6 mL: rinse the column of HLB with 3 mL water and 3 mL phosphate buffered solution (4.6). 4.14 0.22 μm filter.

5.4.4 APPARATUS AND EQUIPMENT

High performance liquid chromatography tandem mass spectrometry.
Analytic balance: sensibility is 0.01 g.
Centrifuge.
Vortex shaker.
Rotary evaporator.
SPE equipment.
pH meter.

5.4.5 SAMPLE PRETREATMENT

(1) Sample preparation

Obtain the combined primary sample of milk or milk powder; mix and label.

The test sample of milk should be stored at −18°C and protected from light. The test sample of milk powder should be stored at room temperature and should be airproofed.

(2) Extraction

Weigh ca. 4 g (accurate to 0.01 g) of the milk sample into a 50 mL centrifuge tube. Weigh ca. 0.5 g (accurate to 0.01 g) of the milk powder sample into 50-mL centrifuge tube; add 4 mL water and mix well. Add 20 mL acetonitrile-water solution, shake for 2 min on a vortex shaker, and centrifuge for 10 min at 3000 rpm. Collect the organic phase and filter with funnel into a heart-shaped bottle. Extract the residue once with 10 mL acetonitrile-water solution and combine organic phase into the same heart-shaped bottle.

(3) Clean-up

Evaporate the solution to ca 7 mL with a rotary evaporator until reduced, in 45°C water bath. Add 2 mL phosphate buffered solution and mix well. Transfer the solution into a column of HLB. Rinse the heart-shaped bottle with 2 mL phosphate buffered solution; when pouring the solution into the column, control the flow rate to less than 2 mL/min. Then rinse the column with 3 mL water and draw to dryness. Then elute the column with 4 mL acetonitrile-water solution. Collect all the elution into a 10-mL tube. Make up to 4.0 mL with water and vortex to homogeneity. After filtrating with a 0.22-μm filter, the final solution is ready for analysis by HPLC-MS/MS.

(4) Blank sample solution preparation

Weigh ca. 4 g (accurate to 0.01 g) of the negative milk sample, and weigh ca. 0.5 g (accurate to 0.01 g) of the negative milk powder sample; the operation is the same as the above-mentioned extraction and cleanup steps.

5.4.6 DETERMINATION

(1) Operating conditions

Column: Phenyl 150 mm × 2.1 mm (i.d.), 5-μm particle size, or equivalent;
Column temperature: 30°C;
Injection volume: 15 μL.
Elution gradient of LC: see Table 5.8.

TABLE 5.8 Elution Gradient of LC

Time (min)	Flow Rate (μL/min)	0.1% Formic Acid Solution	Methanol (%)
0.00	200	80	20
6.00	200	20	80
8.00	200	20	80
8.01	200	80	20
10.0	200	80	20

Scan mode: Electron spray ion source (ESI+);
Detection mode: MRM;
Sheath gas: 30 unit;
Auxiliary gas: 8 unit;
Ion spray voltage (IS): 4000 V;
Capillary temperature: 320°C;
Source CID: 10 V;
Q1 = 0.4, Q3 = 0.7;
Impact gas: high pure Ar;
Impact gas pressure: 1.5 mTorr;
Other MS conditions: see Table 5.9

(2) Qualitative analysis

The qualitative ions must at least include one precursor and two daughter ions. In the same experimental conditions, the variation range of the retention time for the peak of the analyte in the unknown sample and in the matrix standard working solution can only be out of range by ±0.25%; and for the same analysis batch and the same compound, the variation of the ion intensities between the two daughter ions of the unknown sample and the matrix standard working solution at a similar concentration cannot be out of range, as shown in Table 5.4. If conditions apply, then the corresponding analyte must be present in the sample.

(3) Quantitative analysis

Under the best conditions of the apparatus, inject the series of mix matrix standard working solutions separately. The mix matrix standard working curves were made by plotting the responses versus the concentration of standards. Use the curve to quantify each analyte in the unknown sample. The responses of the analyte in the standard working solution and the sample solution should be within the linear range of the instrument's detection. Under the preceding operating conditions, the chromatogram of the standard can be seen in Fig. 5.2.

TABLE 5.9 Retention Time, Collection, Ion Pairs, and Collision Energy

Analyte	Retention Time (min)	Collection (min)	Ion Pairs (*m/z*)	Collision Energy (eV)
Amoxicillin	2.17	0-3.5	366.08/ 113.86*	22
			366.08/ 348.87	10
Ampicillin	5.26	3.5-5.8	350.08/ 105.94	20
			350.08/ 159.91*	13
Piperacillin	6.66	5.8-12	518.07/ 142.92	34
			518.07/ 159.94*	12
Penicillin G	6.85	5.8-12	335.08/ 159.86*	12
			335.08/ 175.91	14
Penicillin V	7.11	5.8-12	351.07/ 113.81	33
			351.07/ 159.92*	11
Oxacillin	7.37	5.8-12	402.08/ 159.85*	12
			402.08/ 242.81	15
Cloxacillin	7.67	5.8-12	436.04/ 160.01*	12
			436.04/ 276.85	16
Nafcillin	7.76	5.8-12	415.10/ 170.90*	36
			415.10/ 198.96	13
Dicloxacillin	8.04	5.8-12	470.00/ 159.98*	13
			470.00/ 310.78	18

Note: **Quantitative ion pair.*

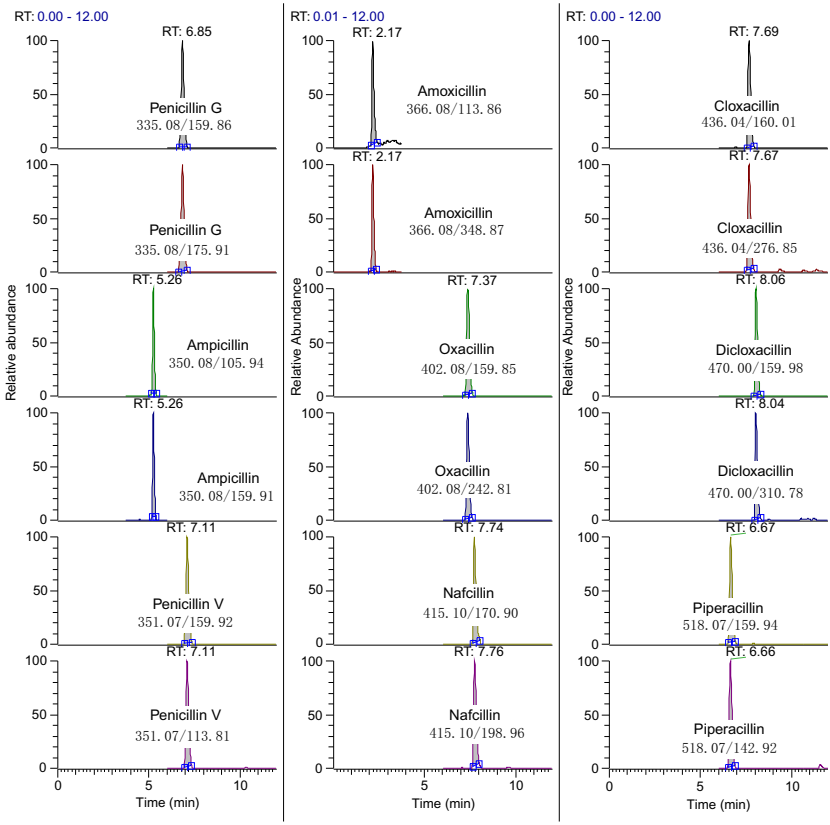

FIG. 5.2 MRM chromatogram of standard.

5.4.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379.1 and GB/T 6379.2. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the repeatability condition, the difference of the absolute value of two independent results must not be above the repeatability limit (r). The content ranges and the repeatability equation of the nine analytes in milk and milk powder are shown in Tables 5.10 and 5.11.

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned, and two independent experiments should be redone.

(2) Reproducibility

Under the reproducibility condition, the difference in the absolute values of two independent results must not be above the reproducibility

TABLE 5.10 Content Ranges and Repeatability and Reproducibility Equations for Milk Sample

Analyte	Content Ranges (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Amoxicillin	2-20	$r = 0.1438m - 0.1230$	$\lg R = 0.8798 \lg m - 0.7535$
Ampicillin	1–10	$\lg r = 0.8003 \lg m - 0.7054$	$\lg R = 0.8472 \lg m - 0.5663$
Piperacillin	2–20	$\lg r = 0.6720 \lg m - 0.4876$	$\lg R = 0.5448 \lg m - 0.2461$
Penicillin G	2–20	$\lg r = 0.5915 \lg m - 0.3613$	$R = 0.1357m + 0.6113$
Penicillin V	2–20	$\lg r = 0.7195 \lg m - 0.5850$	$\lg R = 0.7104 \lg m - 0.5232$
Oxacillin	4–40	$r = 0.1679m - 0.4464$	$R = 0.1625m - 0.0465$
Cloxacillin	2–20	$r = 0.0767m + 0.3501$	$\lg R = 0.7925 \lg m - 0.4350$
Nafcillin	1–10	$r = 0.1312m - 0.0023$	$R = 0.1415m + 0.1296$
Dicloxacillin	4–40	$r = 0.1846m + 0.1196$	$\lg R = 0.7925 \lg m - 0.4491$

Note: m *equals to the average of two results.*

TABLE 5.11 Content Ranges and Repeatability and Reproducibility Equations for Milk Powder Sample

Analyte	Content Ranges (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Amoxicillin	16–160	$r = 0.1456m - 0.9982$	$\lg R = 0.8679 \lg m - 0.5442$
Ampicillin	8–80	$r = 0.1436m + 0.0039$	$\lg R = 0.6306 \lg m - 0.2773$
Piperacillin	16–160	$r = 0.6697m + 1.6895$	$\lg R = 0.6389 \lg m - 0.2682$
Penicillin G	16–160	$\lg r = 0.8869 \lg m - 0.7144$	$\lg R = 0.7058 \lg m - 0.3888$
Penicillin V	16–160	$\lg r = 0.53 \lg m - 0.19$	$\lg R = 0.6895 \lg m - 0.3633$
Oxacillin	32–320	$r = 0.0748m + 1.0403$	$R = 0.0878m + 0.7408$
Cloxacillin	16–160	$\lg r = 0.6879 \lg m - 0.4898$	$\lg R = 1.0076 \lg m - 0.8924$
Nafcillin	8–80	$r = 0.0446m + 2.1090$	$\lg R = 0.3926 \lg m + 0.0716$
Dicloxacillin	32–320	$r = 0.0564m + 3.2365$	$\lg R = 0.7409 \lg m - 0.3706$

Note: m *equals the average of two results.*

limit (R). The content ranges and the reproducibility equation of the five analytes in milk and milk powder are shown in Tables 5.10 and 5.11.

5.4.8 RECOVERY

Under optimized condition, the recoveries of nine penicillins in milk and milk powder using this method are listed in Table 5.12.

TABLE 5.12 Fortifying Levels in Samples and Corresponding Recoveries

Analyte	Milk		Milk Powder	
	Fortifying Levels (μg/kg)	Recovery (%)	Fortifying Levels (μg/kg)	Recovery (%)
Amoxicillin	2	75.5–102.3	16	75.0–106.3
	4	83.2–101.3	32	86.7–103.3
	10	80.3–94.8	80	91.3–103.2
	20	74.4–100.7	160	84.9–98.8
Ampicillin	1	75.4–107.4	8	67.1–102.5
	2	70.8–89.3	16	86.2–102.4
	5	70.6–81.8	40	89.0–96.9
	10	77.9–95.1	80	86.5–100.2
Piperacillin	2	69.8–108.2	16	75.1–109.3
	4	71.1–101.9	32	79.1–105.6
	10	72.9–102.4	80	76.3–98.7
	20	86.8–97.28	160	86.8–98.9
Penicillin G	2	69.5–105.9	16	74.5–101.2
	4	78.1–100.6	32	75.9–104.7
	10	70.2–96.5	80	78.0–99.6
	20	78.1–100.6	160	89.5–98.1
Penicillin V	2	78.5–103.2	16	71.7–107.8
	4	77.5–108.2	32	83.4–105.3
	10	87.7–108.4	80	76.2–97.3
	20	87.3–95.8	160	84.7–100.2

Continued

TABLE 5.12 Fortifying Levels in Samples and Corresponding Recoveries—cont'd

Analyte	Milk		Milk Powder	
	Fortifying Levels (μg/kg)	Recovery (%)	Fortifying Levels (μg/kg)	Recovery (%)
Oxacillin	4	76.7–108.7	32	79.5–98.3
	8	90.0–104.5	64	88.89–106.8
	20	83.8–100.8	160	87.1–109.3
	40	82.6–98.51	320	84.1–98.1
Cloxacillin	2	64.5–102.2	16	70.3–104.8
	4	74.6–103.5	32	74.6–108.8
	10	76.6–95.3	80	81.4–102.2
	20	84.4–98.0	160	85.5–97.1
Nafcillin	1	62.4–114.6	8	74.8–98.0
	2	81.6–98.1	16	70.67–95.0
	5	82.2–95.2	40	83.5–97.4
	10	84.6–98.8	80	89.0–96.9
Dicloxacillin	4	70.6–104.8	32	76.0–106.9
	8	84.4–109.7	64	74.6–106.2
	20	74.6–106.7	160	80.8–101.8
	40	82.6–98.6	320	84.8–98.7

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5.5

Determination of Nine Penicillin Residues in Fugu and Eel—LC-MS-MS Method (GB/T 22952-2008)

5.5.1 SCOPE

This method is applicable to the determination of nine penicillin residues in fugu and eel.

The limit of determination of this standard: for nafcillin, penicillin G, piperacillin, penicillin V, and oxacillin, it is 1.0 μg/kg; for amoxicillin, ampicillin, and dicloxacillin, it is 2.0 μg/kg.

5.5.2 PRINCIPLE

The penicillin residues are extracted from the sample with acetonitrile, using a rotary evaporator to remove acetonitrile; dilute the sample to the volume with water. Finally, residues are determined by LC-MS-MS; an external standard method is used.

5.5.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be analytically pure.

Water: GB/T 6682, the first degree.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
n-Hexane.
n-Hexane saturated with acetonitrile: Mix 100 mL n-Hexane with 50 mL acetonitrile, so that upon saturation, a residual acetonitrile layer is discernible on the bottom of the container.
Ammonia.
Acetonitrile extracted solution: Mix 100 mL acetonitrile with 0.6 mL ammonia.
Standard: amoxicillin (CAS26787-78-0), ampicillin (CAS69-53-4), piperacillin (CAS 59703-84-3), penicillin G (CAS69-57-8), penicillin V (CAS132-98-9), oxacillin (CAS7240-38-2), cloxacillin (CAS642-78-4), nafcillin (CAS7177-50-6), dicloxacillin (CAS13412-64-1), purity ≥99%.
mg/mL stock standard solution of nine penicillins: 1.0 mg/mL. Accurately weigh an adequate amount of each penicillin standard (4.8) and dissolve

in water to prepare a solution of 1.0 mg/mL in concentration as stock standard solutions. Stock standard solutions are stored at −18°C.
Working standard mix solutions in sample matrix: According to the requirement, pipette adequate amount of penicillin standard solutions and dilute with the extraction solution for blank sample to prepare working standard mix solutions in sample matrix of appropriate concentration.
Filter: 0.2 μm.

5.5.4 APPARATUS

Liquid chromatography tandem mass spectrometer: Equipped with electrospray ionization and quadruple detector.

Analytical balance: Capable of weighing to 0.1 mg, 0.01 g.
Microsyringe: 25 μL, 100 μL.
Sample calibration tube: 5 mL; accuracy is 0.1 mL.
Homogenizer.
High-speed centrifuge: with 50-mL centrifuge tubes with stopper, rotate speed >10,000 rpm.

5.5.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Take representative portions from the whole primary sample, about 1 kg, and grind in a blender. Keep the prepared sample in two sample bottles, sealed and labeled. In the course of sample preparation, precautions must be taken to avoid contamination or any factors that may cause a change of residue content.

The test samples should be stored at −18°C.

(2) Preparation of test sample solution

Weigh 5 g of test sample (accurate to 0.01 g) into 50-mL centrifuge tubes and add 20 mL acetonitrile extracted solution. Homogenize for 1 min. Then high-speed centrifuge at 10,000 rpm for 10 min at 10°C. Displace the upper clear solution into another centrifuge tube and repeat the procedure with 15 mL acetonitrile extracted solution; combine the two supertanants and add 10 mL n-Hexane that is saturated with acetonitrile (4.5). Shake for 1 min and discard the n-Hexane. The extract is moved to a 100-mL heart-shaped flask, and rotary evaporated at 40°C to remove the acetonitrile. The residue is reconstituted with 2 mL water, then vortexed and put through a 0.2-μm filter. The filtrate is then ready for LC-MS-MS determination.

(3) Preparation of blank sample added with standard mix solution

According to the above-mentioned extraction and cleanup steps, accurately weigh an adequate amount of nine penicillin working standard mix

TABLE 5.13 Mobile Phase and Flow

Run Time (min)	Flow (μL/min)	1% Acetic Acid (%)	Acetonitrile (%)
0.00	200	95.0	5.0
3.00	200	95.0	5.0
3.01	200	50.0	50.0
13.00	200	50.0	50.0
13.01	200	25.0	75.0
18.00	200	25.0	75.0
18.01	200	95.0	5.0
25.00	200	95.0	5.0

solutions; add to 5.0 g sample. The concentrations of nafcillin, penicillin G, piperacillin, penicillin V, and oxacillin are 1.0, 2.0, 4.0, 10 μg/kg. And the concentrations of amoxicillin, ampicillin, cloxacillin, and dicloxacillin are 2.0, 4.0, 8.0, 20 μg/kg. Add standard mix solution into four samples, which are then ready for LC-MS-MS determination.

5.5.6 DETERMINATION

(1) Operating conditions

Column: ZORBAX SB-C18, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent;
Mobile phase and flow: see Table 5.13
Column temperature: 30°C.
Injection volume: 20 μL.
Ion source mode: ESI.
Scan mode:Positive ion scan.
Monitor mode:Multiple reaction monitor.
Ion spray voltage:5500 V;
Nebulizer gas:0.055 MPa;
Curtain gas:0.079 MPa;
Turbo ionspray gas rate: 6 L/min;
Ion source temperature:400°C;
Qualitative ion pairs, quantitative ion pairs, collision energy, declustering potential: see Table 5.14.

TABLE 5.14 LC-MS-MS Parameters for the Determination of Nine Penicillins

Name	Qualitative ion Pairs (*m*/*z*)	Quantitative Ion Pairs (*m*/*z*)	Collision Energy (V)	Declustering Potential (V)
Amoxicillin	366/114 366/208	366/208	30 19	21
Ampicillin	350/192 350/160	350/160	23 20	20
Piperacillin	518/160 518/143	518/143	35 35	27 25
Penicillin G	335/160 335/176	335/160	20 20	23
Penicillin V	351/160 351/192	351/160	20 15	40
Oxacillin	402/160 402/243	402/160	20 20	23
Cloxacillin	436/160 436/277	436/160	21 22	20
Nafcillin	415/199 415/171	415/199	23 52	23
Dicloxacillin	470/160 470/311	470/160	20 22	20

(2) Determination of qualitative

If under the same experimental conditions, the ratio between the retention times of analytes and internal standards, called relative retention times, of the sample chromatogram peaks are consistent with the standards and the differences are within ±2.5%; and if after background compensation, all the diagnostic ions are present, with the relative ion intensities corresponding to those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 5.4, then the corresponding analyte must be present in the sample.

(3) Determination of quantitative

Under the best analytical conditions, the blank spiked with working standard mix solutions is separately injected. Draw the working curve using a computer or calculator and conducting linear analysis with the peak area as the dependent variable and solution concentration as the independent variable. Calculate the concentrations of the corresponding content from the

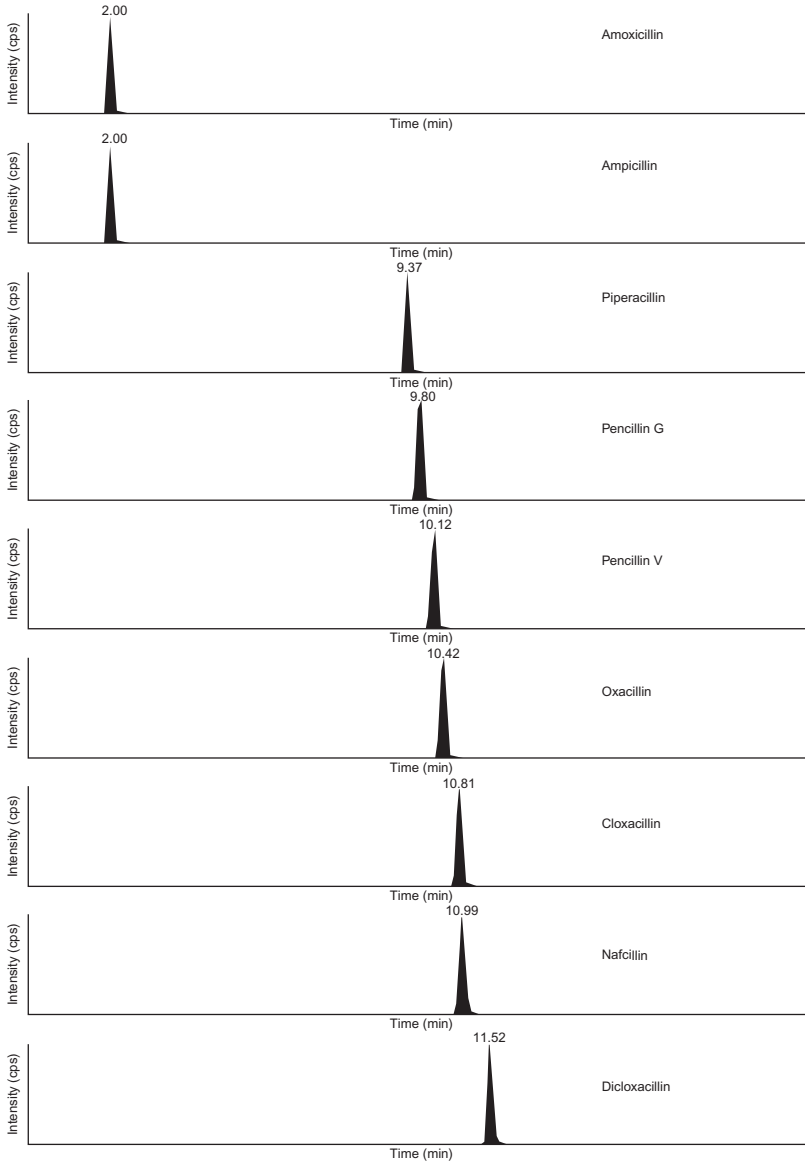

FIG. 5.3 MRM chromatograms of nine penicillins.

working curve; the responses of penicillin in the working standard mix solutions in the sample matrix and the sample solutions should be in the linear range of the instrumental detection. Under these operating conditions, the LC-MS-MS chromatograms of the standards are shown in Fig. 5.3.

TABLE 5.15 Content Ranges and the Repeatability and Reproducibility Equations (μg/kg)

Name	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Nafcillin	1.0–10	$\lg r = 0.8315$ $\lg m - 1.0541$	$\lg R = 0.9712$ $\lg m - 0.6843$
Pencillin G	1.0–10	$\lg r = 0.8373$ $\lg m - 1.0657$	$\lg R = 0.9763$ $\lg m - 0.6907$
Piperacillin	1.0–10	$\lg r = 0.8282$ $\lg m - 1.0580$	$\lg R = 0.9738$ $\lg m - 0.6887$
Oxacillin	1.0–10	$\lg r = 0.8202$ $\lg m - 1.0640$	$\lg R = 0.9772$ $\lg m - 0.6915$
Pencillin V	1.0–10	$\lg r = 0.8481$ $\lg m - 1.0388$	$\lg R = 0.9697$ $\lg m - 0.6697$
Amoxicillin	2.0–20	$\lg r = 0.8303$ $\lg m - 1.0072$	$\lg R = 0.9778$ $\lg m - 0.6846$
Ampicillin	2.0–20	$\lg r = 0.8305$ $\lg m - 1.0055$	$\lg R = 0.9322$ $\lg m - 0.6577$
Cloxacillin	2.0–20	$\lg r = 0.8194$ $\lg m - 1.0017$	$\lg R = 0.9743$ $\lg m - 0.6800$
Dicloxacillin	2.0–20	$\lg r = 0.8384$ $\lg m - 1.0151$	$\lg R = 0.9731$ $\lg m - 0.6789$

Note: m *equals to the average of two results.*

5.5.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379.1 and GB/T6379.2. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the repeatability condition, the difference of absolute value of two independent results cannot be above the repeatability limit (r). The content ranges and the repeatability equations of the nine penicillins are shown in Table 5.15.

If the difference of values exceeds the limit of repeatability (r), the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not

TABLE 5.16 The Test Data of Fortification Concentration and Average Recovery for Nine Penicillins

Name	Fortifying Concentration (μg/kg)	Average Recovery (%)
Nafcillin	1.0	89.2
	2.0	89.6
	4.0	90.8
	10.0	94.8
Penicillin G	1.0	90.2
	2.0	97.0
	4.0	89.7
	10.0	96.4
Piperacillin	1.0	87.6
	2.0	91.5
	4.0	94.0
	10.0	89.9
Oxacillin	1.0	92.3
	2.0	91.8
	4.0	92.1
	10.0	93.5
Penicillin V	1.0	86.2
	2.0	102.6
	4.0	86.9
	10.0	92.4
Amoxicillin	2.0	82.3
	4.0	84.1
	8.0	88.7
	20.0	85.3
Ampicillin	2.0	86.1
	4.0	88.2
	8.0	83.3
	20.0	87.2

Continued

TABLE 5.16 The Test Data of Fortification Concentration and Average Recovery for Nine Penicillins—cont'd

Name	Fortifying Concentration (μg/kg)	Average Recovery (%)
Cloxacillin	2.0	87.1
	4.0	89.6
	8.0	95.6
	20.0	95.7
Dicloxacillin	2.0	86.2
	4.0	92.3
	8.0	90.1
	20.0	91.2

exceed the limit of reproducibility (R); the content range and reproducibility equations for nine penicillins are shown in Table 5.15.

5.5.8 RECOVERY

Under optimized condition, the recoveries of nine penicillins in fugu and eel using this method are listed in Table 5.16.

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5.6

Method for the Determination of Six Penicillin Residues in Honey — LC-MS-MS Method (GB/T 18932.25-2005)

5.6.1 SCOPE

This method is applicable to the determination of penicillin G, penicillin V, nafcillin, oxacillin, cloxacillin, and dicloxacillin residues in honey.

The limit of determination of this method: penicillin G, penicillin V, and oxacillin is 1.0 mg/kg, cloxacillin and dicloxacillin is 2.0 mg/kg, and nafcillin is 0.5 mg/kg.

5.6.2 PRINCIPLE

The penicillin residues in test samples are dissolved in water, sample solutions are cleaned up on a C_{18} SPE cartridge, and the analytes in the extract are determined by LC-MS-MS, using an external standard.

5.6.3 REAGENTS AND MATERIALS

Methanol, acetonitrile, and acetic acid are of LC grade; Penicillin standards: Purity ≥99%; Acetonitrile-water (1+3): Mix 25 mL acetonitrile (4.2) and 75 mL water; Oasis HLB SPE cartridge or equivalent: 500 mg, 6 mL. Condition each cartridge with 5 mL methanol followed by 10 mL water before use.

Stock standard solutions: 1.0 mg/mL. Accurately weigh an adequate amount of each penicillin standard; dissolve in acetonitrile-water (1 + 3, v/v) to prepare a solution with concentration of 1.0 mg/mL as stock standard solutions. Stock standard solutions are stored at −18°C.

Working solutions in honey matrix: according to the requirement, pipette adequate amount of penicillin standard solutions and dilute with the extraction solution from the blank honey sample to prepare working standard mix solutions in honey matrix of appropriate concentration.

5.6.4 APPARATUS

LC-MS-MS: Equipped with ESI source; Analytical balance: Capable of weighing to 0.1 mg, 0.01 g; Vortex mixer; SPE vacuum apparatus; Reservoirs: 50 mL; Microsyringe: 25 μL, 100 μL; Sample tube: 5 mL, accurate to 0.1 mL; Vacuum pump; pH meter: Capable of measuring ±0.02 unit; Nitrogen evaporator.

5.6.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath, warm at $\leq$60°C with occasional shaking until liquefied, mix thoroughly, and promptly cool it to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label.

(2) Extraction and clean-up

Weigh 5 g of test sample (accurate to 0.01 g) into an Erlenmeyer flask; add 25 mL deionized water to each sample and vortex. Mix vigorously for 1 min until honey is completely dissolved. Connect a reservoir to an Oasis HLB cartridge. Load the sample solution into the reservoir and let it drain through the cartridge at about 3 mL/min. Rinse the reservoir and cartridge with 5 mL deionized water and discard all eluates. Dry cartridge for 20 min by using a 65-kPa vacuum. Finally, elute penicillin residues with 3 mL methanol into 5-mL sample tube. Evaporate the extract under a gentle stream of nitrogen in a water bath at 40°C. Dissolve the penicillin residue in 1 mL acetonitrile-water (1+3) and shake well. Then analyze by LC-MS-MS.

5.6.6 DETERMINATION

(1) Operating conditions

LC operating conditions: LC column: SunFire^{TM} C_{18} 3.5 μm, 150 mm × 2.1 mm (i.d.) or equivalent; mobile phase and the gradient elution procedure is listed in Table 5.17; Column temperature: 30°C; Injection volume: 20 μL.

MS operating conditions: Ion source mode: ESI; Scan mode:Negative ion scan; Monitor mode:Multiple reaction monitor; Ion spray voltage: 5500 V;Nebulizer gas: 0.055 MPa; Curtain gas: 0.079 MPa; Turbo ion spray gas rate: 6 L/min; Ion source temperature: 400°C; MRM transitions, declustering potential, focusing potential, collision energy, and collision cell exit potential: see Table 5.18.

TABLE 5.17 Mobile Phase Composition and Gradient Elution Conditions

Run Time (min)	Flow (μL/min)	Water Containing 0.4% Acetic Acid (%)	Acetonitrile (%)
0.00	200	60.0	40.0
15.00	200	35.0	65.0
15.01	200	60.0	40.0
25.01	200	60.0	40.0

TABLE 5.18 LC-MS-MS Parameters for the Determination of Six Penicillins

Analytes	Qualitative ion Pairs (*m*/*z*)	Quantitative ion Pairs (*m*/*z*)	Collision Energy (V)	Declustering Potential (V)	Focusing Potential (V)	Collision Cell Exit Potential (V)
Penicillin G	335/160 335/176	335/160	20 23	45	200	11
Penicillin V	351/160 351/192	351/160	20 15	40	200	11
Oxacillin	402/160 402/243	402/160	22 20	45	200	11
Cloxacillin	436/160 436/277	436/160	22 22	50	200	11
Nafcillin	415/199 415/256	415/199	21 23	45	200	11
Dicloxacillin	470/160 470/311	470/160	21 21	50	200	11

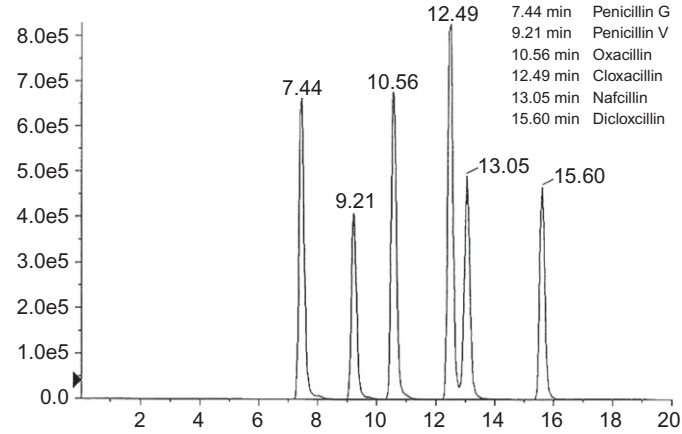

FIG. 5.4 The total ion current chromatogram of six penicillin standards.

TABLE 5.19 The Retention Times and Abundance Ratios of Six Penicillins

Analytes	Qualitative Ion Pairs (*m*/*z*)	Relative Abundance (%)	Retention Times (min)
Penicillin G	335/160 335/176	100 87	7.44
Penicillin V	351/160 351/192	100 9.6	9.21
Oxacillin	402/160 402/243	100 76	10.56
Cloxacillin	436/160 436/277	100 83	12.49
Nafcillin	415/199 415/256	100 13	13.05
Dicloxcillin	470/160 470/311	100 42	15.60

(2) Qualitative analysis

The sample solutions are determined under optimized conditions. In the sample determination, if the retention times of the peak of the sample solutions are the same as the working standard solutions in the honey matrix, and the abundance ratios of selected ion pairs are also the same, the corresponding analyte would be considered to be in the sample.

TABLE 5.20 Analytical Range, Repeatability, and Reproducibility

Analytes	Range (μg/kg)	Repeatability (r)	Reproducibility (R)
Penicillin G	1–40	$\lg r = 1.0027 \lg m - 1.0946$	$\lg R = 0.8860 \lg m - 0.6277$
Penicillin V	1–40	$\lg r = 0.9809 \lg m - 1.1480$	$\lg R = 0.9572 \lg m - 0.6451$
Oxacillin	1–40	$\lg r = 0.7579 \lg m - 0.9166$	$\lg R = 0.8137 \lg m - 0.6173$
Cloxacillin	2–80	$\lg r = 0.9364 \lg m - 1.0862$	$\lg R = 0.8489 \lg m - 0.5625$
Nafcillin	0.5–20	$\lg r = 1.0233 \lg m - 1.1511$	$\lg R = 0.8311 \lg m - 0.6066$
Dicloxacillin	2–80	$\lg r = 1.0171 \lg m - 1.1383$	$\lg R = 0.8018 \lg m - 0.5164$

Note: m *is average value of parallel test results.*

(3) Quantitative analysis

Under optimum analytical conditions, the mixed standard working solutions are separately injected. Draw the working curve using computer or calculator and conduct a linear regression with peak area as the dependent variable vs. concentrations as the independent variable (Fig. 5.4). Using the response ratio measured for an analyte of interest in the test sample, calculate the corresponding concentration from the working curve. For the retention times and the relative ion abundance of penicillin G, penicillin V, nafcillin, oxacillin, cloxacillin, and dicloxacillin, see Table 5.19. For the total ion current chromatogram of penicillin G, penicillin V, nafcillin, oxacillin, cloxacillin, and dicloxacillin see Fig. 5.4.

5.6.7 PRECISION

The precision data of the method for this standard have been determined in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determinations shall not exceed the limit of repeatability (r). Repeatability of this method shall be calculated according to Table 5.20, where m is the average value in micrograms per kilogram obtained from two independent determinations.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

TABLE 5.21 Recovery of Penicillins in Honey

Analytes	Fortification Concentration (μg/kg)	Recovery (%)
Penicillin G	1.0	92.1
	10	91.6
	20	91.9
	40	91.3
Penicillin V	1.0	88.4
	10	88.1
	20	90.7
	40	86.1
Nafcillin	0.5	94.3
	5.0	92.2
	10	92.3
	20	93.2
Oxacillin	1.0	90.9
	10	89.2
	20	92.0
	40	87.8
Cloxacillin	2.0	88.6
	20	87.1
	40	89.0
	80	87.8
Dicloxacillin	2.0	90.3
	20	87.0
	40	88.2
	80	89.0

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determinations shall not exceed the limit of reproducibility (R). Reproducibility of this method shall be calculated by Table 5.20, where m is the average value obtained from two independent determinations.

5.6.8 RECOVERY

Under optimized conditions, the fortifying concentrations of penicillins in honey and their corresponding average recoveries of this method are listed in Table 5.21.

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5.7

Determination of Cefapirin, Cephalexin, Cefalonium, Cefquinome Residues in Milk and Milk Powder—LC-MS-MS Method (GB/T 22989-2008)

5.7.1 SCOPE

This method is applicable to the determination of cefapirin, cephalexin, cefalonium, and cefquinome residues in milk and milk powder.

The limit of determination of this method in milk: cefapirin, cephalexin, cefalonium, and cefquinome is 4.0 μg/kg; the limit of determination of this standard in milk powder: cefapirin, cephalexin, cefalonium, and cefquinome is 32 μg/kg.

5.7.2 PRINCIPLE

Cephalothin antibiotic residues are extracted from the sample with acetonitrile and phosphate buffer solution, and then cleaned up with an SPE cartridge. Finally, determination is by LC-MS-MS; an external standard method is used.

5.7.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be analytically pure.

Water: GB/T 6682, the first degree.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Sodium dihydrogen phosphate (NaH_2PO_4).
Sodium hydroxide: G.R.
Acetic acid.
n-Hexane.
Acetonitrile-water (3+1): Mix 60 mL acetonitrile and 20 mL water.
n-Hexane that is saturated with acetonitrile: Mix 100 mL *n*-Hexane with 50 mL acetonitrile so that, upon saturation, a residual acetonitrile layer is discernible on the bottom of the container.
5 mol/L sodium hydroxide solution: Dissolve 20 g sodium hydroxide and dilute to 100 mL water.
0.10 mol/L sodium dihydrogen phosphate buffer solution: Dissolve 12.0 g sodium dihydrogen phosphate and dilute to 1000 mL water; then adjust to pH = 8.5 with sodium hydroxide solution.
Standard: cefapirin (CAS: 24356-60-3), cephalexin (CAS:16549-56-7), cefalonium (CAS: 5575-21-3), cefquinome (CAS: 118443-89-3), purity ≥99%.
1.0 mg/mL stock standard solution of four cephalothin antibiotics: Accurately weigh each standard, and dissolve with water in different volumetric flasks; the concentration of the solutions is 1.0 mg/mL. Store at −18°C in refrigerator.
Working standard mix solutions in sample matrix: According to the requirement, pipette adequate amount of cephalothin antibiotics stock standard solution and dilute with water to prepare working standard mix solutions of appropriate concentration.

SPE cartridge: Oasis HLB SPE cartridge or equivalent, 500 mg, 6 mL. The SPE columns are conditioned by 5 mL methanol, 5 mL water, and 10 mL sodium dihydrogen phosphate buffer solution; keep wet.
Filter: 0.2 μm.

5.7.4 APPARATUS

Liquid chromatography tandem mass spectrometer: Equipped with electrospray ionization and quadruple detector.

Analytical balance: Capable of weighing to 0.1 mg, 0.01 g.
SPE vacuum apparatus.
Reservoirs: 50 mL
Microsyringe: 25 μL, 100 μL.
Homogenizer.
High-speed centrifuge: with 50-mL centrifuge tubes with stopper, rotate speed >10,000 rpm.
Sample calibration tube: 5 mL, accuracy is 0.1 mL.
Rotary vacuum evaporator.
Nitrogen blowing instrument.

5.7.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Take the representative portions from the whole primary sample, about 1 kg, and grind in a blender. Keep the prepared sample in two sample bottles, sealed and labeled. In the course of sample preparation, precautions must be taken to avoid contamination or any factors that may cause a change of residue content.

The test samples should be stored at −18°C.

(2) Preparation of test sample solution

Milk

Weigh 5 g of test sample (accurate to 0.01 g) into 50-mL centrifuge tubes. Add 20 mL acetonitrile. Homogenize for 1 min. Then high-speed centrifuge at 10,000 rpm for 10 min at 10°C. Displace the upper clear solution into another centrifuge tube and repeat the procedure with 15 mL acetonitrile-water. Combine the two supertanants and add 10 mL *n*-Hexane that is saturated with acetonitrile. Shake for 1 min and discard the n-Hexane. Move the extract to a 100-mL heart-shaped flask, and rotary evaporate at 40°C to remove acetonitrile.

Milk powder

Weigh 5 g of test sample (accurate to 0.01 g) into 50-mL centrifuge tubes and add 4.0 mL water; Make sure it is fully dissolved and add

20 mL acetonitrile. Homogenize for 1 min. Then high-speed centrifuge at 10,000 rpm for 10 min at 10°C. Displace the upper clear solution into another centrifuge tube and repeat the procedure with 15 mL acetonitrile-water; combine the two supertanants and add 10 mL n-Hexane that is saturated with acetonitrile. Shake for 1 min and discard the n-Hexane. Move the extract to a 100-mL heart-shaped flask, and rotary evaporate at 40°C to remove acetonitrile.

(3) Clean-up

Add 20 mL sodium dihydrogen phosphate buffer solution to the sample solution which has removed the acetonitrile, and then adjust the pH value to 8.5 by using sodium hydroxide solution. Connect a reservoir to the Oasis HLB SPE cartridge, decant the sample solution into the reservoir, and let it run through the cartridge at up to 3 mL/min. First, use 5 mL sodium dihydrogen phosphate buffer solution to wash the heart-shaped flask and through the solid extract pillar, and then wash the solid extract pillar using 2 mL water. Discard all the fluid outflow and elute with 2 mL acetonitrile. Collect the eluting solution into a sample calibration tube, nitrogen-drying at 40°C. The residue is reconstituted with 2 mL water, then vortexed and put through a 0.2-μm filter. The filtrate is then ready for LC-MS-MS determination.

(4) Preparation of blank matrix extract

Milk

According to the requirements, pipette an adequate amount of cephalothin antibiotics working standard mix solutions and add to 5.0 g sample. According to the above-mentioned extraction and cleanup steps, with four samples, add the mixed standard work solution; the concentrations of cefapirin, cephalexin, cefalonium, and cefquinome are 4.0, 8.0, 16, and 40 μg/kg. The extract is ready for LC-MS-MS determination.

Milk powder

According to the requirements, pipette an adequate amount of cephalothin antibiotics working standard mix solutions and add to 0.5 g sample. According to the above-mentioned extraction and cleanup steps, with four samples, add mixed standard work solution; the concentrations of cefapirin, cephalexin, cefalonium, and cefquinome are 32, 64, 128, and 320 μg/kg. The extract is ready for LC-MS-MS determination.

5.7.6 DETERMINATION

(1) Operating conditions

Column: ZORBAX SB-C18, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent;

Mobile phase and flow: see Table 5.22.

Column temperature: 30°C.

Injection volume: 20 μL.

TABLE 5.22 Mobile Phase and Flow

Run Time (min)	Flow (μL/min)	1% Acetic Acid (%)	Acetonitrile (%)
0.00	200	95.0	5.0
2.00	200	95.0	5.0
2.01	200	40.0	60.0
8.00	200	40.0	60.0
8.01	200	95.0	5.0
15.00	200	95.0	5.0

TABLE 5.23 LC-MS-MS Parameters for the Determination of Four Cephalothin Antibiotics

Name	Qualitative Ion Pairs (*m*/*z*)	Quantitative Ion Pairs (*m*/*z*)	Collision Energy (V)	Declustering Potential (V)
Cefapirin	424/292 424/152	424/292	23 34	45
Cephalexin	348/158 348/174	348/158	14 22	40
Cefalonium	459/152 459/123	459/152	29 18	35
Cefquinome	529/134 529/396	529/134	21 19	49

Ion source mode: ESI.
Scan mode: Positive ion scan.
Monitor mode: Multiple reaction monitor.
Ion spray voltage: 5500 V;
Nebulizer gas: 0.055 MPa;
Curtain gas: 0.079 MPa;
Turbo ionspray gas rate: 6 L/min;
Ion source temperature: 400°C;
Qualitative ion pairs, quantitative ion pairs, collision energy, declustering potential: see Table 5.23.

(2) Determination of qualitative

Under the same experimental conditions, if the ratios between the retention times of analytes and internal standards, called relative retention times, of the sample chromatogram peaks are consistent with the standards and the differences are within ±2.5%; and if after background compensation, all the diagnostic ions are present, and the relative ion intensities correspond to those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 5.4, then the corresponding analyte must be present in the sample.

(3) Determination of quantitative

Under the best analytical conditions, the mixed standard working solutions are separately injected. Draw the working curve using a computer or calculator and conducting linear regression with the peak area as the dependent variable and solution concentrations as the independent variable. Calculate the concentrations of the corresponding content from the working curve; the responses of cephalothin antibiotics in working standard mix solutions in sample matrix and sample solutions should be in the linear range of the instrumental detection.

5.7.7 PRECISION

The precision data of this part are confirmed according to GB/T 6379.1 and GB/T6379.2. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the repeatability condition, the difference of absolute value of two independent results must not be above the repeatability limit (r). The content ranges and the repeatability equations of the four cephalothin antibiotics are shown in Table 5.24.

If the difference of values exceeds the limit of repeatability (r), the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for four cephalothin antibiotics are shown in Table 5.24.

5.7.8 RECOVERY

Under optimized condition, the recoveries of five cephalothin antibiotics in milk and milk powder using this method are listed in Table 5.25.

TABLE 5.24 Content Ranges and the Repeatability and Reproducibility Equations (μg/kg)

Name	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Cefapirin	4.0–200	$\lg r = 1.0589 \lg m - 1.1748$	$\lg R = 1.0218 \lg m - 0.6945$
Cephalexin	4.0–200	$\lg r = 1.0614 \lg m - 1.1774$	$\lg R = 1.0270 \lg m - 0.7020$
Cefalonium	4.0–200	$\lg r = 1.0609 \lg m - 1.1771$	$\lg r = 1.0609 \lg m - 1.1771$
Cefquinome	4.0–200	$\lg r = 1.0653 \lg m - 1.1887$	$\lg R = 1.0241 \lg m - 0.6985$

Note: m *equals to the average of two results.*

TABLE 5.25 The Test Data of Fortification Concentration and Average Recovery for Five Cephalothin Antibiotics

Name	Milk		Milk Powder	
	Fortifying Concentration (μg/kg)	Average Recovery (%)	Fortifying Concentration (μg/kg)	Average Recovery (%)
Cefapirin	4.0	85.2	32	84.6
	8.0	97.5	64	90.8
	16.0	85.1	128	98.6
	40.0	90.4	320	86.7
Cephalexin	4.0	86.4	32	80.2
	8.0	89.7	64	85.6
	16.0	91.2	128	102.3
	40.0	101.5	320	95.3
Cefalonium	4.0	92.3	32	85.7
	8.0	107.6	64	101.2
	16.0	89.3	128	87.1
	40.0	91.7	320	97.4
Cefquinome	4.0	97.4	32	92.6
	8.0	86.4	64	89.5
	16.0	101.7	128	82.4
	40.0	97.4	320	87.6

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5.8

Determination of Cefazolin, Cefapirin, Cephalexin, Cefalonium, Cefquinome Residues in Fugu and Eel—LC-MS-MS Method (GB/T 22960-2008)

5.8.1 SCOPE

This method is applicable to the determination of cefazolin, cephapirin, cephalexin, cefalonium, and cefquinome residues in fugu and eel.

The limit of determination of this standard: cefazolin is 10 μg/kg; cephapirin, cephalexin, cefalonium, and cefquinome is 2.0 μg/kg.

5.8.2 PRINCIPLE

Cephalothin antibiotic residues are extracted from the sample with acetonitrile, using a rotary evaporator to remove the acetonitrile, get to gratuate with water. Finally, determination is handled by LC-MS-MS; an external standard method is used.

5.8.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be analytically pure.

Water: GB/T 6682, the first degree.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Acetic acid
n-Hexane
n-Hexane that is saturated with acetonitrile: Mix 100 mL *n*-Hexane with 50 mL acetonitrile so that, upon saturation, a residual acetonitrile layer is discernible on the bottom of the container.
Standard: cefazolin (CAS:25953-19-9), cefapirin (CAS:24356-60-3), cephalexin (CAS:16549-56-7), cefalonium (CAS: 5575-21-3), cefquinome (CAS:118443-89-3), purity ≥99%.

Stock standard solutions: 1.0 mg/mL. Accurately weigh an adequate amount of each cephalothin antibiotics standard, and dissolve in water to prepare a solution of 1.0 mg/mL in concentration as stock standard solutions. Stock standard solutions are stored at −18°C.
Working standard mix solutions in sample matrix: According to the requirement, pipette adequate amount of cephalothin antibiotics stock standard solutions and dilute with the extraction solution for blank sample to prepare working standard mix solutions in sample matrix of appropriate concentration.
LC sample filter: 0.2 μm.

5.8.4 APPARATUS

Liquid chromatography tandem mass spectrometer: Equipped with electrospray ionization and quadruple detector.
Analytical balance: Capable of weighing to 0.1 mg, 0.01 g.
Homogenizer.
Microsyringe: 25 μL, 100 μL.
Sample calibration tube: 5 mL, accuracy is 0.1 mL.
High-speed centrifuge: with 50-mL centrifuge tubes with stopper, rotate speed >10,000 rpm.
Nitrogen blowing instrument.

5.8.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Take the representative portions from the whole primary sample, about 1 kg, and grind in a blender. Keep the prepared sample in two sample bottles, sealed and labeled. In the course of sample preparation, precautions must be taken to avoid contamination or any factors that might cause a change of residue content.

The test samples should be stored at −18°C.

(2) Preparation of test sample solution

Weigh 5 g of test sample (accurate to 0.01 g) into 50-mL centrifuge tubes, and add 4.0 mL water. When fully dissolved add 20 mL acetonitrile. Homogenize for 1 min. Then high-speed centrifuge at 10,000 rpm for 10 min at 10°C. Displace the upper clear solution into another centrifuge tube, and repeat the procedure with 15 mL acetonitrile. Combine the two supertanants and add 10 mL n-Hexane that is saturated with acetonitrile. Shake for 1 min and discard the n-Hexane. Move the extract to a 100-mL heart-shaped flask, and rotary evaporate at 40°C to remove acetonitrile. The residue is reconstituted with 2 mL water, then vortexed and put through a 0.2-μm filter. The filtrate is ready for LC-MS-MS determination.

(3) Preparation of blank spiked with standard mixed working solution

Follow the above-mentioned extraction and cleanup steps to accurately weigh an adequate amount of five cephalothin antibiotics. Add to 5.0 g sample. The concentrations of cefazolin are 10, 20, 40, and 100 μg/kg. The concentrations of cephapirin, cephalexin, cefalonium, and cefquinome are 2.0, 4.0, 8.0, 20 μg/kg. Add the mixed standard solutions into four samples, which are then ready for LC-MS-MS determination.

5.8.6 DETERMINATION

(1) Operating condition

Column: ZORBAX SB-C18, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent;
Mobile phase and flow: see Table 5.26.
Column temperature: 30 C.
Injection volume: 20 μL.
Ion source mode: ESI.
Scan mode: Positive ion scan.
Monitor mode: Multiple reaction monitor.
Ion spray voltage: 5500 V;
Nebulizer gas: 0.055 MPa;
Curtain gas: 0.079 MPa;
Turbo ionspray gas rate: 6 L/min;
Ion source temperature: 400°C;
Qualitative ion pairs, quantitative ion pairs, collision energy, declustering potential: see Table 5.27.

(2) Determination of qualitative

Under the same experimental conditions, if the ratios between the retention times of analytes and internal standards, called relative retention times,

TABLE 5.26 Mobile Phase and Flow

Run Time (min)	Flow (μL/min)	1% Acetic Acid (%)	Acetonitrile (%)
0.00	200	95.0	5.0
2.00	200	95.0	5.0
2.01	200	40.0	60.0
8.00	200	40.0	60.0
8.01	200	95.0	5.0
15.00	200	95.0	5.0

TABLE 5.27 LC-MS-MS Parameters for the Determination of Five Cephalothin Antibiotics

Name	Qualitative Ion Pairs (*m*/*z*)	Quantitative Ion Pairs (*m*/*z*)	Collision Energy (V)	Declustering Potential (V)
Cefazolin	456/324 456/156	456/324	17 24	50
Cefapirin	424/292 424/152	424/292	23 34	45
Cephalexin	348/158 348/174	348/158	14 22	40
Cefalonium	459/152 459/123	459/152	29 18	35
Cefquinome	529/134 529/396	529/134	21 19	49

of the sample chromatogram peaks are consistent with the standards and the differences are within ±2.5%, and if, after background compensation, all the diagnostic ions are present, and the relative ion intensities shall correspond to those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 5.4, then the corresponding analyte must be present in the sample.

(3) Determination of quantitative

Under the best analytical conditions, the blanks spiked with working standard mix solutions are separately injected. Draw the working curve using the computer or calculator and conduct linear regression with peak area as the dependent variable and the solution concentrations as the independent variable. Calculate the concentrations of the corresponding content from the working curve; the responses of cephalothin antibiotics in working standard mix solutions in sample matrix and sample solutions should be in the linear range of the instrumental detection.

5.8.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379.1 and GB/T6379.2 The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the repeatability condition, the difference of absolute value of two independent results must not be above the repeatability limit (*r*).

TABLE 5.28 Content Ranges and the Repeatability and Reproducibility Equations (μg/kg)

Name	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Cefazolin	10–100	$\lg r = 0.9719 \lg m - 1.0535$	$\lg R = 0.8908 \lg m - 0.5148$
Cefapirin	2.0–20	$\lg r = 1.2213 \lg m - 1.1577$	$\lg R = 0.1947\ m + 0.1223$
Cephalexin	2.0–20	$\lg r = 1.0504 \lg m - 1.0257$	$\lg R = 0.9167 \lg m - 0.6055$
Cefalonium	2.0–20	$\lg r = 1.0704 \lg m - 1.1173$	$\lg R = 0.8523 \lg m - 0.6099$
Cefquinome	2.0–20	$\lg r = 0.6656 \lg m - 0.7313$	$\lg R = 0.9261 \lg m - 0.6502$

Note: m *equals to the average of two results.*

TABLE 5.29 The Test Data of Fortification Concentration and Average Recovery for Five Cephalothin Antibiotics

Name	Fortifying Concentration (μg/kg)	Average Recovery (%)
Cefazolin	10.0	85.6
	20.0	89.1
	40.0	89.6
	100.0	91.7
Cefapirin	2.0	86.7
	4.0	92.4
	8.0	95.4
	20.0	93.8
Cephalexin	2.0	84.2
	4.0	95.3
	8.0	98.1
	20.0	94.3
Cefalonium	2.0	87.2
	4.0	91.7
	8.0	102.5
	20.0	94.1
Cefquinome	2.0	93.7
	4.0	96.5
	8.0	91.5
	20.0	97.9

The content ranges and the repeatability equations of the five cephalothin antibiotics are shown in Table 5.28.

If the difference of values exceeds the limit of repeatability (r), the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the reproducibility condition, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for five cephalothin antibiotics are shown in Table 5.28.

5.8.8 RECOVERY

Under optimized condition, the recoveries of five cephalothin antibiotics in fugu and eel using this method are listed in Table 5.29.

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5.9

Determination of Cefazolin, Cephapirin, Cephalexin, Cefalonium, Cefquinome Residues in Honey—LC-MS-MS Method (GB/T 22942-2008)

5.9.1 SCOPE

This method is applicable to the determination of cefazolin, cephapirin, cephalexin, cefalonium, cefquinome residues in honey.

The limit of determination of this method: cefazolin is 10 μg/kg; cephapirin, cephalexin, cefalonium, and cefquinome is 2.0 μg/kg.

5.9.2 PRINCIPLE

Cephalothin antibiotic residues are extracted from the sample with sodium dihydrogen phosphate buffer solution, and then cleaned up with the SPE column. Finally the residues are determined by LC-MS-MS, using an external standard method.

5.9.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be analytically pure.

Water: GB/T 6682, the first degree.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Sodium dihydrogen phosphate (NaH_2PO_4).
Sodium hydroxide: GR.
Acetic acid.
5 mol/L sodium hydroxide solution: Dissolve 20 g sodium hydroxide and dilute to 100 mL water.
0.15 mol/L sodium dihydrogen phosphate buffer solution: Dissolve 18.0 g sodium dihydrogen phosphate and dilute to 1000 mL water; then adjust to pH = 8.5 with sodium hydroxide solution.
Standard: cefazolin (CAS:25953-19-9), cefapirin (CAS:24356-60-3), cephalexin (CAS:16549-56-7), cefalonium (CAS: 5575-21-3), cefquinome (CAS:118443-89-3), purity $\geq$99%.
Stock standard solutions: 1.0 mg/mL. Accurately weigh an adequate amount of each cephalothin antibiotics standard, dissolve in water to prepare a solution of 1.0 mg/mL in concentration as stock standard solutions. Stock standard solutions are stored at $-18°C$.
Working standard mix solutions: According to the requirement, pipette adequate amount of cephalothin antibiotics stock standard solutions and dilute with the extraction solution for blank sample to prepare working standard mix solutions in sample matrix of appropriate concentration.
SPE cartridge: Oasis HLB SPE cartridge or equivalent, 500 mg, 6 mL. Condition each cartridge with 5 mL methanol, 5 mL water, followed by 10 mL phosphate buffer before use. Keep wet.
LC sample filter: 0.2 μm.

5.9.4 APPARATUS

Liquid chromatography tandem mass spectrometer: Equipped with electrospray ionization and quadruple detector.

Analytical balance: Capable of weighing to 0.1 mg, 0.01 g.
SPE vacuum apparatus.
Reservoirs: 50 mL

Microsyringe: 25 μL, 100 μL.
Sample calibration tube: 5 mL, accuracy is 0.1 mL.
Homogenizer.

5.9.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath and warm at ≤60°C with occasional shaking until liquefied. Mix thoroughly and promptly cool to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label.

The test samples should be stored at ambient temperature.

(2) Extraction

Weigh 5 g test sample (accurate to 0.01 g) into 150-mL Erlenmeyer flask. Add 25 mL sodium dihydrogen phosphate buffer solution to each sample. Mix vigorously 1 min on vortex mixer, until honey is completely dissolved. Adjust to pH = 8.5 with sodium hydroxide solution. Connect a reservoir to Oasis HLB SPE cartridge. Decant the sample solution into the reservoir, and let it go through the cartridge at up to 3 mL/min. First, use 5 mL sodium dihydrogen phosphate buffer solution to wash the Erlenmeyer flask and through the solid extract pillar, and then wash the solid extract pillar with 2 mL water. Discard all the fluid outflow and elute with 2 mL acetonitrile. Collect the eluating solution into a sample calibration tube, nitrogen-drying at 40°C. The residue is reconstituted with 2 mL water, then vortexed and put through a 0.2-μm filter. The filtrate is ready for LC-MS-MS determination. Do the procedure again to prepare blank matrix extract.

5.9.6 DETERMINATION

(1) Operating conditions

Column: ZORBAX SB-C18, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent;
Mobile phase and flow: see Table 5.26.
Column temperature: 30°C.
Injection volume: 20 μL.
Ion source mode: ESI.
Scan mode: Positive ion scan.
Monitor mode: Multiple reaction monitor.
Ion spray voltage: 5500 V;
Nebulizer gas: 0.055 MPa;
Curtain gas: 0.079 MPa;
Turbo ionspray gas rate: 6 L/min;
Ion source temperature: 400°C;

Qualitative ion pairs, quantitative ion pairs, collision energy, declustering potential: see Table 5.27.

(2) Determination of qualitative

Under the same experimental conditions, if the ratio between the retention times of analytes and internal standards, called relative retention times, of the sample chromatogram peaks are consistent with the standards and the differences are within ±2.5%, and if after background compensation, all the diagnostic ions are present, and the relative ion intensities correspond to those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 5.4, then the corresponding analyte must be present in the sample.

(3) Determination of quantitative

Under the best analytical conditions, the working standard mix solutions are separately injected. Draw the working curve using a computer or calculator and conduct linear regression with the peak area as the dependent variable and the solution concentrations as the independent variable. Calculate the concentrations of the corresponding content from the working curve; the responses of cephalothin antibiotics in working standard mix solutions in sample matrix and sample solutions should be in the linear range of the instrumental detection.

5.9.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379.1 and GB/T6379.2 The values of repeatability and reproducibility are calculated at the 95% confidence level.

TABLE 5.30 Content Ranges and the Repeatability and Reproducibility Equations (μg/kg)

Name	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Cefazolin	10–100	$\lg r = 0.9465$ $\lg m - 0.7255$	$\lg R = 0.9569$ $\lg m - 0.6176$
Cefapirin	2.0–20	$\lg r = 0.9466$ $\lg m - 0.7656$	$\lg R = 0.9680$ $\lg m - 0.6771$
Cephalexin	2.0–20	$\lg r = 0.9428$ $\lg m - 0.7628$	$\lg R = 0.9494$ $\lg m - 0.6452$
Cefalonium	2.0–20	$\lg r = 0.1357\ m$ $+ 0.1326$	$\lg R = 0.9066$ $\lg m - 0.6211$
Cefquinome	2.0–20	$\lg r = 0.9421$ $\lg m - 0.7610$	$\lg R = 0.9280$ $\lg m - 0.6885$

Note: m *equals to the average of two results.*

TABLE 5.31 The Test Data of Fortification Concentration and Average Recovery for Five Cephalothin Antibiotics

Name	Fortifying Concentration (μg/kg)	Average Recovery (%)
Cefazolin	10.0	85.6
	20.0	89.1
	40.0	89.6
	100.0	91.7
Cefapirin	2.0	86.7
	4.0	92.4
	8.0	95.4
	20.0	93.8
Cephalexin	2.0	84.2
	4.0	95.3
	8.0	98.1
	20.0	94.3
Cefalonium	2.0	87.2
	4.0	91.7
	8.0	102.5
	20.0	94.1
Cefquinome	2.0	93.7
	4.0	96.5
	8.0	91.5
	20.0	97.9

(1) Repeatability

Under the repeatability condition, the difference of absolute value of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equations of the five cephalothin antibiotics are shown in Table 5.30.

If the difference of values exceeds the limit of repeatability (r), the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for five cephalothin antibiotics are shown in Table 5.30.

5.9.8 RECOVERY

Under optimized condition, the recoveries of five cephalothin antibiotics in honey using this method are listed in Table 5.31.

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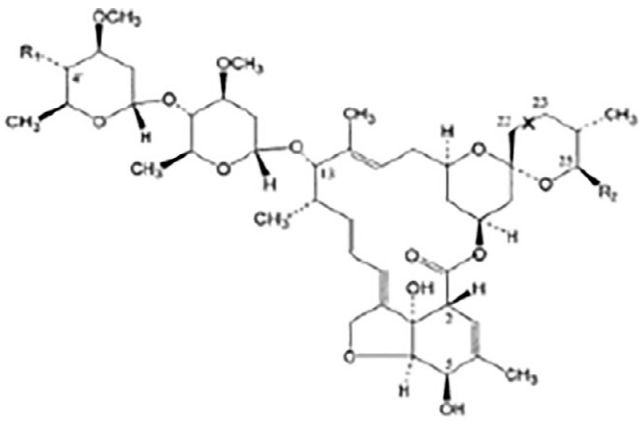
Chapter 6

Macrolides

6.1

Curative Effects and Side Effects of Avermectins

Avermectins are macrocyclic lactone compounds produced by fermentation of the soil bacterium *Streptomyces avermitilis*. They are widely used in agriculture and farm animals for treatment of a broad spectrum of parasitic diseases. The chemical structures of typical avermectins, such as abamectin, ivermectin and doramectin, are shown in Fig. 6.1. Abamectin or avermectin B1, used as an



Abamectin B_{1a}	sec-butyl	-CH=CH-
Abamectin B_{1b}	isopropyl	-CH=CH-
Ivemectin	> 80% sec-butyl < 20% isopropyl	$-CH_2-CH_2-$
Doramectin	cyclohexyl	-CH=CH-

FIG. 6.1 Structures of avermectins.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00006-3

insecticide and acaricide, consists of avermectin B1a as a major component (more than 80%) and avermectin B1b as a minor component (less than 20%). Ivermectin or 22,23-dihydroavermectin B1 is commonly used as an antiparasitic or anthelmintic agent for many farm animals, including cattle, horse, sheep, swine, and dogs. Doramectin or 25-cyclohexylavermectin B1 is usually used for cattle and some farm animals.

The use of avermectins has been associated with neurotoxicity and developmental toxicity. Extralabel use of avermectins in food animals is specifically prohibited by the FDA-CVM.

6.2

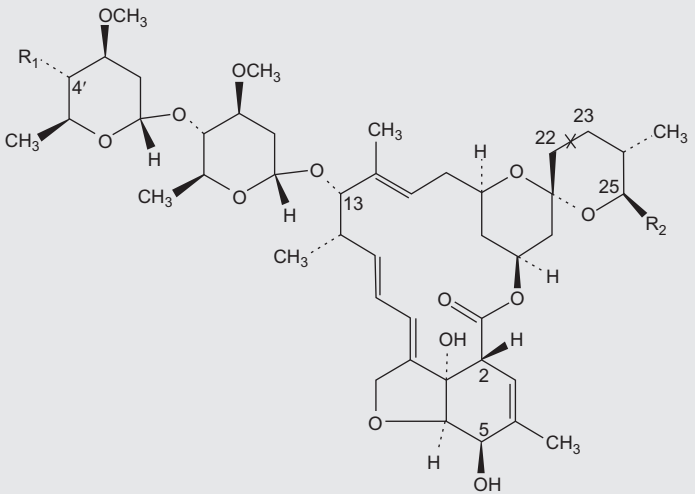
The Kinetics of Avermectins: Disposition and Metabolism

The disposition and metabolism of avermectins in ruminant livestock have been reviewed with particular emphasis on how the routes of administration and formulation affect the persistence of residues in tissues. The biological half-life of ivermectin in plasma is similar in cattle and sheep. Increasing the organic solvent content of subcutaneous formulations slows the release of the drug from the injection site and thereby prolongs its presence in the bloodstream. Because ivermectin and its metabolites are mainly excreted in bile, residues continue to appear in feces for substantially longer periods following subcutaneous injection than after oral dosing.

6.3

Maximum Residue Limits for Avermectins (Table 6.1)

TABLE 6.1 Maximum Residue Limits for Avermectins

Compound Name	Molecular Structure	MRLs (μg/kg)	
Avermectins		America	10 (in musle) 100 (in liver)
		China	20 (in musle)
		EU	20 (in musle)
		Japan	10 (in musle)

6.4

Determination of Avermectin Residues in Bovine Liver and Muscles—LC-MS-MS Method (GB/T 20748-2006)

6.4.1 SCOPE

This method is applicable to the determination of ivermectin, abamectin, doramectin, and eprinomectin residues in bovine liver and muscle tissues.

The limits of quantification of the method are 4.0 μg/kg for ivermectin, abamectin, doramectin, and eprinomectin.

6.4.2 PRINCIPLE

Avermectin residues in bovine liver and muscle tissues are extracted with acetonitrile, followed by a clean-up step on an aluminum-n solid phase extraction cartridge, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS-MS).

6.4.3 REAGENTS AND MATERIALS

Acetonitrile, HPLC grade; Acetic acid; Tertiary amine; Neutral aluminum oxide, Brockmann Activity 1; Aluminum oxide SPE: fill an empty SPE with a little absorbent cotton wool, 2 g neutral aluminum oxide, 4 g anhydrous sodium sulfate, before use; Anhydrous sodium sulfate: Ignite at 650°C for 4 h and store in a desiccator.

Standard: Ivermectin, abamectin, doramectin, eprinomectin: Purity $\geq$99%.

Stock standard solutions: Accurately weigh adequate amounts of ivermectin, abamectin, doramectin, eprinomectin standards in a volume flask; dissolve in acetonitrile to make up standard of 100 μg/mL. Store at −18°C.The standard solution of eprinomectin remains stable for 3 months, and ivermectin, abamectin, and doramectin remain stable for 1 year.

Mixed standard solution: Accurately measure 0.500 mL each of ivermectin, abamectin, doramectin, and eprinomectin stock solution into a 100-mL volumetric flask and make to volume with acetonitrile. The concentration of the solution is 0.500 μg/mL. The solution should be stored at −18°C; it will remain stable for 3 months.

Matrix extracted working standard solutions: According to the requirements, matrix extacted working standard solutions at different concentration levels are obtained by combining mixed standard solution with matrix extracted solution. Prepare just before use.

6.4.4 APPARATUS

Liquid chromatography-triple quadrupole tandem mass spectrometer equipped with APCI ionization; Homogenizer; Centrifuge: 4000 rpm; Ultrasonic water bath; Vortex mixer; KD container, 25 mL; Vacuum manifold for solid phase extract; Rotary vacuum evaporator; 0.45-μm membrane filter.

6.4.5 SAMPLE PRETREATMENT

(1) Sample preparation

Sample, about 500 g, is blended and homogenized thoroughly in a blender, placed in clean containers as the test sample, sealed, and labeled.

(2) Extraction

Accurately weigh 2 g of the test sample (accurate to 0.01 g) into a 50-mL centrifuge tube. Add 8 mL of acetonitrile and homogenize for 20 s at 8000 rpm. Then centrifuge 5 min at 4000 rpm. Transfer the supernatant into a 50-mL colorimetric tube. Add 8 mL acetonitrile to another centrifuge tube and rinse the homogenizer probe for 10 s. Transfer the elution to the first centrifuge tube and pound to pieces with a glass stirring rod. Then vortex to mix for 30 s and centrifuge at 4000 rpm for 5 min. Combine the supernatants in the 50-mL colorimetric tube. Add 5 mL acetonitrile to the first centrifuge tube and pound to pieces with glass stirring rod again. Vortex to mix for 30 s and centrifuge at 4000 rpm for 5 min. Combine the supernatants in the 50-mL colorimetric tube for clean-up.

(3) Clean-up

Pass the sample extract solution through the neutral aluminum oxide column at a rate of 1 to 2 mL/min. After the drop over, rinse the column with 2 mL of acetonitrile. Collect all the eluate in a KD container. Evaporate to dryness with rotary evaporator at 40°C. Add 1.00 mL acetonitrile to dissolve the residue and sonicate for 10 min. Then filter with 0.45-μm membrane and it is ready for LC-MS-MS determination.

6.4.6 DETERMINATION

(1) Operation conditions

Column: Intersil C8-3: 150 mm × 4.6 mm (i.d.) with 5-μm particle size or equivalent; Mobile phase: Methanol-water (9 + 1), add 1 mL tertiary

amine per liter mobile phase; Flow rate: 1.0 mL/min; Column temperature: 40°C; Injection volume: 50 μL; Ion source: APCI; Scan mode: Negative mode; Monitor mode: multiple reaction monitoring; Nebulizer gas, curtain gas, heater gas and collision gas are high purity nitrogen or equivalent, with the flow rate of each gas optimized to reach the requirement of the sensitivity of the mass spectrometer.

Ion spray voltage (IS), declustering potential (DP), and collision energy (CE) should be optimized to reach the requirement of the sensitivity of the mass spectrometer.

Quality ions, quantity ions, declustering potential, and collision energy are shown in Table 6.2.

(2) Qualitative determination

The qualitative ions for each analyst include at least one precursor ion and two product ions. Under the same determination conditions, the retention time of the analyte in the sample shall match that of the calibration standard within the tolerances ±2.5%. If the relative intensities of the detected ions of each analyst correspond to those of the calibration standard at comparable concentrations, within the tolerances shown in Table 6.3, then the corresponding analyte must be present in the sample.

(3) Quantitative determination

Under the best working conditions of LC-MS-MS, inject mix standard working solutions and calculate the linear regression curve using the peak area of the analyte versus the concentration of the analyte. Quantify the sample solution by the standard working curve. The responses of the analyte in the standard working solution and the sample solution should be

TABLE 6.2 Mass Parameters of Four Avermetins

Analytes	Quality Ions (m/z)	Quantity Ions (m/z)	Dwell Time (ms)	DP (V)	CE (V)
Ivermectin	873.7/567.2	873.7/567.2	200	−44	−28
	873.7/837.5				−28
Abamectin	871.7/565.2	871.7/565.2	200	−47	−36
	871.7/853.5				−31
Doramectin	897.6/591.2	897.6/591.2	200	−44	−38
	897.6/879.4				−28
Eprinomectin	912.5/876.6	912.5/876.6	200	−47	−23
	912.5/565.3				−38

TABLE 6.3 Maximum Permitted Tolerances for Relative Ion Intensities During Confirmation

Relative intensity	>50%	>20% to 50%	>10% to 20%	≤10%
Permitted tolerances	±20%	±25%	±30%	±50%

within the linear range of the instrument detection. Quantification is by external standard. The MRM chromatograms of four avermetin standards are shown in Fig. 6.2.

6.4.7 PRECISION

The method for the determination of precision for this standard is according to GB/T6379. Repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

For two independent test results carried out under repeatability conditions, the absolute difference value would be $\leq r$ (repeatability limit). The fortified levels range and repeatability equations of analysis are shown in Tables 6.4 and 6.5.

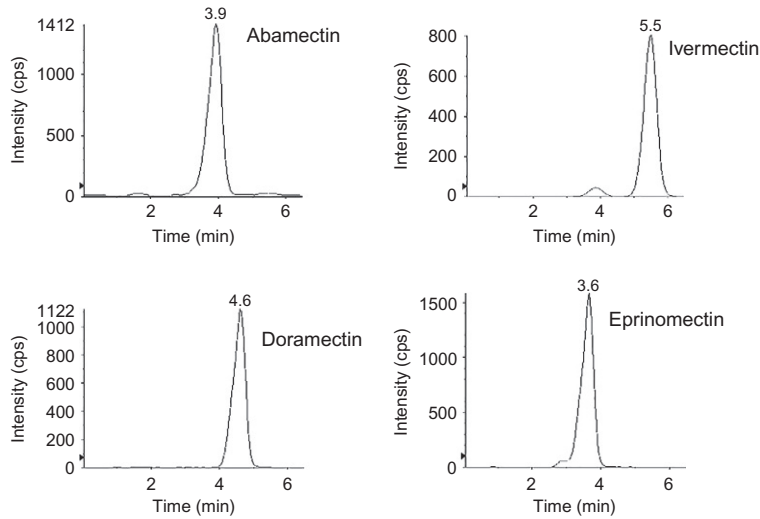


FIG. 6.2 MRM chromatograms of avermectins.

TABLE 6.4 The Analytical Range Repeatability and Reproducibility Equations (Matrix Is Muscle)

Analytes	Fortified Levels Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Ivermectin	2–16	$\lg r = 0.85 \lg m - 0.506$	$\lg R = 0.999 \lg m - 0.511$
Abamectin	2–16	$r = 0.419m$	$\lg R = 0.865 \lg m - 0.306$
Doramectin	2–16	$r = 0.152m + 0.38$	$\lg R = 0.813 \lg m - 0.291$
Eprinomectin	2–16	$r = 0.471m$	$\lg R = 0.826 \lg m - 0.328$

Note: m *is the average of two test results.*

TABLE 6.5 The Range of Fortified Levels, and the Repeatability and Reproducibility Equations (Matrix Is Liver)

Analytes	Fortified Levels Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Ivermectin	2–16	$\lg r = 0.656 \lg m - 0.499$	$R = 0.184m + 0.222$
Abamectin	2–16	$r = 0.0562m + 0.390$	$R = 0.053m + 0.726$
Doramectin	2–16	$r = 0.0979m + 0.236$	$\lg R = 0.833 \lg m - 0.492$
Eprinomectin	2–16	$r = 0.116m + 0.293$	$R = 0.351m$

Note: m *is the average of two test results.*

If the absolute difference value exceeds r, the two independent test results should be rejected and the tests redone.

(2) Reproducibility

For two independent test results carried out under reproducibility conditions, the absolute difference value would be $\leq R$ (reproducibility limit). The analytical range and reproducibility equations of analysis are shown in Tables 6.4 and 6.5.

6.4.8 RECOVERY

Under optimized conditions, the recoveries of ivermectin, abamectin, doramectin, and eprinomectin using this method are listed in Table 6.6.

TABLE 6.6 The Recoveries of Ivermectin, Abamectin, Doramectin, and Eprinomectin

Matrix	Analytes	Fortified Level (μg/kg)	Average Recovery (%)
Muscle	Ivermectin	4.0	76.4
		8.0	72.7
		16	92.6
	Abamectin	4.0	80.2
		8.0	96.5
		16	96.8
	Doramectin	4.0	78.9
		8.0	93.5
		16	77.6
	Eprinomectin	4.0	82.2
		8.0	71.3
		16	92.0
Liver	Ivermectin	4.0	84.2
		8.0	85.5
		16	87.9
	Abamectin	4.0	79.7
		8.0	87.7
		16	92.9
	Doramectin	4.0	79.8
		8.0	80.1
		16	84.8
	Eprinomectin	4.0	107
		8.0	112
		16	110

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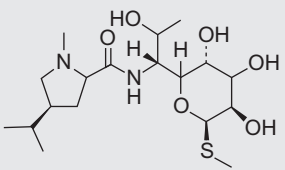
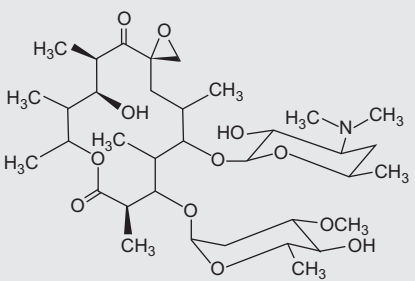
6.5

Curative Effect and Side Effects of Lincomycin, Oleandomycin, Erythromycin, Tilmicosin, Tylosin, Clindamycin, Spiramycin, Kitasamycin and Josamycin

Macrolide antibiotics are a very important class of antibacterial compounds widely used in human and veterinary practice. Erythromycin (EM) and oleandomycin (OM) are 14-membered ring macrolide antibiotics. Josamycin (JM), kitasamycin (KT), spiramycin (SPM), tylosin (TS), and tilmicosin (TLM) belong to the class of 16-membered macrolide antibiotics. Macrolide antibiotics are considered to be medium-spectrum antibiotics. They are highly active against a wide range of Gram-positive bacteria such as *Mycoplasma* and *Chlamydia*. The macrolides are most effective against diseases produced by *Mycoplasmas*. These drugs are well absorbed after oral administration and are distributed extensively in tissues, especially lungs, liver, and kidneys, with high tissue/plasma ratios. Therefore, these macrolides have been widely used in the rearing of food-producing animals to prevent and treat diseases. The veterinary drugs that can be authorized as a medicinal product intended for food-producing animals within the European Union (EU) are regulated by Council Regulation 2377/90/EC and its amendments. With the exception of erythromycin, spiramycin and tylosin are banned for use as feed additives, because the presence of antibiotic residues in foods could directly affect sensitive individuals who develop allergic reactions to their use; they can also indirectly contribute to the development of resistant bacteria as a result of widespread usage.

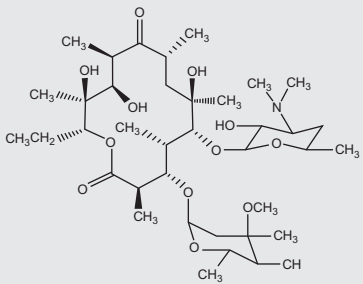
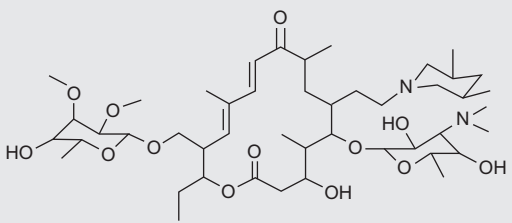
Chemical Structures and Maximum Residue Limits for Lincomycin, Oleandomycin, Erythromycin, Tilmicosin, Tylosin, Clindamycin, Spiramycin, Kitasamycin, and Josamycin (Table 6.7)

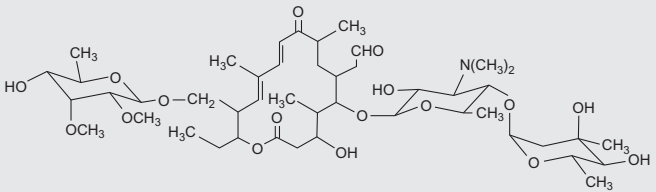
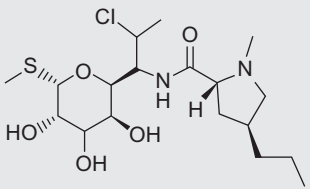
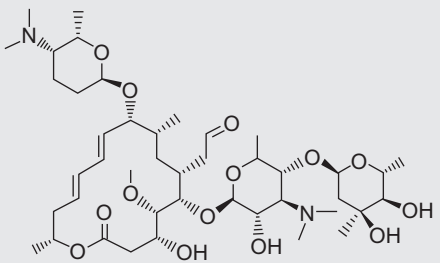
TABLE 6.7 Chemical Structures and Maximum Residue Limits

Compound	Molecular structure	Molecular weight	Cas. No	MRLs (μg/kg)
Lincomycin		406.54	859-18-7	America: 100 (swine muscle) China:100 (muscle), 50 (fat,eggs) EU: 100 (muscle), 50 (fat, eggs) Japan: 50 (cattle fat), 100 (pork fat), 200 (muscle) Canada: 100 (chicken muscle), 500 (pig liver) Australia: 20 (milk), 100 (chicken liver, kidney), 200 (muscle)
Oleandomycin		687.87	2751-09-9	America: 150 (swine) China: — EU: ? Japan: 50 (cattle fat, muscle, kidney, milk), 100 (pig muscle) Canada: — Australia: 100 (liver, kidney)

Continued

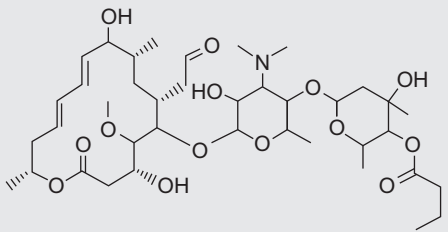
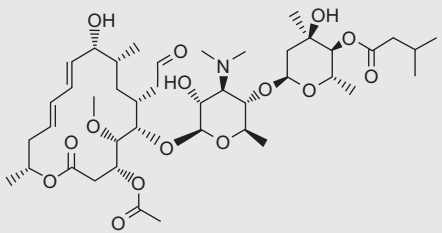
TABLE 6.7 Chemical Structures and Maximum Residue Limits—cont'd

Compound	Molecular structure	Molecular weight	Cas. No	MRLs (μg/kg)
Erythromycin		733.93	0114-07-8	America: 100 (tissue), 0 (milk) China: 40 (milk), 400 (muscle) EU: 200 (muscle), 40 (milk) Japan: 50 (muscle), 40 (milk) Canada: 50 (milk), 100 (muscle) Australia: 40 (milk), 300 (muscle)
Tilmicosin		869.13	108050-54-0	America: 100 (muscle) China: 50 (muscle, fat, milk) EU: 50 (other mammals muscle, fat, fish) Japan: 50 (milk, fish, other mammal muscle), 100 (sheep, cattle, pig muscle) Canada: — Australia: 1000 (Cattle, pig edible offal excluding liver and kidney)

Tylosin		916.11	74610-55-2	America: 200 (muscle), 50 (milk) China: 50 (milk), 100 (muscle) EU: 100 (muscle), 50 (milk) Japan: 50 (pig, cattle, chicken muscle, fat, liver, milk) Canada: 200 Australia: 50
Clindamycin	$C_{18}H_{33}ClN_2O5_S$ 	424.99	21462-39-5	America: — China: — EU: — Japan: — Canada: —
Spiramycin		843.06	8025-81-8	America: — China: 200 (muscle) EU: 200 (bovine, chicken muscle), 250 (porcine muscle) Japan: 100 (cattle edible offal excluding liver and kidney, other poultry, muscle), 200 (cattle, pig muscle) Canada: —

Continued

TABLE 6.7 Chemical Structures and Maximum Residue Limits—cont'd

Compound	Molecular structure	Molecular weight	Cas. No	MRLs (μg/kg)
Kitasamycin		771.94	1392-21-8	America: — China: EU: — Japan: 200 (muscle, liver, kidney) Canada: — Australia: 200 (muscle, liver, kidney)
Josamycin		827.99	16846-24-5	America: — China: — EU: — Japan: 40 (pig, chicken muscle, liver, kidney) Canada: — Australia: —

6.7

Determination of 9 Macroide and Lincosamide Residues in Livestock and Poultry Muscles—LC-MS-MS Method (GB/T 20762-2006)

6.7.1 SCOPE

This method is applicable to the determination of lincomycin, oleandomycin, erythromycin, tilmicosin, tylosin, clindamycin, spiramycin, kitasamycin, and josamycin residues in livestock and poultry muscle.

The limit of determination of this method for lincomycin, oleandomycin, erythromycin, tilmicosin, tylosin, clindamycin, spiramycin, kitasamycin, and josamycin: 1.0 μg/kg.

6.7.2 PRINCIPLE

The drugs are extracted from muscle tissues with acetonitrile. The extracts are concentrated after divesting of fat with hexane. The extracts are then reconstituted in phosphate solution and cleaned up on a Oasis HLB column. The drugs are eluted from the columns with methanol. The eluate is dried and the residues reconstituted in ammonium formate solution to constant volume. The solution is analyzed by LC-MS-MS and quantified using an internal standard.

6.7.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be reagent grade; "water" is deionized water. Methanol, acetonitrile: HPLC grade; Hexane; Ammonium formate. Disodium hydrogen phosphate; Sodium hydroxide; Sodium chloride; Filtration membrane: 0.2 μm; Sodium chloride solution: 2%. Dissolve 10.0 g sodium chloride in 500 mL water.

Phosphate buffer: 0.1 M. Dissolve 6.0 g disodium hydrogen phosphate in 450 mL water. Adjust pH to 8.0 with NaOH saturated solution and dilute to 500 mL. Prepare fresh daily as required.

Methanol-water solution (2+3): Mix 400 mL methanol and 600 mL water. Prepare fresh daily as required.

Ammonium formate solution: 10 mmol/L. Dissolve 0.63 g ammonium formate in 1000 mL water. Filter the solution with 0.2-μm aqueous phase filter membrane.

Lincomycin, oleandomycin, erythromycin, tilmicosin, tylosin, clindamycin, spiramycin, kitasamycin, josamycin, and roxithromycin standards: purity ≥95%.

Stock standard solutions of macrolides: 1.0 mg/mL. Accurately weigh 10 mg of each drug standard into a 10-mL volumetric flask, dissolve in a small volume of methanol, and make up to volume with methanol. Store at 4°C.

Mixed stock standards solution of macrolides: 10.0 μg/mL. Pipette 0.1 mL each stock standard solutions of macrolide into 10.0-mL volumetric flask and make up to volume with methanol. Prepare weekly and store at 4°C.

Mixed working standard solution of macrolides: 1.0 μg/mL. Pipette 1 mL each stock standard solution of macrolides into 10.0-mL volumetric flask and make up to volume with methanol. Prepare fresh daily as required.

Internal standard stock solution: 1.0 mg/mL. Accurately weigh 10.0 mg roxithromycin into 10.0-mL volumetric flask, dissolve in a small volume of methanol, and make up to volume with methanol. Store at 4°C.

Mixed concentration internal standard stock solution: 10.0 μg/mL. Pipette 0.1 mL internal standard stock solution into 10-mL volumetric flask and make up to volume with methanol. Prepare fresh daily as required. Store at 4°C.

Internal standard working solution: 1.0 μg/mL. Pipette 1 mL midst concentration internal standard stock solution into 10-mL volumetric flask and make up to volume with methanol. Prepare fresh daily as required.

Matrix standard working solutions: Pipette respectively 1.0 μL, 2.0 μL, 5.0 μL, 10.0 μL, and 50.0 μL mixed working standard solutions, add 10.0 μL internal standard working solution, dilute to 1.0 mL with blank sample extracts, and prepare into matrix standard working solutions of a series of concentrations: 1.0 μL, 2.0 μL, 5.0 μL, 10.0 μL, and 50.0 μL. Prepare them just before use.

Oasis HLB solid extraction column: 500 mg, 6 mL. Condition each column with 10 mL methanol followed by 10 mL water before use.

6.7.4 APPARATUS

LC-MS-MS: Equipped with ESI source; Analytical balance: Capable of weighing to 0.01 g, 0.0001 g; Solid phase extraction apparatus; Nitrogen evaporator; Centrifuge tubes: 50 mL, polypropylene with screw cap; Centrifuge.

6.7.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Take representative samples of 1 kg from the total samples, shred and mix them thoroughly, break into two equal parts and load respectively into a clean container, to serve as test samples after being sealed. Clearly mark or label the containers. Precautions should be taken to prevent the samples from being contaminated or from any change of the residue content in the

sampling and sample preparation operations. The test samples should be stored below −20°C.

(2) Extraction

Weigh 5 g test sample (accurate to 0.01 g) into 50-mL centrifuge tube. Add 10.0 μL internal standard working solution and 15.0 mL acetonitrile, vortex to mix, and shake for 10 min in high speed horizontal shaker. Centrifuge at 4200 rpm for 5 min. Transfer the supernatant to a second centrifuge tube. Add 2.0 g sodium chloride and 10.0 mL hexane, vortex to mix, and shake for 10 min in high speed horizontal shaker. Repeat centrifuge at 4200 rpm for 10 min. Carefully transfer the 12.0 mL mesosphere of acetonitrile to another centrifuge tube. Evaporate the extract under nitrogen at 55°C to minimum volume.

(3) Clean-up

Reconstitute residues with 7 mL phosphate buffer and add the extract to the Oasis HLB solid extraction column. After the extract has all flowed out, wash the column with 10 mL water and 5 mL methanol-water solution. Discard all eluates. Dry the cartridge by drawing air through it for 1 h by vacuum pump. Finally, elute the macrolides with 10 mL methanol into a 15-mL conical flask. Evaporate the eluates under nitrogen in a water bath at 55°C to minimum volume. Reconstitute residues in 1.0 mL ammonium formate solution to constant volume. Repeat the steps to prepare blank sample. Filtrate the solution with 0.2-μm filter membrane and conduct determination by LC-MS-MS.

6.7.6 DETERMINATION

(1) Operation conditions

Chromatographic column: Intersil C18, 3 μm, 150 mm × 2.1 mm, or equivalent; Injection volumes: 40 μL; Flow rate: 0.5 mL/min; Column temperature: 25°C; Mobile phase: A: 10 mmol/L ammonium formate solution; B: acetonitrile. See Table 6.8 for gradient conditions.

Ion source: ESI source; Scan mode: Positive scan; Monitor mode: Multiple reaction monitor (MRM); Ionspray voltage: 5500 V; Nebulizer gas: 0.069 MPa; Curtain gas: 0.69 MPa; Turbo ionspray gas rate: 6 L/min; Source temperature: 350°C; Precursor/product ion combinations, collision energy, and declustering potential: see Table 6.9.

(2) Qualitative analysis

Under identical test conditions, the difference of the ratios of the retention times of the analyte in the sample solutions and that of the analyte in the matrix standard working solutions shall be within ±2.5%. Moreover, the ratio difference of the relative abundance of each qualifying ion in the analyte of the sample solutions and that of the analyte in the matrix standard working solutions of nearly identical concentrations shall not

TABLE 6.8 Gradient Conditions

Time (min)	Flow Rate (mL/min)	A	B
0.0	0.50	95	5
1.0	0.50	95	5
6.0	0.50	40	60
6.1	0.50	5	95
10.0	0.50	5	95
10.1	0.50	95	5
20.0	0.50	95	5

TABLE 6.9 Precursor/Product Ion Combinations, Collision Energy, and Declustering Potential, of Nine Macrolides

Analytes	Precursor Ion Combinations (m/z)	Product Ion Combinations (m/z)	Collision Energy (V)	Declustering Potential (V)
Lincomycin	407.2/126.1 407.2/359.2	407.2/126.1	37 24	50 50
Oleandomycin	688.4/158.2 688.4/544.3	688.4/158.2	70 42	30 30
Erythromycin	734.3/158.2 734.3/576.3	734.3/158.2	42 28	50 50
Tilmicosin	869.4/174.2 869.4/132.1	869.4/174.2	62 70	90 90
Tylosin	916.4/174.2 916.4/145.1	916.4/174.2	54 55	80 80
Clindamycin	425.2/126.1 425.2/377.3	425.2/126.1	45 28	53 53
Spiramycin	843.3/142.2 843.3/174.2	843.3/142.2	48 50	60 60
Kitasamycin	772.3/215.2 772.3/109.1	772.3/215.2	43 42	70 70
Josamycin	828.3/174.2 828.3/109.1	828.3/174.2	45 45	80 80

exceed the range specified in Table 6.3; the analyte is then judged to be existent in the sample.

(3) Quantitative analysis

In the optimum working conditions of the instrument, a standard working curve is drawn, based on the matrix standard working solution concentration as ordinate and peak area as abscissa. Quantitative analysis is conducted using the working curve of the matrix standard working solutions, trying to make the response values of nine macrolide antibiotics fall within the linear range of the instrumental detection. Under the previously discussed chromatic and mass spectrometric conditions, the reference response times for nine macrolide antibiotics are shown in Table 6.10 The chromatograms for multiple response monitoring (MRM) of nine macrolide antibiotics are given in Fig. 6.3.

6.7.7 PRECISION

The precision of the method has been determined in accordance with the stipulations of GB/T 6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for phenylbutazone in muscle tissues are shown in Table 6.11.

TABLE 6.10 Reference Retention Times for Nine Macrolide Antibiotics

Analytes	Retention Time (min)
Lincomycin	6.96
Oleandomycin	7.36
Erythromycin	8.06
Tilmicosin	8.36
Tylosin	8.48
Clindamycin	8.56
Spiramycin	8.67
Kitasamycin	8.83
Josamycin	9.14

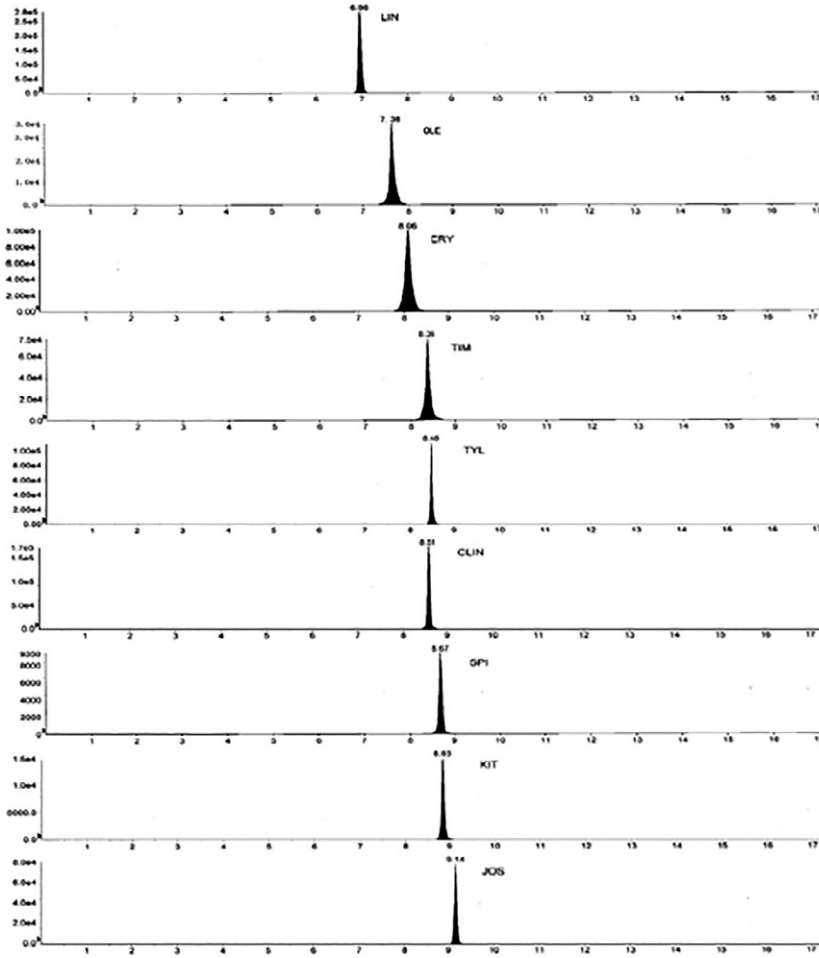


FIG. 6.3 Chromatograms for multiple reaction monitoring (MRM) of nine macrolide antibiotic standards.

If the difference of values exceeds the limit of repeatability (r), the testing results should be discarded and two individual testing determinations shall be reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for phenylbutazone in muscle tissues are shown in Table 6.11.

TABLE 6.11 Range, Repeatability, and Reproducibility

Analytes	Range (μg/kg)	Repeatability (r) (μg/kg)	Reproducibility (R) (μg/kg)
Lincomycin	1.0–10.0	$\lg r = 1.0940 \lg m - 1.4214$	$\lg R = 0.9331 \lg m - 0.8335$
Oleandomycin	1.0–10.0	$\lg r = 1.0632 \lg m - 1.4236$	$R = 0.1461m - 0.0124$
Erythromycin	1.0–10.0	$\lg r = 0.8820 \lg m - 1.3150$	$\lg R = 1.0196 \lg m - 0.8527$
Tilmicosin	1.0–10.0	$\lg r = 1.1543 \lg m - 1.4568$	$R = 0.1157m + 0.0094$
Tylosin	1.0–10.0	$\lg r = 1.1137 \lg m - 1.4373$	$\lg R = 1.3973 \lg m - 1.1955$
Clindamycin	1.0–10.0	$r = 0.0436m - 0.0137$	$\lg R = 1.4436 \lg m - 1.1911$
Spiramycin	1.0–10.0	$\lg r = 1.1521 \lg m - 1.4711$	$\lg R = 0.9930 \lg m - 0.8379$
Kitasamycin	1.0–10.0	$\lg r = 0.7018 \lg m - 1.2580$	$\lg R = 1.0171 \lg m - 1.0072$
Josamycin	1.0–10.0	$\lg r = 0.9971 \lg m - 1.4369$	$\lg R = 0.9865 \lg m - 0.7972$

Note: The m *is average value of parallel test results.*

6.7.8 RECOVERY

Under optimized conditions, the fortifying concentrations of lincomycin, oleandomycin, erythromycin, tilmicosin, tylosin, clindamycin, spiramycin, kitasamycin, and josamycin and the corresponding average recoveries of this method are listed in Table 6.12.

TABLE 6.12 The Fortifying Concentrations and Corresponding Recoveries

Analytes	Fortifying Concentration (μg/kg)	Recovery (%)
Lincomycin	1.0	88.0
	2.0	88.2
	5.0	87.1
	10.0	84.5
Oleandomycin	1.0	84.9
	2.0	85.1
	5.0	85.7
	10.0	86.4

Continued

TABLE 6.12 The Fortifying Concentrations and Corresponding Recoveries—cont'd

Analytes	Fortifying Concentration (μg/kg)	Recovery (%)
Erythromycin	1.0	84.9
	2.0	82.7
	5.0	86.7
	10.0	88.6
Tilmicosin	1.0	86.9
	2.0	87.8
	5.0	84.8
	10.0	89.4
Tylosin	1.0	86.6
	2.0	77.2
	5.0	84.0
	10.0	85.5
Clindamycin	1.0	89.5
	2.0	82.0
	5.0	88.8
	10.0	87.0
Spiramycin	1.0	86.8
	2.0	86.6
	5.0	88.4
	10.0	86.8
Kitasamycin	1.0	83.3
	2.0	88.2
	5.0	87.4
	10.0	87.1
Josamycin	1.0	85.9
	2.0	84.2
	5.0	86.9
	10.0	82.9

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6.8

Determination of Ivermectin, Abamectin, Doramectin, and Eprinomectin Residues in Milk and Milk Powder—LC-MS-MS Method (GB/T 22968-2008)

6.8.1 SCOPE

This method is applicable to the determination of ivermectin, abamectin, doramectin, and eprinomectin residues in milk and milk powder.

The limit of quantitation of the method for ivermectin, abamectin, doramectin, and eprinomectin in milk is 5 μg/kg, and it is 40 μg/kg for milk powder.

6.8.2 PRINCIPLE

Ivermectin, abamectin, doramectin, and eprinomectin residues are extracted from milk with acetonitrile-dichloromethane; the extract is defatted using

n-hexane. The residues are determined by LC-MS and quantified by peak area, and an external standard method is used.

Ivermectin, abamectin, doramectin, and eprinomectin residues are extracted from milk powder with acetonitrile, and the extract is defatted using *n*-hexane. The residues are determined by LC-MS and quantified by peak area, and an external standard method is used.

6.8.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents are analytically pure; "water" is first grade water prescribed by GB/T 6682.

Acetonitrile: HPLC grade.
Methanol.
Dichloromethane.
n-Hexane: saturated by acetonitrile before use.
Sodium chloride.
Acetonitrile-dichloromethane (4+1): mix 80 mL acetonitrile and 20 mL dichloromethane.
Saturated sodium chloride aqueous solution.
Standard: ivermectin, abamectin, doramectin, and eprinomectin, purity ≥99%
Standard stock solution: 100 μg/mL.
Separately, accurately weigh adequate amounts of ivermectin, abamectin, doramectin, and eprinomectin standards (accurate to 0.1 mg). Dissolve in acetonitrile and prepare a stock solution of 100 μg/mL. Store at −18°C. The standard solution of eprinomectin is stable for 3 months and the standard solutions of ivermectin, abamectin, and doramectin are stable for 1 year.
Mixed standard solution: 0.500 μg/mL.
Accurately transfer 0.500 mL of each stock solution into a 100 mL volumetric flask and dilute to volume with acetonitrile. The concentration of the solution is 0.500 μg/mL. The solution is stable for 3 months at −20°C.
Working standard solution with matrix:

According to the requirement, accurately measure an adequate volume of mixed standard solution, and dilute with blank sample extraction solution to prepare an appropriate working standard solution with matrix. Prepare just before use.

Membrane filter: 0.2 μm, for organic solvent.

6.8.4 APPARATUS

LC-MS-MS, with ESI ion-source.
Electronic balance: 0.01 g and 0.001 g sensitivity.
Centrifuge: ≥4000 rpm.

Ultrasonic bath.
Vortex mixer.
Nitrogen evaporator.

6.8.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Milk

Place about 250 g milk in clean containers as the test sample, which is sealed and labeled. The test sample should be stored at 4°C.

Milk powder

Place about 250 g milk powder in clean containers as the test sample, which is sealed and labeled.

In the course of sample preparation, precautions should be taken to avoid contamination or any factors that may cause a change of residue content.

(2) Extraction

Milk

Accurately weigh 2 g of the test sample (accurate to 0.01 g) into a 25-mL centrifuge tube. Add 5 mL saturated sodium chloride aqueous solution and homogenize for 1 min. Add 7.5 mL × 2 of acetonitrile-dichloromethane mixed solvent and homogenize for 1 min. Then centrifuge for 5 min at 4000 rpm. Transfer the supernatant into a 50-mL tube and use the nitrogen evaporator to evaporate to dryness below 45°C with nitrogen gas. Add 2.00 mL of acetonitrile to dissolve the residue and homogenize for 1 min by vortex mixer. Add 3 mL of *n*-hexane (4.4), homogenize for 1 min and let stand for 30 min. Filter the lower solution through a 0.2-μm organic filter for LC-MS-MS determination.

Milk powder

Accurately weigh 0.5 g of the test sample (accurate to 0.01 g) into a 10-mL centrifuge tube. Add 3 mL of acetonitrile and homogenize for 5 min by ultrasonic bath. Then centrifuge for 5 min at 4000 rpm. Transfer the supernatant into a 15-mL centrifuge tube with cap. Extract the residue with 2 mL of acetonitrile repeatedly. Mix the supernatant in the 15-mL centrifuge tube and dilute to 5.0 mL with acetonitrile. Add 3 mL of *n*-hexane, homogenize for 1 min, and let stand for 30 min. Filter the lower solution through a 0.2-μm organic filter for LC-MS-MS determination.

6.8.6 DETERMINATION

(1) Conditions

Column: Intersil C8-3, 5 μm, 150 mm × 4.6 mm (i.d.), or other equivalent;
Column temperature: 40°C;
Mobile phase: Methanol + water, gradient eluent, see Table 6.13;
Flow rate: 0.8 mL/min;

TABLE 6.13 Elution Gradient of LC

Time (min)	Methanol (%)	Water (%)
0.00	75	25
3.00	100	0
10.00	100	0
10.01	75	25
15.00	75	25

Injection volume: 25 μL.
Ion source: ESI;
Scan polarity: Negative mode;
Scan mode: Multiple reaction monitoring (MRM);
Nebulizer gas, curtain gas, heater gas, and collision gas are high purity nitrogen or other equal gas; optimize the flow rate of gas to reach the requirement of the sensitivity of mass spectrometry. DP, EP, CE should be optimized to the best sensitivity;
Monitor ions and reference MS parameters are listed in Table 6.14.

(2) Qualitative determination

The qualitative ions for each analyst include at least one precursor ion and two product ions. Under the same determination conditions, the ratio of the chromatographic retention time of the analyte shall correspond to that of the calibration solution at a tolerance of ±2.5%.The relative intensities

TABLE 6.14 Monitor Ions and Reference MS Parameter

Compound	Qualitative Ion (m/z)	Quantitative Ion (m/z)	Dwell Time (ms)	DP (V)	CE (V)
Ivermectin	873.7/567.8	873.7/229.2	100	−75	−37
	873.7/229.2				−50
Abamectin	871.7/565.2	871.7/565.2	100	−80	−40
	871.7/229.3				−54
Doramectin	897.6/591.4	897.6/591.2	100	−70	−38
	897.6/229.0				−51
Eprinomectin	912.5/270.0	912.5/565.4	100	−82	−49
	912.5/565.4				−37

of the qualitative ions of each analyte shall correspond to those of the calibration standard at comparable concentrations, within the tolerances shown in Table 6.3; then the corresponding analyte must be present in the sample.

(3) Quantitative determination

On the optimal condition of equipment, inject working standard solution with matrix. Using linear regression, construct a standard curve by plotting concentration in nanograms per milliliter (x), versus peak area (y). The response of the sample must be within the standard curve. Reconstituted ion chromatograms of ivermectin, abamectin, doramectin, and eprinomectin can be found in Figs. 6.4 to 6.7.

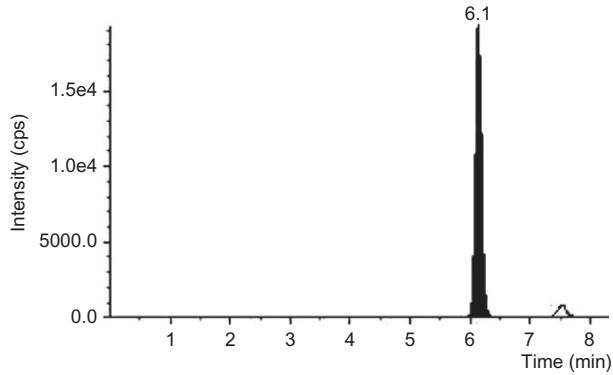

FIG. 6.4 Reconstituted ion chromatogram of ivermectin.

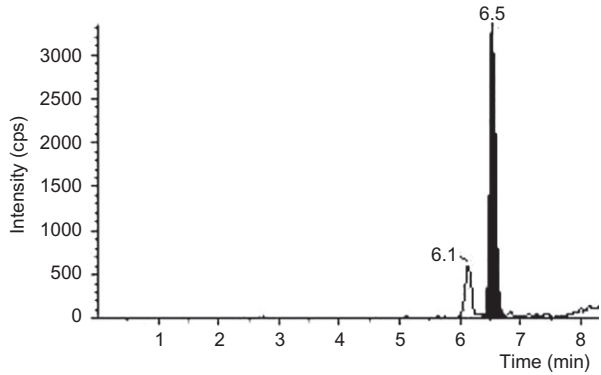

FIG. 6.5 Reconstituted ion chromatogram of abamectin.

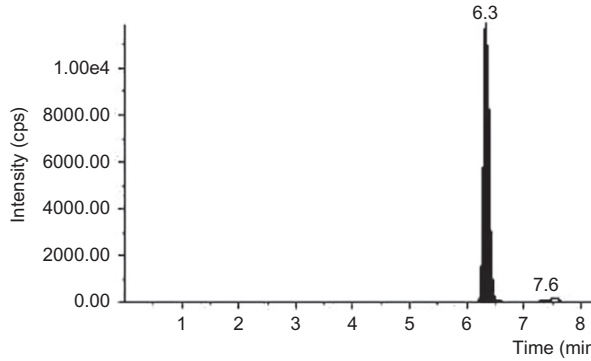

FIG. 6.6 Reconstituted ion chromatogram of doramectin.

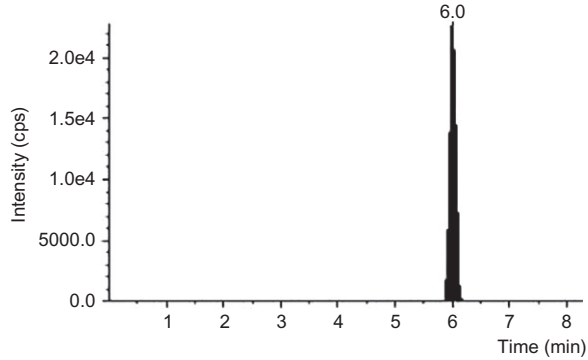

FIG. 6.7 Reconstituted ion chromatogram of eprinomectin.

6.8.7 PRECISION

The precision data is in accordance with GB/T 6379.1 and GB/T 6379.2. Repeatability and reproducibility are calculated according to the 95% confidence level.

(1) Repeatability

In repeatability conditions, the absolute difference between two independent test results does not exceed the repeatability limit (r). The scope of content and repeatability equations for analytes are shown in Tables 6.15 and 6.16.

If the absolute difference is more than the margin threshold repeatability limit, the test results should be discarded and the tests should be redone.

(2) Reproducibility

In reproducibility conditions, the absolute difference between two independent test results does not exceed the reproducibility limit (R). The scope of content and reproducibility equations for analytes are shown in Tables 6.15 and 6.16.

TABLE 6.15 The Scope of Content, Repeatability Equations, and Reproducibility Equations for Analytes (Milk)

Compound	Scope of Content (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Ivermectin	5–50	$r=0.332m-0.971$	$R=0.346m+0.182$
Abamectin	5–50	$r=0.271m-0.318$	$R=0.272m+0.323$
Doramectin	5–50	$r=0.259m-0.015$	$R=0.308m+0.599$
Eprinomectin	5–50	$r=0.230m+0.085$	$R=0.408m-1.24$

Remarks: m *is arithmetic average of two tests.*

TABLE 6.16 The Scope of Content, Repeatability Equations, and Reproducibility Equations for Analytes (Milk Powder)

Compound	Scope of Content (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Ivermectin	40–400	$r=0.215m-0.662$	$R=0.188m-0.262$
Abamectin	40–400	$r=0.220m+0.113$	$R=0.266m+0.333$
Doramectin	40–400	$r=0.229m-0.122$	$R=0.196m+0.026$
Eprinomectin	40–400	$r=0.111m+0.437$	$R=0.147m+0.179$

Remarks: m *is arithmetic average of two tests.*

6.6.8 RECOVERY

Under optimized condition, the recoveries of ivermectin, abamectin, doramectin, and eprinomectin residues in milk and milk powder using this method are listed in Table 6.17.

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TABLE 6.17 The Spiking Level and Recovery of Ivermectin, Abamectin, Doramectin, and Eprinomectin

Matrix	Compound	Spiking Level (μg/kg)	Recovery (%)
Milk	Ivermectin	5	100.6
		10	97.2
		20	103.0
		50	90.1
	Abamectin	5	82.4
		10	97.2
		20	87.7
		50	88.3
	Doramectin	5	87.6
		10	93.7
		20	98.3
		50	95.3
	Eprinomectin	5	84.5
		10	96.0
		20	90.2
		50	90.1
Milk powder	Ivermectin	40	96.7
		80	105.0
		160	86.0
		400	86.6
	Abamectin	40	81.8
		80	83.3
		160	84.9
		400	80.6
	Doramectin	40	88.9
		80	89.1
		160	89.7
		400	87.3
	Eprinomectin	40	86.6
		80	86.7
		160	89.0
		400	85.5

6.9

Determination of Ivermectin, Abamectin, Doramectin, and Eprinomectin Residues in Fugu, Eel, and Baked Eel—LC-MS-MS Method (GB/T 22953-2008)

6.9.1 SCOPE

This method is applicable to the determination of ivermectin, abamectin, doramectin, and eprinomectin residues in balloonfish, eel, and roasted eel.

The limit of quantitation of the method for ivermectin, abamectin, doramectin, and eprinomectin in balloonfish, eel, and roast eel is 5 μg/kg.

6.9.2 PRINCIPLE

Ivermectin, abamectin, doramectin, and eprinomectin residues are extracted from balloonfish, eel, and roasted eel with acetonitrile. The acetonitrile is defatted by n-hexane and purified through a neutral alumina column. The residues are determined by LC-MS and quantified by peak area, and an external standard method is applied.

6.9.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents are analytically pure; "water" is first grade water prescribed by GB/T 6682.

Acetonitrile: HPLC grade.
Methanol.
N-Hexane, saturated by acetonitrile before use.
Neutral Aluminum oxide, Brockmann Activity 1.
Anhydrous sodium sulfate: Ignite at 650°C for 4 h and store in a desiccator.
Neutral alumina SPE cartridge: Fill a vacant SPE cartridge in turn with a little defatted cotton, 2 g neutral aluminum oxide and 4 g anhydrous sodium sulfate. Prepare just before use.
Standard: ivermectin, abamectin, doramectin, and eprinomectin, purity $\geq$99%.
Standard stock solution: 100 μg/mL.

Separately, accurately weigh an adequate amount of ivermectin, abamectin, doramectin, and eprinomectin standards (accurate to 0.1 mg). Dissolve in acetonitrile and prepare a stock solution of 100 μg/mL. Store at −18°C; the standard solution of eprinomectin is stable for 3 months and the standard solutions of ivermectin, abamectin, and doramectin are stable for 1 year.
Mixed standard solution: 0.500 μg/mL.
Accurately transfer 0.500 mL of each stock solution into a 100-mL volumetric flask and dilute to volume with acetonitrile. The concentration of the solution is 0.500 μg/mL. The solution is stable for 3 months at −20°C.
Working standard solution with matrix: According to the requirement, accurately measure an adequate volume of mixed standard solution, and dilute with blank sample extraction solution to prepare an appropriate working standard solution with matrix. Prepare just before use.
Membrane filter: 0.2 μm, for organic solvent.

6.9.4 APPARATUS

LC-MS-MS, with ESI ion-source.
Electronic balance: 0.01 g and 0.001 g sensitivity.
Tissue blender.
Homogenizer: ≥8000 rpm.
Centrifuge: ≥4000 rpm.
Ultrasonic bath.
Vortex mixer.
Solid phase extractor with vacuum manifold.
Nitrogen evaporator.

6.9.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Sample, about 500 g, is blended and homogenized thoroughly in a blender and placed in clean containers as the test sample, which is then sealed and labeled. The test sample should be stored at −18°C.

In the course of sample preparation, precautions should be taken to avoid contamination or any factors that might cause a change of residue content.

(2) Extraction

Accurately weigh 2 g of the test sample (accurate to 0.01 g) into a 50-mL centrifuge tube. Add 8 mL acetonitrile and homogenize for 20 s at 8000 rpm.Then centrifuge for 5 min at 4000 rpm. Transfer the supernatant into a 50-mL centrifuge tube. Add 8 mL acetonitrile to another centrifuge tube and rinse the homogenizer probe for 10 s. Transfer the rinse solution to the first centrifuge tube and pound to pieces with glass rod. Then vortex to mix for 30 s and centrifuge at 4000 rpm for 5 min. Combine the supernatants in the 50-mL centrifuge tube. Add 6 mL acetonitrile to the first centrifuge tube and pound to pieces with glass rod again. Vortex to mix for 30 s

and centrifuge mixture at 4000 rpm for 5 min.Combine the supernatants in the 50-mL centrifuge tube, dilute to the mark of 25.0 mL with acetonitrile, and mix well for clean-up.

(3) Clean-up

Add 10 mL n-hexane saturated by acetonitrile to a 50-mL centrifuge tube with extraction solution, vortex to mix for 1 min, and centrifuge mixture at 4000 rpm for 5 min. Decant the n-hexane. Repeat this operation. The acetonitrile layer stand for clean.

Pass 10.0 mL sample extract solution through a neutral alumina column at a rate of 1–2 mL/min. Rinse the column with 2 mL × 2 of acetonitrile. Collect all eluent in a nitrogen evaporator tube. Evaporate to dryness below 50° C. Add 1.00 mL of acetonitrile to dissolve the residue and mix in an ultrasonic bath for 10 min. Filter the solution through a 0.2-μm organic filter for LC-MS-MS determination.

6.9.6 DETERMINATION

(1) Operating conditions

Column: Intersil C8-3, 5 μm, 150 mm × 4.6 mm (i.d.), or equivalent;
Column temperature: 40°C;
Mobile phase: Methanol + water, gradient eluent, see Table 6.18;
Flow rate: 0.8 mL/min;
Injection volume: 25 μL;
Ion source: ESI;
Scan polarity: Negative mode;
Scan mode: Multiple reaction monitoring (MRM);

Nebulizer gas, curtain gas, heater gas, and collision gas are high purity nitrogen or other equal gas; optimize the flow rate of gas to reach the requirement of the sensitivity of mass spectrometry. DP, EP, CE should be optimized to the best sensitivity;

Monitor ions and reference MS parameters are listed in Table 6.19.

TABLE 6.18 Elution Gradient of LC

Time (min)	Methanol (%)	Water (%)
0.00	75	25
3.00	100	0
10.00	100	0
10.01	75	25
15.00	75	25

TABLE 6.19 Monitor Ions and Reference MS Parameters

Compound	Qualitication Ion (m/z)	Quantification Ion (m/z)	Dwell Time (ms)	DP (V)	CE (V)
Ivermectin	873.7/567.8	873.7/229.2	100	−75	−37
	873.7/229.2				−50
Abamectin	871.7/565.2	871.7/565.2	100	−80	−40
	871.7/229.3				−54
Doramectin	897.6/591.4	897.6/591.2	100	−70	−38
	897.6/229.0				−51
Eprinomectin	912.5/270.0	912.5/565.4	100	−82	−49
	912.5/565.4				−37

(2) Qualitative determination

The qualitative ions for each analyst include at least one precursor ion and two product ions. Under the same determination conditions, the ratio of the chromatographic retention time of the analyte shall correspond to that of the calibration solution at a tolerance of ±2.5%.The relative intensities of the qualitative ions of each analyte shall correspond to those of the calibration standard at comparable concentrations, within the tolerances shown in Table 6.3, then the corresponding analyte must be present in the sample.

(3) Quantitative determination

Under optimal conditions of the equipment, inject the working standard solution with matrix (4.10). Using linear regression analysis, construct a standard curve by plotting the concentration in nanograms per milliliter (x), vs. peak area (y). The response of the sample must fall within the standard curve.

6.9.7 PRECISION

The precision data is in accordance with GB/T 6379.1 and GB/T 6379.2. Repeatability and reproducibility are calculated according to a 95% confidence level.

(1) Repeatability

In repeatability conditions, the absolute difference between two independent test results does not exceed the repeatability limit (r). The scope of content and repeatability equations for analytes are shown in Tables 6.20 to 6.22.

TABLE 6.20 The Scope of Content, Repeatability Equations, and Reproducibility Equations for Analytes (for Muscle of Balloonfish)

Compound	Scope of Content (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Ivermectin	5–50	$r=0.243m-0.242$	$R=0.273m$
Abamectin	5–50	$r=0.325m-0.76$	$R=0.26172m+0.277$
Doramectin	5–50	$r=0.228m+0.072$	$\lg R=0.965\lg m-0.591$
Eprinomectin	5–50	$\lg r=1.39\lg m-1.05$	$R=0.376m-1.43$

Remarks: m *is arithmetic average of two tests.*

TABLE 6.21 The Scope of Content, Repeatability Equations, and Reproducibility Equations for Analytes (for Muscle of Eel)

Compound	Scope of Content (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Ivermectin	5–50	$r=0.109m+0.367$	$R=0.217m-0.188$
Abamectin	5–50	$r=0.234m-0.702$	$R=0.191m+0.149$
Doramectin	5–50	$r=0.229m-0.363$	$R=0.262m-0.587$
Eprinomectin	5–50	$r=0.199m-0.563$	$R=0.203m-0.240$

Remarks: m *is arithmetic average of two tests.*

TABLE 6.22 The Scope of Content, Repeatability Equations, and Reproducibility Equations for Analytes (for Roast Eel)

Compound	Scope of Content (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Ivermectin	5–50	$\lg r=0.883\lg m-0.583$	$\lg R=1.15\lg m-0.875$
Abamectin	5–50	$r=0.252m-0.771$	$R=0.288m-0.775$
Doramectin	5–50	$r=0.239m-0.020$	$R=0.232m+0.214$
Eprinomectin	5–50	$r=0.295m-1.200$	$R=0.248m-0.478$

Remarks: m *is arithmetic average of two tests.*

If the absolute difference is more than the margin threshold repeatability limit, the test results should be discarded and the tests redone.

(2) Reproducibility

In reproducibility conditions, the absolute difference between two independent test results does not exceed the reproducibility limit (R). The scope of content and reproducibility equations for analytes are shown in Tables 6.20 to 6.22.

6.9.8 RECOVERY

The spiking level and recovery of ivermectin, abamectin, doramectin, and eprinomectin are shown in Table 6.23.

TABLE 6.23 The Spiking Level and Recovery of Ivermectin, Abamectin, Doramectin and Eprinomectin

Matrix	Compound	Spiking Level (μg/kg)	Recovery (%)
Muscle of balloonfish	Ivermectin	5	101.2
		10	104.1
		20	103.0
		50	99.7
	Abamectin	5	104.9
		10	96.4
		20	104.4
		50	102.7
	Doramectin	5	92.2
		10	96.4
		20	93.2
		50	96.1
	Eprinomectin	5	101.7
		10	85.7
		20	86.4
		50	89.9

TABLE 6.23 The Spiking Level and Recovery of Ivermectin, Abamectin, Doramectin and Eprinomectin—cont'd

Matrix	Compound	Spiking Level (μg/kg)	Recovery (%)
Muscle of eel	Ivermectin	5	95.7
		10	99.2
		20	100.0
		50	104.6
	Abamectin	5	95.2
		10	102.0
		20	99.4
		50	101.6
	Doramectin	5	80.1
		10	97.3
		20	102.0
		50	93.0
	Eprinomectin	5	83.2
		10	92.6
		20	86.1
		50	96.8
Roast eel	Ivermectin	5	96.7
		10	104.0
		20	92.0
		50	103.0
	Abamectin	5	105.0
		10	98.3
		20	106.0
		50	106.0
	Doramectin	5	85.3
		10	97.0
		20	91.3
		50	101.0
	Eprinomectin	5	87.9
		10	90.2
		20	89.8
		50	104.0

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6.10

Determination of Spiramycin, Pirlimycin, Oleandomycin, Tilmicosin, Erythromycin, and Tylosin Residues in Milk and Milk Powder—LC-MS-MS Method (GB/T 22988-2008)

6.10.1 SCOPE

This method is applicable to the determination of residues in milk and milk powder.

The limit of quantification for spiramycin, pirlimycin, oleandomycin, tilmicosin, erythromycin, and tylosin in milk is 1 μg/kg and in milk powder is 8 μg/kg.

6.10.2 PRINCIPLE

The residues in the test sample are extracted with acetonitrile and the extractions are cleaned up with solid-phase extraction (SPE). Determination is made using LC-MS-MS using an external standard method.

6.10.3 REAGENTS AND MATERIALS

Unless specifically noted, all reagents used should be of analytical grade; "water" is deionized water.

Acetonitrile, HPLC grade.
Methanol, HPLC grade.
Acetic acid, HPLC grade.
Ammonium formate, HPLC grade.

Disodium hydrogen phosphate.
Sodium hydrate.
Methanol-water (30+70, V/V): mix 30 volume units methanol and 70 volume units water.
5 mol/L sodium hydrate aqueous solution: weigh 20 g sodium hydrate and dissolve in 100 mL water.
0.1 mol/L phosphate buffer: weigh 6 g disodium hydrogen phosphate, dissolve in 400 mL water, adjust pH of the solution to 5.0 with sodium hydrate aqueous solution and make the volume up to 500 mL with water.
0.1 mol/L ammonium formate aqueous solution: weigh 0.63 g ammonium formate and dissolve in 1000 mL water.
Standards of spiramycin (CAS, 8025-81-8), pirlimycin (CAS79548-73-5), -oleandomycin (CAS, 7060-74-4), tilmicosin (CAS, 108050-54-0), erythromycin (CAS, 114-07-8), and tylosin (CAS, 1401-69-0): purity $\geq$98.0%.
Stock standard solution: Respectively, accurately weigh appropriate amount of standards and dissolve in 100 mL methanol to make a solution concentration of approximately 100 mg/mL.
Mixed standard solution: Accurately measure 1.00 mL stock standard solution respectively into a 100-mL umber volumetric flask; dilute with methanol to 100 mL and mix to homogeneity. The concentration of the solution is 1 μg/mL.
0.45 μm filter.
HLB column or equivalent: 500 mg, 6 mL; condition the column with 3 mL methanol, 3 mL water, and 3 mL phosphate buffer in sequence before using.

6.10.4 APPARATUS

High-performance LC-MS-MS.
Centrifuge.
Vortex shaker.
Rotary vacuum evaporator.
Nitrogen evaporator.
Vacuum manifold processing station.

6.10.5 SAMPLE PREPARATION

(1) Preparation of test sample

Milk

About 250 g representative samples should be taken from all samples and placed in clean sample containers, sealed, and labeled. The test samples should be stored at −4°C and kept away from light.

Milk powder

About 250 g representative samples should be taken from all samples and placed in clean sample containers, sealed, and labeled. The test samples should be stored at room temperature and kept away from light.

(2) Extraction

Milk

Weigh approximate 5 g (accurate to 0.01 g) of the test sample into a 50-mL centrifuge tube. Add 20 mL acetonitrile 2 min by vortex shaker. Centrifuge the tube for 10 min at 3000 rpm and filter the extraction into a heart-shaped flask. Then extract again using 10 mL acetonitrile and mix the extraction into the same flask.

Milk powder

Weigh approximately 1 g of the test sample, add 4 mL water, and vortex to homogeneity. Add 20 mL acetonitrile 2 min by vortex shaker. Centrifuge the tube for 10 min at 3000 rpm and filter the extraction into a heart-shaped flask. Then extract again using 10 mL acetonitrile and mix the extraction into the same flask.

(3) Clean-up

Evaporate the extractions to approximately 4 mL at 45°C using a rotary vacuum evaporator. Add 2 mL phosphate buffer and mix well. Transfer the solution into the reservoir above the conditioned HLB cartridge. Then dissolve the residues with another 2 mL phosphate buffer twice and pour the solution into the same reservoir. Pass the solution through the cartridge at a speed of less than 2 mL/min. After drop over, wash the cartridge with 3 mL water and 2 mL methanol-water in turn and dry the column by vacuum pump. Finally, elute the cartridge with 6 mL acetonitrile into a 10-mL tube with scale. Evaporate elution to approximately 1 mL with nitrogen evaporator at 45°C and meter the volume of the solution to 2 mL with ammonium formate aqueous solution. Then the solution is passed through a 0.45-μm filter and is ready for analysis.

(4) Preparation of blank matrix solution

For milk, accurately weigh 5 g negative sample, and for milk powder, accurately weigh 1 g negative sample (accurate to 0.01 g) to prepare the blank matrix solution, according to the above-mentioned extraction and cleanup steps.

6.10.6 DETERMINATION

(1) HPLC operating conditions

Column: Phenyl 150 mm × 2.1 mm (i.d.), 5 μm particle size, or equivalent;
Column temperature: 30°C;
Injection volume: 15 μL;
Mobile phase: The elution gradient and flow rate are listed in Table 6.24.
Ionization mode: ESI+;
Scan mode: MRM;
Sheath gas: 15 unit;
Auxiliary gas: 20 unit;
Ion spray voltage: 4000 V;
Capillary temperature: 320°C;

TABLE 6.24 Elution Gradient of LC

Time (min)	Flow Rate (μL/min)	0.1% Formic Acid Solution (%)	Methanol (%)
0.00	200	80	20
6.00	200	20	80
8.00	200	20	80
8.10	200	80	20
10.0	200	80	20

Source CID: 10V;
Width of Q1 and Q3: 0.7;
Collision gas: Argon;
Collision gas pressure: 1.5 mTorr;
Other mass operating conditions are listed in Table 6.25.

TABLE 6.25 The Scan Segment, Ion Pairs, and Collision Energy of the Analytes

Analyte	Retention Time (min)	Ion Pairs (m/z)	Collision Energy (eV)
Spiramycin	4.73	843.50/173.87[a]	36
		843.50/540.09	29
Pirlimycin	4.78	411.12/111.98[a]	25
		411.12/363.02	14
Oleandomycin	5.62	688.46/158.20[a]	35
		688.46/544.30	20
Tilmicosin	5.83	869.57/173.83[a]	41
		869.57/155.89	37
Erythromycin	6.12	734.18/157.88[a]	33
		734.18/576.13	20
Tylosin	6.33	916.36/173.80[a]	38
		916.36/772.18	28

[a]*Quantitative ion pair.*

(2) Qualitative analysis

The qualitative ions must at least include one precursor and two daughter ions. Under the same experimental conditions, the variation range of the retention time for the peak of the analyte in the unknown sample and in the matrix standard working solution can be out of range by only ±0.25%; and for the same analysis batch and the same compound, the variation of the ionization between the two daughter ions of the unknown sample and the matrix standard working solution at similar concentrations cannot be out of range of Table 6.3; then the corresponding analyte must be present in the sample.

(3) Quantitative analysis

Under optimum conditions of the apparatus, inject the series of mix matrix standard working solutions separately. The mix matrix standard working curves are made by plotting the responses vs. the concentration of standards. Use the curve to quantify each analyte in the unknown sample. The responses of the analyte in the standard working solution and the sample solution should be within the linear range of the instrument detection. Under these operating conditions, the chromatograms of the standard can be found in Fig. 6.8.

6.10.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the repeatability condition, the difference of absolute value of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equations of the five analytes in milk and milk powder are shown in Tables 6.26 and 6.27.

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned, and two independent experiments should be redone.

(2) Reproducibility

Under the reproducibility condition, the difference of absolute value of two independent results is not above the reproducibility limit (R). The content ranges and the reproducibility equations of the five analytes in milk and milk powder are shown in Tables 6.26 and 6.27.

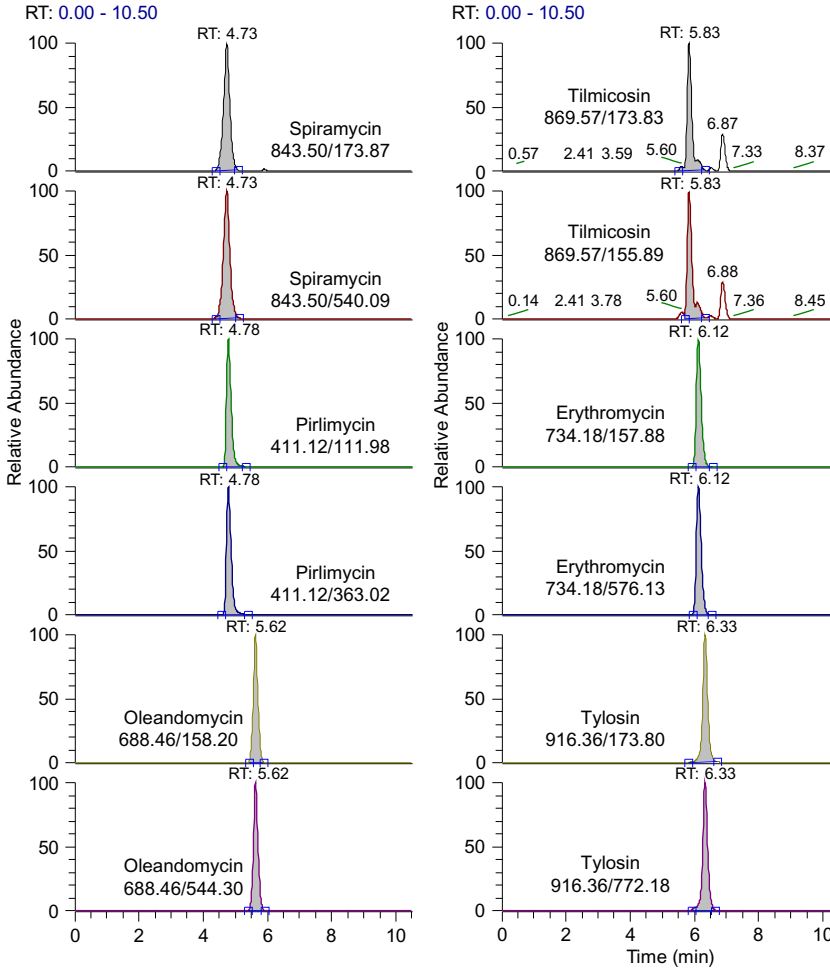

FIG. 6.8 MRM chromatograms of standard working solution.

6.10.8 RECOVERY

Under optimized condition, the recoveries of the spiramycin, pirlimycin, oleandomycin, tilmicosin, erythromycin, and tylosin in milk and milk powder using this method are listed in Table 6.28.

TABLE 6.26 Content Ranges, Repeatability and Reproducibility Equations for Milk Sample

Analyte	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Spiramycin	1–10	$\lg r = 1.1344 \lg m - 0.8765$	$\lg R = 0.8221 \lg m - 0.4995$
Pirlimycin	1–10	$r = 0.1762m - 0.0356$	$R = 0.2104m + 0.1712$
Oleandomycin	1–10	$\lg r = 0.9909 \lg m - 0.8121$	$\lg R = 1.1010 \lg m - 0.5519$
Tilmicosin	1–10	$r = 0.135m + 0.0002$	$R = 0.4061m - 0.3118$
Erythromycin	1–10	$\lg r = 0.8294 \lg m - 0.9144$	$\lg R = 0.8442 \lg m - 0.6306$
Tylosin	1–10	$\lg r = 1.1755 \lg m$-0.8883	$\lg R = 0.9544 \lg \mathrm{m} - 0.5613$

Note: m *equals to the average of two results.*

TABLE 6.27 Content Ranges, Repeatability and Reproducibility Equations for Milk Powder Sample

Analyte	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Spiramycin	8–80	$\lg r = 0.9740 \lg m - 0.6973$	$\lg R = 0.8565 \lg m - 0.4092$
Pirlimycin	8–80	$r = 0.1811m - 0.0675$	$R = 0.2055m + 1.1866$
Oleandomycin	8–80	$r = 0.1387m + 0.2924$	$R = 0.3701m - 1.4134$
Tilmicosin	8–80	$r = 0.1374m + 0.2364$	$R = 0.3727m - 2.1249$
Erythromycin	8–80	$\lg r = 0.8404 \lg m - 0.6594$	$\lg R = 0.8448 \lg m - 0.5026$
Tylosin	8–80	$\lg r = 1.0699 \lg m - 0.8131$	$\lg R = 0.9585 \lg m - 0.5348$

Note: m *equals to the average of two results.*

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TABLE 6.28 The Recovery Range of the Analytes in Milk and Milk Powder at Four Fortifying Levels

	Milk		Milk Powder	
Analyte	Spiked Level (μg/kg)	Recover Range (%)	Spiked Level (μg/kg)	Recover Range (%)
Spiramycin	1	78.0–87.0	8	79.1–98.0
	2	78.0–95.0	16	79.4–98.1
	5	82.2–97.2	40	79.8–105
	10	78.0–96.4	80	79.6–105
Pirlimycin	1	77.0–97.0	8	77.8–96.6
	2	73.0–94.5	16	70.0–94.4
	5	81.2–100	40	68.3–92.0
	10	78.3–92.0	80	78.4–94.5
Oleandomycin	1	78.0–105	8	74.8–98.1
	2	75.0–93.5	16	85.6–105
	5	82.2–98.0	40	80.3–99.8
	10	74.5–96.0	80	79.3–105
Tilmicosin	1	85.0–108	8	76.9–109
	2	72.5–94.5	16	81.3–98.1
	5	77.6–96.6	40	83.8–111
	10	72.4–93.6	80	81.1–101
Erythromycin	1	74.0–95.0	8	73.4–89.4
	2	81.5–96.5	16	85.0–94.4
	5	77.0–93.6	40	76.0–101
	10	79.4–91.1	80	77.0–94.6
Tylosin	1	77.0–88.0	8	81.0–105
	2	76.0–89.0	16	78.1–96.9
	5	79.2–95.0	40	83.8–107
	10	74.0–92.6	80	78.6–98.5

6.11

Determination of Lincomycin, Oleandomycin, Erythromycin, Tilmicosin, Tylosin, Spiramycin, Kitasamycin, and Josamycin Residues in Fugu and Eel—LC-MS-MS Method (GB/T 22964-2008)

6.11.1 SCOPE

This method is applicable to the determination of lincomycin, oleandomycin, erythromycin, tilmicosin, tylosin, spiramycin, kitasamycin, and josamycin residues in fugu and eel.

The limit of determination of this method for lincomycin, oleandomycin, erythromycin, tilmicosin, tylosin, spiramycin, kitasamycin, and josamycin is 2.0 μg/kg in fugu; lincomycin, erythromycin, tylosin, kitasamycin is 2.0 μg/kg, and eandomycin, tilmicosin, spiramycin, josamycin is 5.0 μg/kg in eel.

6.11.2 PRINCIPLE

The eight macrolide antibiotics (lincomycin, oleandomycin, erythromycin, tilmicosin, tylosin, spiramycin, kitasamycin, and josamycin) residues were extracted from fugu and eel with a Tris buffer. The residues are cleaned up on an Oasis HLB column. Elute the residues from the columns with methanol. Then concentrate the eluate and bring to constant volume. The solution is then ready for determination by LC-MS-MS, and is quantified by an internal standard of roxithromycin.

6.11.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be analytically pure.

Water: first grade water regulated by GB/T 6682.
Methanol: HPLC grade.
Ammonium acetate.
Acetonitrile: HPLC grade.
Chlorhydric acid.
Tris hydroxymethylaminomethane (tris): $C_4H_{11}NO_3$.

Calcium chloride: $CaCl_2 \cdot 2H_2O$.
Methanol water solution (2+3): Mix 400 mL methanol and 600 mL water.
0.01 mol/L Ammonium acetate solution: Dissolve 0.77 g ammonium acetate into a 1000-mL volumetric flask, bring to volume with water, and mix.
Constant volume solution: Mix 0.01 mol/L Ammonium acetate solution and Acetonitrile in the proportion of 17:3.
Tris solution: Dissolve 12.0 g tris (4.6) and 7.35 g $CaCl_2 \cdot 2H_2O$ in 1000 mL water. Adjust pH to 9 with chlorhydric acid.
Standards: Lincomycin (CAS 7179-49-9), oleandomycin (CAS 7060-74-4), erythromycin (CAS 59319-72-1), tilmicosin (CAS 108050-54-0), tylosin (CAS 74610-55-2), spiramycin (CAS 8025-81-8), kitasamycin (CAS 1392-21-8), josamycin (CAS 16846-24-5), and roxithromycin (CAS 80214-83-1); purity $\geq$95%.
Stock standard solutions of macrolide: 1.0 mg/mL. Accurately weigh 10 mg of each standard into different 10-mL volumetric flasks, dissolve in a small volume of methanol, and make up to volume with methanol. Store at 4°C.
Standard stock solutions of macrolide: 10.0 μg/mL. Pipette 0.1 mL each stock standard solutions of macrolide into different 10.0-mL volumetric flasks and make up to volume with methanol. Prepare weekly and store at 4°C.
Standard working solutions of macrolide: 2.0 μg/mL. Pipette 2 mL each standard stock solutions of macrolide into different 10.0-mL volumetric flasks and make up to volume with methanol.
Internal standard stock solution: 1.0 mg/mL. Accurately weigh 10.0 mg roxithromycin into 10.0-mL volumetric flask, dissolve in a small volume of methanol, and make up to volume with methanol. Store at 4°C.
Midst concentration internal standard stock solution: 10.0 μg/mL. Pipette 0.1 mL internal standard stock solution into 10-mL volumetric flask and make up to volume with methanol. Store at 4°C.
Internal standard working solution: 1.0 μg/mL. Pipette 1 mL midst concentration internal standard stock solution into 10-mL volumetric flask and make up to volume with methanol. Prepare fresh daily as required.
Matrix mixed standard working solution:
Matrix mixed standard working solution of eight macrolides (lincomycin, oleandomycin, erythromycin, tilmicosin, tylosin, spiramycin, kitasamycin, and josamycin) for fugu determination: pipette respectively 1.0 μL, 2.0 μL, 5.0 μL, and 25.0 μL standard working solutions, add 20.0 μL internal standard working solutions, dilute to 1.0 mL with blank sample extracts, and prepare into matrix standard working solutions of a series of concentrations 2.0 ng/mL, 4.0 ng/mL, 10.0 ng/mL, 50.0 ng/mL of lincomycin, oleandomycin, erythromycin, tilmicosin, tylosin, spiramycin, kitasamycin, and josamycin.
Matrix mixed standard working solution of macrolide for eel determination: pipette respectively 1.0 μL, 2.0 μL, 5.0 μL, and 25.0 μL standard working

solutions of lincomycin, erythromycin, tylosin, kitasamycin, and 2.5 μL, 5.0 μL, 10.0 μL, 25.0 μL standard working solutions of eandomycin, tilmicosin, spiramycin, and josamycin; add 20.0 μL internal standard working solutions, dilute to 1.0 mL with blank sample extracts, and prepare into matrix standard working solutions of a series of concentrations 2.0 ng/mL, 4.0 ng/mL, 10.0 ng/mL, 50.0 ng/mL of lincomycin, erythromycin, tylosin, kitasamycin, and matrix standard working solutions of a series of concentrations 5.0 ng/mL, 10.0 ng/mL, 20.0 ng/mL, 50.0 ng/mL of eandomycin, tilmicosin, spiramycin, and josamycin.
Oasis HLB solid extraction column: 500 mg, 6 mL. Condition each column with 10 mL methanol followed by 10 mL water before use.
Filtration membrane: 0.2 μm.

6.11.4 APPARATUS

LC-MS-MS: Equipped with ESI source.
Analytical balance: Capable weighing to 0.01 g, 0.0001 g.
Solid phase extraction apparatus.
Nitrogen evaporator.
Centrifuge tubes: 50 mL, polypropylene with screw cap.
Centrifuge: centrifugal force $\geq$20,000 g.
Ultrasonic Cleaning Instrument.
pH meter.
Shaker.

6.11.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Take representative samples of 1 kg from the total samples, shred and mix them thoroughly, break into two equal parts and load respectively into a clean container. These will serve as test samples after being sealed, and should be clearly marked or labeled. Precautions should be taken to prevent the samples from being contaminated or from encountering any change of the residue content in the sampling and sample preparation operations.

The test samples should be stored below −18°C.

(2) Extraction

Weigh 5 g test sample (accurate to 0.01 g) into 50-mL centrifuge tube (5.5). Add 20.0 μL Internal standard working solution and 10.0 mL tris solution, vortex to mix, and shake for 10 min on high speed horizontal shaker, and centrifuge at 12,000 g for 10 min. Transflux the supernatant to the Oasis HLB solid extraction column with a ratio of 1.0 mL/min. Repeat addition of 10.0 ml Tris solution, vortex to mix, shake for 10 min on high speed horizontal shaker, centrifuge at 12,000 g for 10 min and transflux the supernatant to the Oasis HLB solid extraction column at the ratio of 1.0 mL/min.

(3) Clean-up

After the extract all flows out, wash the column with 10 mL water and 10 mL methanol-water solution. Discard all eluates. Dry the cartridge by drawing air through it for 1 h by vacuum pump. Finally, elute macrolides with 10 mL methanol into 15-mL N blowpipe. Evaporate the extract under nitrogen in a water bath at 50°C to minimum. Accurately add 1.0 mL constant volume solution to reconstitute residues in an ultrasonic cleaning instrument. Repeat the steps to prepare blank sample with negative sample. Filtrate the solution with 0.2-μm filter membrane and move on to determination using LC-MS-MS.

6.11.6 DETERMINATION

(1) Operation conditions

Chromatographic column: Atlantis C_{18}, 3 μm, 150 mm × 2.1 mm i.d. or equivalent;
Mobile phase A: Acetonitrile;
Mobile phase B: 0.1% methanoic acid-water solution;
Mobile phase C: Methanol;
Gradient conditions acetonitrile. See Table 6.29;
Flow rate: 0.2 mL/min;
Column temperature: 30°C;
Injection volume: 20 μL.
Ion source: ESI source;
Scan mode: Positive scan;
Monitor mode: Multiple reaction monitor (MRM);
Ionspray voltage: 5500 V;
Nebulizer gas: 0.24 MPa;
Turbo ionspray gas rate: 0.4 L/min;
Source temperature: 550°C;
collision voltage: 20 V;

TABLE 6.29 Gradient Conditions

Time (min)	A (%)	B (%)	C (%)
0.00	90.0	5.0	5.0
5.00	5.0	90.0	5.0
9.00	5.0	90.0	5.0
9.10	90.0	5.0	5.0
17.0	90.0	5.0	5.0

Precursor/product ion combinations, collision energy and declustering potential: see Table 6.30.

(2) Qualitative analysis

Under identical test conditions, the difference of the ratios of the retention time of the analyte in the awaiting sample solutions and those of the analyte in the matrix standard working solutions shall fall within ±2.5%. Moreover, the ratio difference of the relative abundance of each qualifying ion in the analyte of the awaiting sample solutions and that of the analyte in the matrix standard working solutions of nearly identical concentrations shall not exceed the range specified in Table 6.3; the corresponding analyte is then judged to be existent in the sample.

(3) Quantitative analysis

In the optimum working conditions of the instrument, sample injections with matrix standard working solution are made and a standard working curve is drawn, based on the ratio of testing sample solution peak area with internal standard peak area as ordinate and the ratio of testing sample solution concentration with internal standard concentration as abscissa.

TABLE 6.30 Precursor/Product Ion Combinations, Collision Energy and Declustering Potential of Nine Macrolides

Macrolide	Precursor Ion Combinations (m/z)	Product Ion Combinations (m/z)	Collision Energy (V)	Declustering Potential (V)
Lincomycin	407/126 407/359	407/126	37 24	50 50
Oleandomycin	688/158 688/544	688/158	70 42	30 30
Erythromycin	734/158 734/576	734/158	42 28	50 50
Tilmicosin	869/174 869/132	869/174	62 70	90 90
Tylosin	916/174 916/145	916/174	54 55	80 80
Spiramycin	843/142 843/174	843/142	48 50	60 60
Kitasamycin	772/215 772/109	772/215	43 42	70 70
Josamycin	828/174 828/109	828/174	45 45	80 80

Quantitative analysis is conducted using the working curve of the matrix standard working solutions, trying to make the response values of eight macrolide antibiotics fall within the linear range of the instrumental detection.

Under the previously mentioned chromatic and mass spectrometric conditions, the reference response times for eight macrolide antibiotics are shown in Table 6.31.

The chromatograms for multiple response monitoring of eight macrolide antibiotics are given in Figs. 6.9–6.16.

TABLE 6.31 Reference Retention Times for Macrolide Antibiotics

Macrolide	Retention Time (min)
Lincomycin	6.96
Oleandomycin	7.36
Erythromycin	8.06
Tilmicosin	8.36
Tylosin	8.48
Spiramycin	8.67
Kitasamycin	8.83
Josamycin	9.14

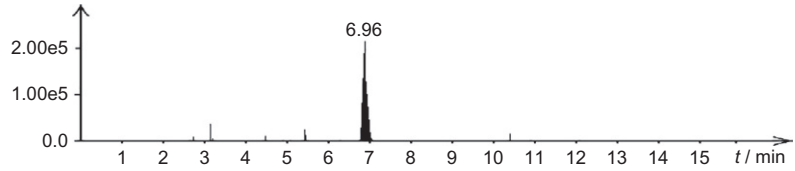

FIG. 6.9 Chromatogram for multiple reaction monitoring of lincomycin standards.

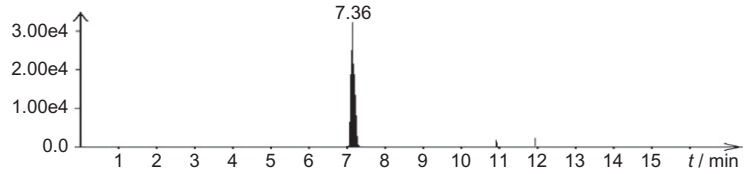

FIG. 6.10 Chromatogram for multiple reaction monitoring of oleandomycin standards.

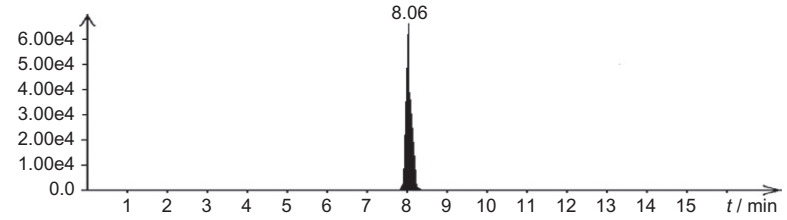

FIG. 6.11 Chromatogram for multiple reaction monitoring of erythromycin standards.

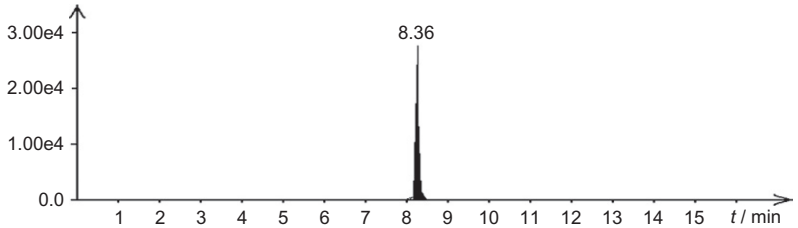

FIG. 6.12 Chromatogram for multiple reaction monitoring of tilmicosin standards.

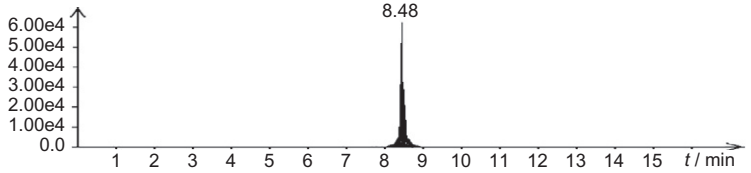

FIG. 6.13 Chromatogram for multiple reaction monitoring of tylosin standards.

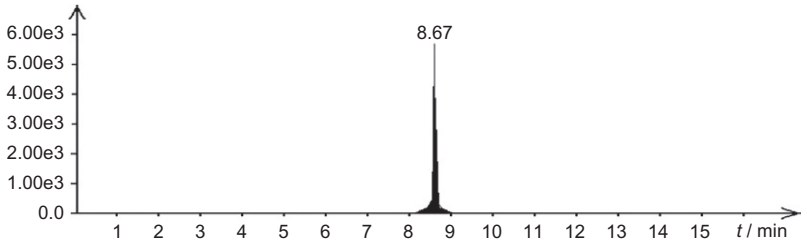

FIG. 6.14 Chromatogram for multiple reaction monitoring of spiramycin standards.

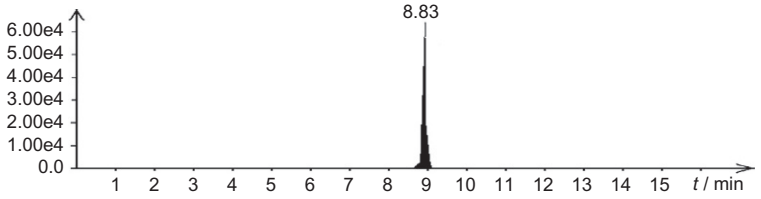

FIG. 6.15 Chromatogram for multiple reaction monitoring of kitasamycin standards.

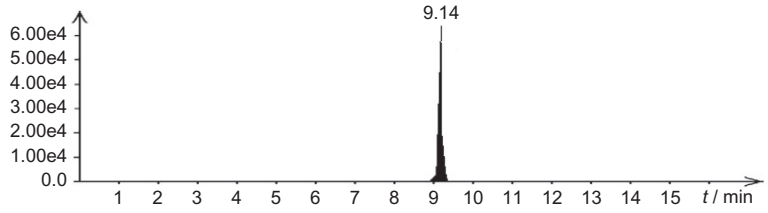

FIG. 6.16 Chromatogram for multiple reaction monitoring of josamycin standards.

6.11.7 PRECISION

The precision data of the method for this standard have been determined in accordance with the stipulations of GB/T 6379.The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for macrolides in fugu and eel tissues are shown in Table 6.32.

If the difference of values exceeds the limit of repeatability (r), the testing results should be discarded and two individual testing determinations shall be reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for phenylbutazone in muscle tissues are shown in Table 6.32.

TABLE 6.32 Range, Repeatability, and Reproducibility (μg/kg)

Macrolide	Range	Repeatability (r)	Reproducibility (R)
Lincomycin	2.0–50.0	$\lg r = 1.0319 \lg m - 1.0199$	$\lg R = 1.1879 \lg m - 0.6782$
Oleandomycin	2.0–50.0	$\lg r = 0.7337 \lg m - 0.7835$	$\lg R = 0.8225 \lg m - 0.4402$
Erythromycin	2.0–50.0	$\lg r = 0.9962 \lg m - 1.0136$	$\lg R = 0.9399 \lg m - 0.4795$
Tilmicosin	2.0–50.0	$\lg r = 1.2925 \lg m - 1.2341$	$\lg R = 1.0931 \lg m - 0.5760$
Tylosin	2.0–50.0	$\lg r = 0.8905 \lg m - 1.0104$	$\lg R = 0.9828 \lg m - 0.5994$
Spiramycin	2.0–50.0	$\lg r = 1.0276 \lg m - 1.1067$	$\lg R = 0.8969 \lg m - 0.4389$
Kitasamycin	2.0–50.0	$\lg r = 0.9403 \lg m - 1.0077$	$\lg R = 0.9771 \lg m - 0.5434$
Josamycin	2.0–50.0	$\lg r = 0.8163 \lg m - 0.9362$	$\lg R = 0.9073 \lg m - 0.5037$

Note: The m *is average value of parallel test results.*

6.11.8 RECOVERY

Under optimized condition, the concentrations and the average recoveries of eight macrolide antibiotics are given in Table 6.33.

TABLE 6.33 Test Data of Spiked Concentrations and the Average Recoveries of Eight Macrolide Antibiotics

Macrolide	Fortifying Concentration (μg/kg)	Recovery (%)
Lincomycin	2.0	91.0
	4.0	93.9
	8.0	92.5
	20.0	87.2
Oleandomycin	2.0	106.6
	4.0	103.1
	8.0	90.3
	20.0	91.2
Erythromycin	2.0	98.1
	4.0	93.0
	8.0	85.3
	20.0	93.4
Tilmicosin	2.0	86.8
	4.0	94.2
	8.0	86.8
	20.0	88.6
Tylosin	2.0	85.8
	4.0	88.9
	8.0	88.7
	20.0	91.9
Spiramycin	2.0	96.2
	4.0	87.8
	8.0	92.4
	20.0	94.4

TABLE 6.33 Test Data of Spiked Concentrations and the Average Recoveries of Eight Macrolide Antibiotics—cont'd

Macrolide	Fortifying Concentration (μg/kg)	Recovery (%)
Kitasamycin	2.0	96.4
	4.0	99.1
	8.0	84.9
	20.0	83.4
Josamycin	2.0	89.6
	4.0	89.1
	8.0	83.6
	20.0	89.4

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6.12

Determination of Lincomycin, Erythromycin, Spiramycin, Tilmicosin, Tylosin, Josamycin, Kitasamycin, Oleandomycin Residues in Honey—LC-MS-MS Method (GB/T 22941-2008)

6.12.1 SCOPE

This method is applicable to the determination of lincomycin, erythromycin, spiramycin, tilmicosin, tylosin, josamycin, kitasamycin, and oleandomycin residues in honey.

The limit of determination of this method of lincomycin, erythromycin, spiramycin, tilmicosin, tylosin, josamycin, kitasamycin, and oleandomycin is 2.0 μg/kg.

6.12.2 PRINCIPLE

The lincomycin, erythromycin, spiramycin, tilmicosin, tylosin, josamycin, kitasamycin, and oleandomycin drugs are extracted from honey with tris buffer (pH 9); after filtering, the honey solution is cleaned up by Oasis HLB or equivalent solid extraction columns, the drugs are eluted with methanol and dryness, the dregs are dissolved with acetonitrile 0.01 mol/L ammonium acetate solution, and it is then put through a 0.2-μm filter membrane. The solution is then used for determination by LC-MS-MS, using an internal standard method.

6.12.3 REAGENTS AND MATERIALS

Water: GB/T 6682, First-level.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Tris-hydroxymethyl-amino-methane (Tris): G.R.
Calcium chloride ($CaCl_2 \cdot 2H_2O$): G.R.
Ammonium acetate: G.R.
Hydrochloric acid: G.R.
Rinse solution: methanol-water (2+3, v/v). Mix 40 mL methanol and 60 mL water.
0.01 mol/L Ammonium acetate solution: Weigh 0.77 g ammonium acetate and dissolve in 1000 mL water.
0.2 mol/L Tris buffer solution: Weigh 12.0 g Tris-hydroxymethyl-amino-methane and 7.35 g calcium chloride into a 1000-mL volumetric flask; dissolve in 800 mL water and adjust pH to 9.0 by hydrochloric acid. Dilute solution to volume with water.
Dissolve solution: Acetonitrile 0.01 mol/L ammonium acetate solution (3+17, v/v). Mix 15 mL acetonitrile and 85 mL 0.01 mol/L ammonium acetate solution.
Macrolide standards: lincomycin (CAS 7179-49-9), erythromycin (CAS 59319-72-1), spiramycin (CAS 8025-81-8), tilmicosin (CAS 108050-54-0), tylosin (CAS 74610-55-2), josamycin (CAS 16846-24-5), kitasamycin (CAS 1392-21-8), and oleandomycin (CAS 7060-74-4): purity $\geq$99%.
Standard stock solutions of macrolides: 0.1 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the standard stock solutions of 0.1 mg/mL in concentration. The standard stock solutions are stored at 4°C.

Standards working solution: 1.0 μg/mL. Pipette 0.1 mL standard stock solution into 10-mL volumetric flask and dilute solution to volume with methanol. The standard working solutions are stored at 4°C.
Internal standards: Roxithromycin (CAS 80214-83-1) and clindamycin (CAS 21462-39-5). Purity ≥99%。
Internal standard stock solution of macrolides: 0.1 mg/mL. Accurately weigh appropriate amount of each internal standard, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the internal standard stock solutions of 0.1 mg/mL in concentration. The internal standard stock solutions are stored at 4°C.
Mixed internal standard working solutions of macrolides: 1.0 μg/mL. Pipette 0.1 mL each internal standards stock solutions into 10-mL volumetric flasks, respectively. Dilute the internal standards solution to volume with methanol. The internal standard working solutions are stored at 4°C.
Base standard working solutions: Pipette different volume mixed standard working solutions of macrolides and 20 μL mixed internal standard working solutions of macrolides. Prepare the base standard working solutions of 2.0 ng/mL, 5.0 ng/mL, 10.0 ng/mL, 20.0 ng/mL, 50.0 ng/mL different concentrations for macrolides with honey control sample extract. Prepare fresh daily.
Oasis HLB solid extraction columns or equivalent: 200 mg, 6 mL. Condition each column with 5 mL methanol followed by 10 mL water.
Filter membrane: 0.2 μm.

6.12.4 APPARATUS

LC-MS-MS: Equipped with ESI source.
Analytical balance: capable of weighing to 0.1 mg, 0.01 g.
Nitrogen evaporator.
Vortex mixer.
Reservoirs and adapters to fit SPE columns: 50 mL
Vacuum pump: Vacuum should attain 80 kPa.
Solid phase extraction vacuum apparatus.
Conical tube: 10 mL.

6.12.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath and warm at ≤60°C with occasional shaking until liquefied. Mix thoroughly and promptly cool it to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label.

The test samples should be stored at ambient temperature.

(2) Extraction

Weigh 2-g test sample (accurate to 0.01 g) into 150-mL Erlenmeyer flask. Add 20 μL mixed internal standard working solutions and 25 mL Tris buffer solution to each sample. Mix vigorously 1 min on vortex mixer, until honey is completely dissolved.

(3) Clean-up

Connect a reservoir with glass wool plug to Oasis HLB solid extraction columns. The honey sample solution is decanted into the reservoir. Let the solution pass through the Oasis HLB columns at reduced pressure, adjusting the flow rate to $\leq$3 mL/min. Wait till sample solution has thoroughly drained, and then rinse the cartridge respectively with 5 mL water and 5 mL rinse solution. Discard all the effluents. Dry the cartridge by drawing air through it for 20 min. Finally, elute macrolides with 5 mL methanol into the 10-mL conical tube. The elution solution is blown to dryness on nitrogen evaporator at 50°C. Accurately add 1 mL mobile phase to dregs, and then put it through 0.2-μm filter membrane and move on to determination by LC-MS-MS.

6.12.6 DETERMINATION

(1) LC Operation conditions

Chromatographic column: Atlantis C18, 3 μm, 150 mm × 2.1 mm (i.d.) or equivalent;
Flow: 0.2 mL/min;
Column temperature: 35°C;
Injection volumes: 20 μL;
Mobile phase: A: Acetonitrile, B: 0.1% Formic acid solution, C: Methanol. Gradient elution condition: see Table 6.34.
Ion source: ESI source;
Scan mode: Positive scan;
Monitor mode: Multiple reaction monitor;
Ionspray voltage: 5500 V;

TABLE 6.34 Gradient Elution Condition

Time (min)	A (%)	B (%)	C (%)
0.00	90.0	5.0	5.0
5.00	5.0	90.0	5.0
9.00	5.0	90.0	5.0
9.10	90.0	5.0	5.0
17.0	90.0	5.0	5.0

Nebulizer gas: 0.076 MPa;
Curtain gas: 0.069 MPa;
Turbo ionspray gas rate: 6 L/min;
Source temperature: 450°C;
Precursor/product ion combinations, declustering potential, collision energy: see Table 6.35.

(2) Qualitative determination

Select one precursor and more than two daughters. In the same conditions, if the ratio error of the chromatographic retention time of the analyte and the standard corresponds within ±2.5% compared with the matrix standard solutions, and the relative ion intensity of the clenbuterol in the sample and matrix standard solutions accords with Table 6.3, then this sample has the macrolide.

(3) Quantitation determination

The base standard working solutions of different concentrations for macrolides are prepared with honey control sample extract on base. Then inject 20 μL of the different concentration working standard solutions, respectively, in duplication under LC and MS conditions. Draw these

TABLE 6.35 Precursor/Product Ion Combinations, Declustering Potential, Collision Energy

Drug	Precursor Ion Combinations (m/z)	Production ion Combinations (m/z)	Collision Energy (V)	Declustering Potential (V)
Lincomycin	407/126 407/359	407/126	37 24	50 50
Erythromycin	688/158 688/544	688/158	42 23	30 30
Spiramycin	734/158 734/576	734/158	42 28	50 50
Tilmicosin	869/174 869/132	869/174	62 70	90 90
Tylosin	916/174 916/145	916/174	54 55	80 80
Josamycin	425/126 425/377	425/126	45 25	53 53
Kitasamycin	843/142 843/174	843/142	48 50	60 60
Oleandomycin	772/215 772/109	772/215	42 70	70 70

5-point standard curves of macrolides (peak area vs. clenbuterol concentration). Calculate the concentrations of the corresponding content from the working curve. The responses of macrolides in the standard working solution and sample solution should be in the linear range of the instrumental detection. Under LC and MS conditions, for the reference retention time of the macrolides, see Table 6.36.

6.12.7 PRECISION

The precision data of the method for this standard have been determined in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for lincomycin, erythromycin, spiramycin, tilmicosin, tylosin, josamycin, kitasamycin, and oleandomycin in honey are shown in Table 6.37.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations should be reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for lincomycin, erythromycin, spiramycin, tilmicosin, tylosin, josamycin, kitasamycin, and oleandomycin in honey are shown in Table 6.37.

TABLE 6.36 Reference Retention Time of Macrolides

Drug	Retention Time (min)
Lincomycin	7.61
Erythromycin	9.96
Spiramycin	8.91
Tilmicosin	9.56
Tylosin	10.04
Josamycin	11.33
Kitasamycin	10.62
Oleandomycin	9.72

TABLE 6.37 Content Range, Repeatability and Reproducibility (Unit μg/kg)

Drug	Fortifying Concentration Range	Repeatability (r)	Reproducibility (R)
Lincomycin	2.0–50.0	$\lg r = 1.0510 \lg m - 1.2650$	$\lg R = 0.8656 \lg m - 0.6520$
Erythromycin	2.0–50.0	$\lg r = 1.0335 \lg m - 1.2251$	$\lg R = 1.1108 \lg m - 0.9901$
Spiramycin	2.0–50.0	$\lg r = 0.8702 \lg m - 1.1014$	$\lg R = 1.1028 \lg m - 1.0160$
Tilmicosin	2.0–50.0	$\lg r = 1.0934 \lg m - 1.3491$	$\lg R = 0.9621 \lg m - 0.8070$
Tylosin	2.0–50.0	$\lg r = 0.9707 \lg m - 1.1463$	$\lg R = 0.8157 \lg m - 0.6801$
Josamycin	2.0–50.0	$\lg r = 0.9379 \lg m - 1.1770$	$\lg R = 0.8571 \lg m - 0.7565$
Kitasamycin	2.0–50.0	$\lg r = 1.0208 \lg m - 1.3386$	$\lg R = 0.9598 \lg m - 0.9004$
Oleandomycin	2.0–50.0	$\lg r = 0.9757 \lg m - 1.2742$	$\lg R = 0.8863 \lg m - 0.5849$

Note: The m *is average value of parallel test results.*

6.12.8 RECOVERY

Under optimized condition, the recoveries of lincomycin, erythromycin, spiramycin, tilmicosin, tylosin, josamycin, kitasamycin, and oleandomycin residues in honey using this method are listed in Table 6.38.

TABLE 6.38 The Data of Macrolides Fortifying Concentrations and Recoveries

Drug	Fortifying Concentration (μg/kg)	Recovery (%)
Lincomycin	2.0	100.0
	5.0	99.4
	10.0	95.7
	50.0	94.9
Erythromycin	2.0	85.7
	5.0	86.2
	10.0	87.7
	50.0	90.8

Continued

TABLE 6.38 The Data of Macrolides Fortifying Concentrations and Recoveries—cont'd

Drug	Fortifying Concentration (μg/kg)	Recovery (%)
Spiramycin	2.0	83.9
	5.0	83.7
	10.0	82.8
	50.0	87.7
Tilmicosin	2.0	84.9
	5.0	87.6
	10.0	90.4
	50.0	89.3
Tylosin	2.0	86.4
	5.0	84.4
	10.0	86.7
	50.0	88.8
Josamycin	2.0	80.6
	5.0	86.0
	10.0	77.7
	50.0	84.0
Tilmicosin	2.0	79.3
	5.0	83.4
	10.0	79.3
	50.0	83.0
Tylosin	2.0	83.0
	5.0	83.8
	10.0	84.6
	50.0	83.0

6.13

Determination of Lincomycin, Erythromycin, Tilmicosin, Tylosin, Spiramycin, Clindamycin, Kitasamycin, and Josamycin Residues in Royal Jelly and Lyophilized Royal Jelly Powder—LC-MS-MS Method (GB/T 22946-2008)

6.13.1 SCOPE

This method is applicable to the determination of lincomycin, erythromycin, tilmicosin, tylosin, clindamycin, spiramycin, kitasamycin, and josamycin residues in royal jelly and lyophilized powder.

The limit of determination of this method for lincomycin, erythromycin, tilmicosin, tylosin, clindamycin, spiramycin, kitasamycin, and josamycin is 2.0 μg/kg in royal jelly; lincomycin, erythromycin, clindamycin, tylosin, josamycin is 2.0 μg/kg; and tilmicosin, spiramycin, and kitasamycin is 5.0 μg/kg in lyophilized powder.

6.13.2 PRINCIPLE

The eight macrolide antibiotics (lincomycin, erythromycin, tilmicosin, tylosin, clindamycin, spiramycin, and kitasamycin) residues are extracted from royal jelly and lyophilized powder with Tris solution. The residues are cleaned up on an Oasis HLB column. Elute the residues from the columns with methanol. The eluate is concentrated and brought to constant volume. The solution is then used for determination by LC-MS-MS and quantified by an external standard.

6.13.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be analytically pure.

Water: first grade water regulated by GB/T 6682.
Methanol: HPLC grade.
Ammonium acetate
Acetonitrile: HPLC grade.
Chlorhydric acid
Tris hydroxymethylaminomethane (tris): $C_4H_{11}NO_3$

Calcium chloride: $CaCl_2 \cdot 2H_2O$.
Methanol water solution (2+3): Mix 400 mL methanol and 600 mL water.
0.01 mol/L Ammonium acetate solution: Dissolve 0.77 g ammonium acetate into a 1000-mL volumetric flask, bring to volume with water and mix.
Constant volume solution: Mix 0.01 mol/L Ammonium acetate solution and Acetonitrile in volume proportion of 17:3.
Tris solution: Dissolve 12.0 g tris and 7.35 g $CaCl_2 \cdot 2H_2O$ (3.7) in 1000 mL water. Adjust pH to 9 with chlorhydric acid.
Standards: Lincomycin (CAS 7179-49-9), erythromycin (CAS 59319-72-1), tilmicosin (CAS 108050-54-0), tylosin (CAS 74610-55-2), clindamycin (CAS 21462-39-5), spiramycin (CAS 8025-81-8), kitasamycin (CAS 1392-21-8), josamycin (CAS 16846-24-5), and roxithromycin (CAS 80214-83-1), purity $\geq$95%
Stock standard solutions of macrolide: 1.0 mg/mL. Accurately weigh 10 mg of each standard into different 10-mL volumetric flasks, dissolve in a small volume of methanol, and make up to volume with methanol. Store at 4°C.
Standard stock solutions of macrolide: 10.0 μg/mL. Pipette 0.1 mL each stock standard solutions of macrolide into different 10.0-mL volumetric flasks and make up to volume with methanol. Prepare weekly and store at 4°C.
Standard working solutions of macrolides: 2.0 μg/mL. Pipette 2 mL each standard stock solutions of macrolide into different 10.0-mL volumetric flasks and make up to volume with methanol.
Oasis HLB solid extraction column: 500 mg, 6 mL. Condition each column with 10 mL methanol followed by 10 mL water before use.
Filtration membrane: 0.2 μm.

6.13.4 APPARATUS

LC-MS-MS: Equipped with ESI source.
Analytical balance: Capable of weighing to 0.01 g, 0.0001 g.
Solid phase extraction apparatus.
Nitrogen evaporator.
Centrifuge tubes: 50 mL, polypropylene with screw cap.
Centrifuge: centrifugal force $\geq$20,000 g.
Ultrasonic cleaning instrument.
pH meter.
Shaker.

6.13.5 SAMPLE PRETREATMEN

(1) Preparation of rest sample

Take representative samples of 200 g from the total samples, mix them thoroughly, break into two equal parts and load respectively into a clean

container. These will serve as test samples after being sealed and shall be clearly marked or labeled. Precautions should be taken to prevent the samples from getting any contamination or any change of residue content in the sampling and sample preparation operations.

The royal jelly should be stored below −18°C. The lyophilized powder should be sealed for storage to prevent absorption of moisture.

(2) Extraction

Weigh 2 g test sample of royal jelly (1 g lyophilized powder), accurate to 0.01 g, into 50-mL centrifuge tube. Add 10.0 mL Tris solution, vortex to mix, and shake for 10 min on high speed horizontal shaker, and centrifuge at 18,000 g for 10 min. Transflux the supernatant to the Oasis HLB solid extraction column with a ratio of 1.0 mL/min. Repeat addition of 10.0 mL Tris solution, vortex to mix, shake for 10 min on high speed horizontal shaker, centrifuge at 18,000 g for 10 min and transflux the supernatant to the Oasis HLB solid extraction column with a ratio of 1.0 mL/min.

(3) Clean-up

After the extract all flowing out, wash the column with 10 mL water and 10 mL Methanol water solution. Discard all eluates. Dry the cartridge by drawing air through it for 1 h by vacuum pump. Finally elute macrolides with 10 mL methanol into 15 mL N blowpipe. Evaporate the extract under nitrogen in water bath at 50°C to minimum. Accurately add 1.0 mL constant volume solution to reconstitute residues in ultrasonic cleaning instrument. Repeat the steps to prepare blank sample with negative sample. Filtrate the solution with 0.2-μm filter membrane and detect use LC-MS-MS.

(4) Matrix mixed standard working solutions for royal jelly determination

Pipette respectively 1.0 μL, 2.0 μL, 5.0 μL, and 25.0 μL standard working solutions of eight macrolides, add homological negative samples, according to above-mentioned extraction and cleanup steps to prepare into matrix mix standard working solutions of a series of concentrations 2.0 ng/mL, 4.0 ng/mL, 10.0 ng/mL, 50.0 ng/mL of lincomycin, erythromycin, tilmicosin, tylosin, clindamycin, spiramycin, kitasamycin, and josamycin.

(5) Matrix mixed standard working solutions for lyophilized powder determination

Pipette respectively 1.0 μL, 2.0 μL, 5.0 μL and 25.0 μL standard working solutions of lincomycin, erythromycin, tylosin, clindamycin, and josamycin and 2.5 μL, 5.0 μL, 10.0 μL, 25 μL standard working solutions of tilmicosin, spiramycin, and kitasamycin; add homological negative samples, according to above-mentioned extraction and cleanup steps to prepare into matrix mix standard working solutions of a series of concentrations 2.0 ng/mL, 4.0 ng/mL, 10.0 ng/mL, 50.0 ng/mL of lincomycin, erythromycin, clindamycin, tylosin, and josamycin and 5.0 ng/mL, 10.0 ng/mL, 20.0 ng/mL, 50.0 ng/mL of tilmicosin, spiramycin, and kitasamycin.

6.13.6 DETERMINATION

(1) LC Operation conditions

Chromatographic column: Atlantis C_{18}, 3 μm, 150 mm × 2.1 mm (i.d.), or equivalent;
Mobile phase A: Acetonitrile, B: 0.1% Formic acid water solution C: Methanol. Gradient conditions acetonitrile. See Table 6.39;
Flow rate: 0.2 mL/min;
Column temperature: 30°C;
Injection volume: 20 μL.
Ion source: ESI source;
Scan mode: Positive scan;
Monitor mode: Multiple reaction monitor (MRM);
Ionspray voltage: 5500 V;
Nebulizer gas: 0.24 MPa;
Turbo ionspray gas rate: 0.4 L/min;
Source temperature: 550°C;
Room collision export voltage: 20 V;
Precursor/product ion combinations, collision energy and declustering potential, see Table 6.40.

(2) Qualitative analysis

In identical test conditions, the difference of the ratios of the retention time of the analyte in the awaiting sample solutions (7.2) and that of the analyte in the matrix standard working solutions shall fall within ±2.5%. Moreover, the ratio difference of the relative abundance of each qualifying ion in the analyte of the awaiting sample solutions and that of the analyte in the matrix standard working solutions of nearly identical concentrations shall not exceed the range specified in Table 6.3, and the corresponding analyte is then judged to be existent in the sample.

(3) Quantitative analysis

In optimum working conditions of the instrument, a standard working curve is drawn, based on the matrix standard working solution

TABLE 6.39 Gradient Conditions

Time (min)	A (%)	B (%)	C (%)
0.00	90.0	5.0	5.0
5.00	5.0	90.0	5.0
9.00	5.0	90.0	5.0
9.10	90.0	5.0	5.0
17.0	90.0	5.0	5.0

TABLE 6.40 Precursor/Product Ion Combinations, Collision Energy and Declustering Potential, of Eight Macrolides

Macrolide	Precursor ion Combinations (m/z)	Product Ion Combinations (m/z)	Collision Energy (V)	Declustering Potential (V)
Lincomycin	407/126 407/359	407/126	37 24	50 50
Erythromycin	734/158 734/576	734/158	42 28	50 50
Tilmicosin	869/174 869/132	869/174	62 70	90 90
Tylosin	916/174 916/145	916/174	54 55	80 80
Clindamycin	425/126 425/377	425/126	45 28	53 53
Spiramycin	843/142 843/174	843/142	48 50	60 60
Kitasamycin	772/215 772/109	772/215	43 42	70 70
Josamycin	828/174 828/109	828/174	45 45	80 80

concentration as ordinate and peak area as abscissa. Quantitative analysis is conducted using the working curve of the matrix standard working solutions, trying to make the response values of eight macrolide antibiotics fall within the linear range of the instrumental detection.

Under the previously mentioned chromatic and mass spectrometric conditions, the reference response times for eight macrolides antibiotics are shown in Table 6.41.

6.13.7 PRECISION

The precision data of the method for this standard have been determined in accordance with the stipulations of GB/T 6379. The values of repeatability and reproducibility obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not

TABLE 6.41 Reference Retention Time for Macrolides Antibiotics

Macrolide	Retention Time (min)
Lincomycin	6.96
Erythromycin	8.06
Tilmicosin	8.36
Tylosin	8.48
Clindamycin	8.51
Spiramycin	8.67
Kitasamycin	8.83
Josamycin	9.14

exceed the limit of repeatability (r); the content range and repeatability equations for macrolides are shown in Table 6.42.

If the difference of values exceeds the limit of repeatability (r), the testing results should be discarded and two individual testing determinations shall be reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for macrolides are shown in Table 6.42.

TABLE 6.42 Range, Repeatability and Reproducibility (μg/kg)

Macrolide	Range	Repeatability (r)	Reproducibility (R)
Lincomycin	2.0–50.0	$\lg r = 0.9392 \lg m - 1.0304$	$\lg R = 0.8741 \lg m - 0.5182$
Erythromycin	2.0–50.0	$\lg r = 0.7974 \lg m - 0.9811$	$\lg R = 1.0506 \lg m - 0.5775$
Tilmicosin	2.0–50.0	$\lg r = 1.0318 \lg m - 0.9859$	$\lg R = 0.9853 \lg m - 0.5561$
Tylosin	2.0–50.0	$\lg r = 1.0569 \lg m - 0.9993$	$\lg R = 0.8513 \lg m - 0.5616$
Clindamycin	2.0–50.0	$\lg r = 1.0798 \lg m - 1.1495$	$\lg R = 0.8854 \lg m - 0.4146$
Spiramycin	2.0–50.0	$\lg r = 0.9125 \lg m - 0.9751$	$\lg R = 1.0432 \lg m - 0.5917$
Kitasamycin	2.0–50.0	$\lg r = 1.1001 \lg m - 1.0250$	$\lg R = 1.1453 \lg m - 0.7665$
Josamycin	2.0–50.0	$\lg r = 1.4389 \lg m - 1.4185$	$\lg R = 1.1227 \lg m - 0.6965$

Note: The m *is average value of parallel test results.*

6.13.8 RECOVERY

Under optimized condition, the concentrations and the average recoveries of eight macrolide antibiotics are given in Table 6.43.

TABLE 6.43 Test Data of Spiked Concentrations and the Average Recoveries of Eight Macrolides Antibiotics

Macrolide	Fortifying Concentration (μg/kg)	Recovery (%)
Lincomycin	2.0	94.9
	5.0	94.9
	10.0	101.9
	20.0	88.8
Erythromycin	2.0	97.1
	5.0	98.0
	10.0	96.6
	20.0	94.1
Tilmicosin	2.0	94.2
	5.0	98.7
	10.0	92.7
	20.0	85. 8
Clindamycin	2.0	89.6
	5.0	86. 8
	10.0	96.6
	20.0	91.9
Tylosin	2.0	98.4
	5.0	98.1
	10.0	93.7
	20.0	90.5
Spiramycin	2.0	87.6
	5.0	93.1
	10.0	95.8
	20.0	92.2

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Chapter 7

Nitrofurans

7.1

Curative Effects and Side Effects of Nitrofurans

Furazolidone, furaltadone, nitrofurazone, and nitrofurantoin are veterinary drugs that belong to the nitrofuran group, which have been used in the treatment of infections caused by *Escherichia coli* and *Salmonella* in pigs, poultry, and fishes. It has been demonstrated that a proportion of the bound residues of furazolidone and furaltadone possess intact side-chains that have molecular characteristics in common with the parent compounds. These side-chains can be released from the bound metabolites under mildly acidic conditions such as may occur in the stomach of the consumer. It has been suggested that the furazolidone side-chain, 3-amino-2-oxazolidinone (AOZ), can be metabolized into β-hydroxyethylhydrazine, which is a mutagenic and carcinogenic compound. Because no safe limit for the presence of these drugs in food products for human consumption could be assigned, nitrofurans have been banned for food-producing animals by the European Union (EU) since 1995.

7.2

Pharmacokinetics of Nitrofurans

Nitrofurans are rapidly metabolized and are not detected a few hours after administration. Their metabolites, however, remain bound to tissue proteins for several months. Metabolites of each nitrufuran are shown in Fig. 7.1 (Table 7.1).

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00007-5

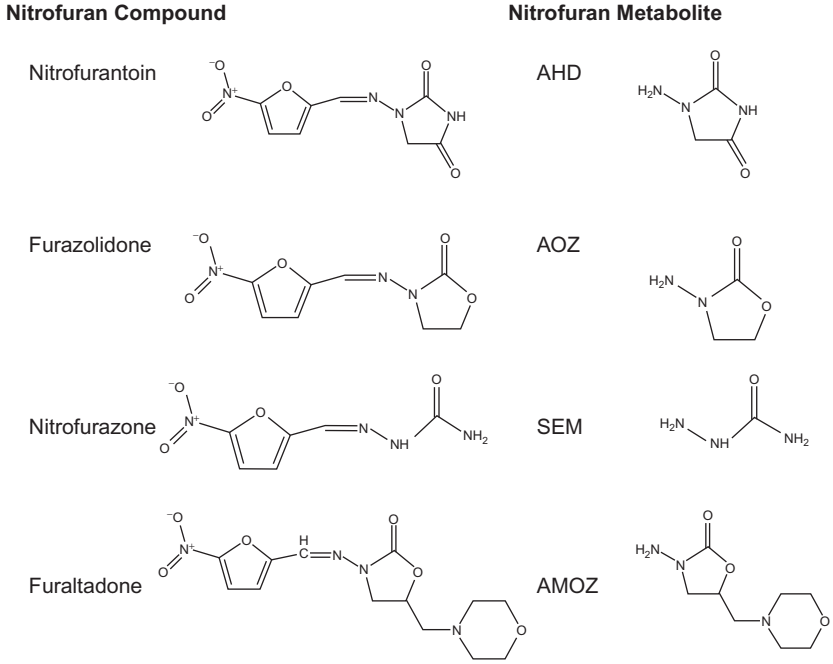


FIG. 7.1 Metabolites considered for the analysis of the residues of the four nitrofurans in food from animal origin.

TABLE 7.1 Chemical Structures for Nitrofuran Metabolites

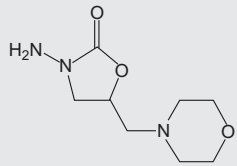
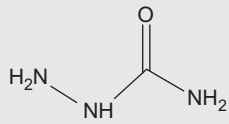
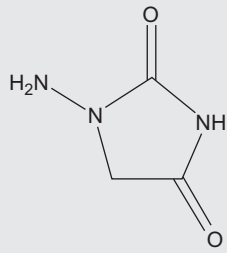
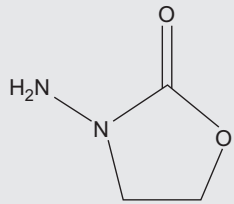
Compound Names	Molecular Structure	Molecular Weight	Cas. No	MRL (μg/kg)
5-Morpholinomethyl-3-amino-2-oxazolidinone		201.22	43056-63-9	America: banned China: banned EU: banned Japan: banned Canada: banned
Semicarbazide		75.07	57-56-7	America: banned China: banned EU: banned Japan: banned Canada: banned

TABLE 7.1 Chemical Structures for Nitrofuran Metabolites—cont'd

Compound Names	Molecular Structure	Molecular Weight	Cas. No	MRL (μg/kg)
1-Aminohydantoin		115.09	282756-7	America: banned China: banned EU: banned Japan: banned Canada: banned
3-Amino-2-oxazolidinone		102.09	80-65-9	America: banned China: banned EU: banned Japan: banned Canada: banned

7.3

Determination of Residues of the Metabolites of Nitrofurans in Pork, Beef, Chicken, Porcine Liver, and Aquatic Products—LC-MS-MS Method (GB/T 20752-2006)

7.3.1 SCOPE

This method is applicable to the determination of 3-amino-5-morpholino-methyl-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone in muscle tissues of porcine, bovine, poultry, hepatic, and aquatic products (fisheries, shrimp, crab, and shellfish).

The limit of determination of this method for 3-amino-5-morpholinomethyl-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone is 0.5 μg/kg.

7.3.2 PRINCIPLE

The metabolites of nitrofuran in sample undergo hydrolysis and derivatization with 2-nitrobenzaldehyde. The solution obtained is cleaned up with Oasis HLB or equivalent extraction. Residues are analyzed by LC-MS-MS.

7.3.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be guaranteed pure; "water" is deionized water.

Methanol: HPLC grade; Acetonitrile: HPLC grade; Ethyl acetate: HPLC grade; Dipotassium hydrogen phosphate, K_2HPO_4; Acetate acid; Dimethyl sulfoxide; Hydrochloric acid; Sodium hydroxide; 2-Nitrobenzaldehyde (2-NBA), $C_7H_5NO_3$: purity ≥99%. Dipotassium hydrogen phosphate solution: 0.1 mol/L. Place 17.4 g dipotassium hydrogen phosphate into a 1000-mL volumetric flask and dissolve in water. Dilute solution to volume with water; Hydrochloric acid solution: 0.2 mol/L. Combine 17 mL hydrochloric acid into 1000-mL volumetric flask. Dilute solution to volume with water; Sodium hydroxide: 1 M. Place 40 g sodium hydroxide into 1000-mL volumetric flask and dissolve in water. Dilute solution to volume with water; Derivatized solutions: 0.05 M. Place 0.075 g 2-Nitrobenzaldehyde in 10 mL dimethyl sulfoxide. Prepare daily before use; Dilute solution: 10 mL acetonitrile + 0.3 mL acetate acid solution; dilute with water into 100-mL volumetric flask.

3-Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), semicarbazide (SEM), 1-aminohydantoin (AHD), and 3-amino-2-oxazolidinone (AOZ) standards: purity ≥99%; Internal standards of four metabolites of nitrofuran are D_5-AMOZ, $^{13}C^{15}N$-SEM, $^{13}C_3$-AHD, D_4-AOZ: purity ≥99%; 0.2-μm syringe filter.

Standard stock solutions of 3-amino-5-morpholinomethyl-2-oxazolidinone, semicarbazide, 1-aminohydantoin and 3-amino-2-oxazolidinone: 1.0 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volume of methanol, then dilute with methanol separately to prepare the standard stock solutions of 1.0 mg/mL in concentration. Keep at −18°C away from exposure to any light. Storage life is 6 months.

Standard working solutions of 3-amino-5-morpholinomethyl-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone: 0.1 μg/mL. Accurately pipette appropriate amount of each standard stock solution; mix and dilute with methanol separately to prepare the standard working solutions of 0.1 μg/mL in concentration. Keep at −18°C. Storage life is 3 months; return to room temperature before use.

Standard stock solutions of internal standards: 1.0 mg/mL. Accurately weigh appropriate amount of each drug internal standard, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the internal standard stock solutions of 1.0 mg/mL in concentration. Keep at −18°C. Storage life is 3 months.

Standard working solutions of internal standards: 0.1 μg/mL. Accurately pipette appropriate amount of each internal standard stock solution. Mix and dilute with methanol separately to prepare the internal standard working solutions of 0.1 μg/mL in concentration. Keep at −18°C. Storage life is 3 months; return to room temperature before use.

Oasis HLB solid extraction columns: 60 mg, 3 mL. Condition each column with 5 mL methanol followed by 10 mL water before use.

7.3.4 APPARATUS

LC-MS-MS: Equipped with ESI source; Analytical balance: Capable of weighing to 0.1 mg, 0.01 g; Homogenizer; Solid phase extraction vacuum apparatus; Nitrogen evaporator; Incubator; Vacuum pump: Vacuum to 80 kPa; Microsyringes: 25 μL, 100 μL; Brown centrifuge tubes: 25 mL, 50 mL; pH meter: capable of measuring ±0.02 unit; Reservoirs and adapters to fit SPE columns: 50 mL; Centrifuge.

7.3.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Comminute tissue (muscle or liver) using the disintegrator, and take 0.5 kg as the test sample. The test samples should be stored at ambient temperature.

(2) Hydrolysis, derivatization, and extraction

Weigh 2-g samples (accurate to 0.01 g) into 50-mL brown centrifuge tubes; add 10 mL methanol-H2O (2+1) and homogenize for 1 min and then rinse the homogenizer with an additional 5 mL methanol-H2O (2+1) and mix in the centrifuge tubes. Centrifuge at 4000 rpm for 5 min and discard the supernatant. Add standard solutions of the four metabolites of nitrofuran. Form 0.5 ng/mL, 1.0 ng/mL, 2.0 ng/mL, 4.0 ng/mL, 10 ng/mL solutions of the four metabolites of nitrofuran. Add internal standard as 2 ng/mL of the four metabolites of nitrofuran.

Add 10 mL 0.2 mol/L hydrochloric acid solution and 0.3 mL derivatized solution to each sample, homogenize for 1 min, and then rinse the homogenizer with an additional 10 mL 0.2 mol/L hydrochloric acid solution and mix in the centrifuge tubes. The reaction mixture is kept for 16 h at 37°C.

(3) Clean-up

After this period of time, samples are removed and allowed to cool to room temperature. A pH value of about 7.4 is adjusted by addition of 5 mL

of 0.1 mol/L dipotassium hydrogen phosphate solution and an amount of 1.0 mol/L sodium hydroxide. Centrifuge at 4000 rpm for 10 min. The solution is decanted into a preconditioned Oasis HLB solid extraction column with reservoir, and the flow rate is adjusted to $\leq$2 mL/min. Let the solution pass through the Oasis HLB solid extraction column. Wait until the solution has thoroughly drained and then rinse evaporate tube with 10 mL water and pass it through the Oasis HLB solid extraction column. Discard all the effluents. Dry the cartridge by drawing air through it for 10 min under a 65 kPa vacuum. Finally, elute the derivatives with 5 mL ethyl acetate into 25-mL brown centrifuge tubes. Evaporate the elute solution to dryness on a nitrogen evaporator at 40°C. Accurately add 1 mL dilute solution. Filter with a 0.2-μm syringe filter and determine using LC-MS-MS.

7.3.6 DETERMINATION

(1) Operation conditions

LC column: Atlantis-C_{18}, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent; Column temperature: 35°C; Injection volumes: 40 μL; Mobile phase and flow rate: see Table 7.2.

Ion source: ESI source; Scan mode: Positive scan; Monitor mode: Multiple reaction monitor; Ion spray voltage: 5000 V; Turbo ion spray gas rate: 7 L/min; Source temperature: 480°C; Focusing potential: 150 V; Collision cell exit potential: 11 V; Declustering potential (DP): 45 V; Precursor/product ion combinations, collection time (dwell) and collision energy: see Table 7.3.

(2) Qualitative analysis

Select one precursor and more than two daughters. Under the same conditions, if the ratio error of the chromatographic retention time of the analyte and the internal standard is between ±2.5% compared with the matrix standard solutions, and if the relative ion intensity of the metabolite of nitrofuran in the sample and matrix standard solutions accords with Table 7.4, then this sample has the nitrofuran.

TABLE 7.2 Mobile Phase and Flow Rate

Time (min)	Flow Rate (μL/min)	0.3% Acetate Acid Solution (%)	Acetonitrile + 0.3% Acetate Acid (%)
0.00	200	80	20
3.00	200	50	50
8.00	200	50	50
8.01	200	80	20
16.00	200	80	20

TABLE 7.3 Precursor/Product Ion Combinations, Collection Time (Dwell) and Collision Energy

Derivatized Metabolite of Nitrofuran and Internal Standard	Qualifying Parent/ Production (m/z)	Quantifying Parent/ Production (m/z)	Time (ms)	Collision Energy (V)
2-NP-AMOZ	335/291 335/128	335/291	100	18 16
2-NP-SEM	209/192 209/166	209/166	150	17 15
2-NP-AHD	249/134 249/178	249/134	200	19 22
2-NP-AOZ	236/134 236/192	236/134	100	19 17
2-NP-D5-AMOZ	340/296	340/296	100	18
2-NP-13C15N-SEM	212/168	212/168	100	15
2-NP-13C3-AHD	252/134	252/134	100	32
2-NP-D4-AOZ	240/134	240/134	100	22

TABLE 7.4 Maximum Permitted Tolerances for Relative Ion Intensities

Relative ion intensities	$K > 50\%$	$20\% < K < 50\%$	$10\% < K < 20\%$	$K \leq 10\%$
Maximum permitted tolerances	±20%	±25%	±30%	±50%

(3) Quantitative analysis

Internal standard method: Use the software for the instrument.

External standard method: Inject the different concentrations of mixed matrix calibration standard solution of 3-amino-5-morpholinomethyl-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone, respectively, in duplication under LC and MS conditions. Draw the standard curves of the four metabolites of nitrofuran (concentration vs. peak area). Calculate the concentrations of the corresponding content from the working curves. The responses of the four metabolites of nitrofuran in the sample solutions should be in the linear range of the instrumental detection. The MRM chromatograms of the analyte standard and internal standards are shown in Fig. 7.2.

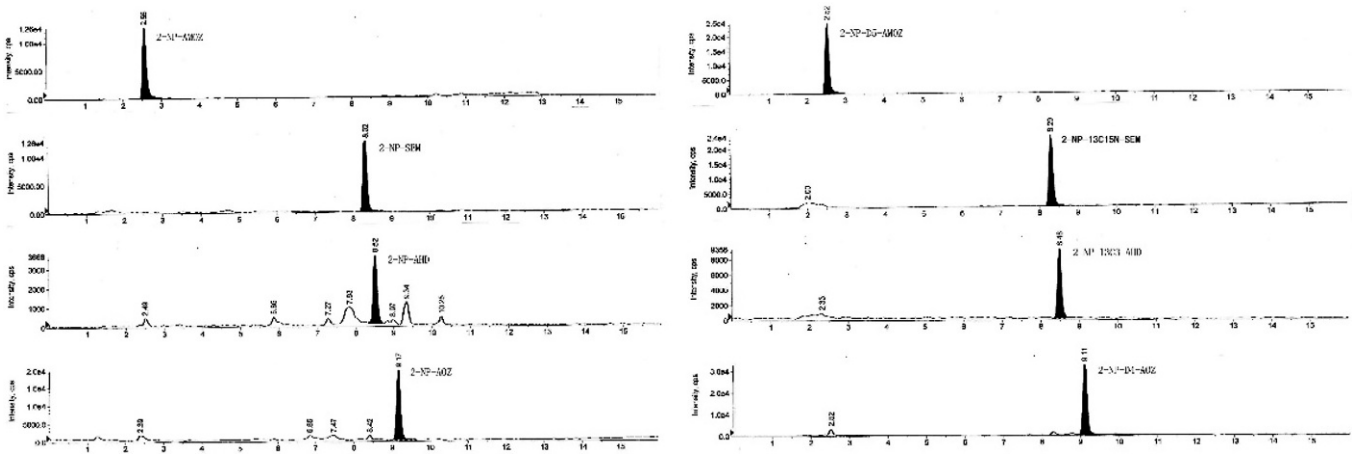


FIG. 7.2 MRM chromatogram of the analyte standard and internal standards

TABLE 7.5 Analytical Range, Repeatability, and Reproducibility

Metabolite of Nitrofuran	Content Range (μg/kg)	Limit of Repeatability (r)	Limit of Reproducibility (R)
5-Morpholinomethyl-3-amino-2-oxazolidinone	0.2–4	$\lg r = 0.9656 \lg m - 1.0897$	$\lg R = 0.9625 \lg m - 0.7128$
Semicarbazide	0.5–10	$\lg r = 0.9660 \lg m - 1.1445$	$\lg R = 0.9152 \lg m - 0.6513$
1-Aminohydantoin	0.5–10	$\lg r = 0.9844 \lg m - 1.0935$	$R = 0.2058 m + 0.0119$
3-Amino-2-oxazolidinone	0.2–4	$r = 0.0735 m + 0.0016$	$\lg R = 1.0813 \lg m - 0.7982$

Note: m*: The average values obtained from two independent determination results.*

7.3.7 PRECISION

The precision of the method has been determined in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r). For repeatability and content range of this method, see Table 7.5.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations shall be reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); for reproducibility and content range of this method, see Table 7.5.

If the difference of values exceeds the limit of reproducibility, the testing results should be discarded and two individual testing determinations shall be reconducted and completed.

7.3.8 RECOVERY

Under optimized conditions, the recoveries of 3-amino-5-morpholinomethyl-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone using this method are listed in Table 7.6.

TABLE 7.6 The Recoveries ($n = 10$) of 3-Amino-5-morpholinomethyl-2-oxazolidinone, Semicarbazide, 1-Aminohydantoin, and 3-Amino-2-oxazolidinone

Derivatized Metabolite of Nitrofuran	Fortifying Concentration (μg/kg)	Average Recovery (%) in Different						
		Muscle Tissues of Porcine	Muscle Tissues of Bovine	Muscle Tissues of Poultry	Muscle Tissues of Hepatic	Fisheries	Shrimps	Shellfishes
2NP-AMOZ	0.2	88.2	90.1	91.2	85.4	89.5	82.3	84.5
	0.5	90.3	88.2	92.3	83.2	87.9	84.1	87.8
	1.0	85.6	85.5	88.6	82.4	90.1	85.2	88.2
	2.0	87.8	90.6	85.9	84.1	85.6	83.1	85.6
2NP-SEM	0.2	89.1	88.9	89.7	82.4	88.9	81.2	82.3
	0.5	87.6	87.5	88.6	86.6	87.8	86.6	84.5
	1.0	86.8	86.8	86.9	82.1	85.6	85.8	86.2
	2.0	85.4	85.4	85.8	83.5	87.2	82.6	82.1
2NP-AHD	0.5	84.2	83.2	88.9	82.3	88.2	82.1	82.1
	1.0	87.8	85.9	89.6	81.6	85.7	83.2	82.8
	2.0	88.1	87.1	87.5	84.6	84.6	84.2	83.6
	5.0	83.6	82.6	86.8	84.7	82.8	81.6	84.2
2NP-AOZ	0.2	91.2	90.2	92.1	88.2	90.2	88.2	88.2
	0.5	90.3	91.0	90.5	87.3	85.3	87.9	89.1
	1.0	88.6	88.9	89.6	85.6	91.2	86.2	85.4
	2.0	86.2	85.6	88.2	84.8	88.6	85.4	83.2

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FURTHER READING

[1] Leitner A, Zollner P, Linder WJ. J Chromatogr A 2001;939:49.
[2] McCracken RJ, Kennedy DG. J Chromatogr 1997;691:87.

7.4

Determination for the Residues of Furaltadon, Nitrofurazone, Nitrofurantoin, and Furazolidone Metabolites in Milk and Milk Powder—LC-MS-MS Method (GB/T 22987-2008)

7.4.1 SCOPE

This method is applicable to the determination of AMOZ, SEM, AHD, and AOZ residues in milk and milk powder.

The limit of determination of this method for AMOZ, SEM, AHD, and AOZ is 0.2 μg/kg in milk; the limit of determination of this method for AMOZ, SEM, AHD, and AOZ is 0.2 μg/kg in milk.

7.4.2 PRINCIPLE

The metabolites of AMOZ, SEM, AHD, and AOZ in sample undergo hydrolysis and derivatization with 2-nitrobenzaldehyde. The solution obtained is cleaned up with Oasis HLB or equivalent extraction. Determine the residues by LC-MS-MS.

7.4.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be guaranteed pure; "water" is deionized water.

Water: GB/T 6682, the first degree.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Ethyl acetate: HPLC grade.
Dipotassium hydrogen phosphate, K_2HPO_4.
Acetate acid.
Dimethyl sulfoxide.
Hydrochloric acid.
Trichloroacetic acid, $C_2HCl_3O_2$.
Sodium hydroxide.
2-Nitrobenzaldehyde (2-NBA), $C_7H_5NO_3$: purity $\geq$99%.
Dipotassium hydrogen phosphate solution: 0.1 mol/L. Place 17.4 g dipotassium hydrogen phosphate into 1000-mL volumetric flask and dissolve in water. Dilute solution to volume with water.
Hydrochloric acid solution: 0.2 mol/L. Combine 17 mL hydrochloric acid into 1000-mL volumetric flask. Dilute solution to volume with water.
Sodium hydroxide: 1 mol/L. Place 80 g sodium hydroxide into 500-mL volumetric flask and dissolve in water. Dilute solution to volume with water.
Trichloroacetic acid: 0.5 mol/L. Place 37.5 g trichloroacetic acid in 500-mL volumetric flask and dissolve in water. Dilute solution to volume with water.
Derivatized solutions: 0.05 mol/L. Place 0.075 g 2-Nitrobenzaldehyde in 10 mL dimethyl sulfoxide. Freshly prepare each day before use.
Dilute solution: 10 mL acetonitrile +0.3 mL acetate acid solution; dilute with water in 100-mL volumetric flask.
Standard: AMOZ (CAS:43056-63-9), SEM (CAS:563-41-7), AHD (CAS:2827-56-7), and AOZ (CAS:80-65-9), purity $\geq$99%.
Internal standards of four metabolites of nitrofuran are D_5-AMOZ, $^{13}C^{15}N$-SEM, $^{13}C_3$-AHD, and D_4-AOZ: purity $\geq$99%.
Standard stock solutions: 1.0 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the standard stock solutions of 1.0 mg/mL in concentration. Keep at $-18°C$ away from exposure to any light.
Standard working solutions: 0.1 μg/mL. Accurately pipette appropriate amount of each standard stock solution. Mix and dilute with methanol separately to prepare the standard working solutions of 0.1 μg/mL in concentration. Keep at $-18°C$ and away from exposure to any light.
Standard stock solutions of internal standards: 1.0 mg/mL. Accurately weigh appropriate amount of each drug internal standard, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the

internal standard stock solutions of 1.0 mg/mL in concentration. Keep at −18°C and away from exposure to any light.
Standard working solutions of internal standards: 0.1 μg/mL. Accurately pipette appropriate amount of each internal standard stock solution. Mix and dilute with methanol separately to prepare the internal standard working solutions of 0.1 μg/mL in concentration. Keep at −18°C and away from exposure to any light.
Oasis HLB solid extraction columns: 60 mg, 3 mL. Condition each column with 5 mL methanol followed by 10 mL water before use.
0.2 μm syringe filter.

7.4.4 APPARATUS

LC-MS-MS: Equipped with ESI source.
Analytical balance: Capable of weighing to 0.1 mg, 0.01 g.
Homogenizer.
Solid phase extraction vacuum apparatus.
Nitrogen evaporator.
Incubator.
Vacuum pump: Vacuum to 80 kPa.
Microsyringes: 25 μL, 100 μL.
Brown centrifuge tubes: 25 mL, 50 mL.
pH Meter: capable of measuring ±0.02 unit.
Reservoirs and adapters to fit SPE columns: 50 mL.
Centrifugal machine: rotate speed > 4000 rpm.

7.4.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Take the representative portions from the whole primary sample, about 1 kg, and grind in a blender. Keep the prepared sample in two sample bottles, sealed and labeled. In the course of sample preparation, precautions must be taken to avoid contamination or any factors that may cause a change of residue content.

The test samples should be stored at −18°C.

(2) Mixed matrix calibration standard solution: experiment sample solution

Drod proteinic hydrolysis and derivatization of milk

Weigh 8-g milk samples (accurate to 0.01 g) into 50-mL brown centrifuge tubes. Add 15 mL trichloroacetic acid solutions and 0.2 mL derivatized solution and add adequate standard working solutions of internal standards as 2 ng/mL, homogenize for 1 min, and then rinse the homogenizer with an additional 10 mL 0.2 mol/L hydrochloric acid solution. Mix in the centrifuge tubes. Keep the reaction mixture for 16 h at 37°C.

Drod proteinic hydrolysis and derivatization of milk powder

Weigh 1-g milk samples (accurate to 0.01 g) into 50-mL brown centrifuge tubes. Add 8 mL water, revolve mix, and add 15 mL trichloroacetic acid solutions and 0.2 mL derivatized solution. Add adequate standard working solutions of internal standards as 2 ng/ml, homogenize for 1 min, and then rinse the homogenizer with an additional 10 mL 0.2 mol/L hydrochloric acid solution and mix in the centrifuge tube. Keep the reaction mixture for 16 h at 37°C.

(3) Clean-up

After this period of time, samples are removed and allowed to cool to room temperature. A pH value of about 7.4 is adjusted by addition of 5 mL of 0.1 mol/L dipotassium hydrogen phosphate solution and an amount of 1.0 mol/L sodium hydroxide. Centrifuge at 4000 rpm for 10 min. The solution is decanted into a preconditioned Oasis HLB solid extraction column with reservoir, and the flow rate is adjusted to $\leq$2 mL/min. Let solution pass through the Oasis HLB solid extraction column. Wait until the solution has thoroughly drained, and then rinse the evaporate tube with 10 mL water and pass through the Oasis HLB solid extraction column. Discard all the effluents. Dry the cartridge by drawing air through it for 10 min under 65 kPa vacuum. Finally, elute derivatives with 5 mL ethyl acetate into 25-mL brown centrifuge tubes. Evaporate the elute solution to dryness on a nitrogen evaporator at 40°C. Accurately add 1 mL dilute solution. Filter with 0.2-μm syringe filter and proceed to determination with LC-MS-MS.

(4) Mixed matrix calibration standard solution

Mixed matrix calibration standard solution of milk

Weigh 5 × 8 g negative milk samples (accurate to 0.01 g) into 50-mL brown centrifuge tubes. Add standard solutions of the four metabolites of nitrofuran. Form 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL, 1.0 ng/mL, 2.0 ng/mL solutions of the four metabolites of nitrofuran. Add internal standard as 2 ng/mL of the four metabolites of nitrofuran. This should be the same as the above-mentioned extraction and cleanup steps.

Mixed matrix calibration standard solution of milk powder

Weigh 5 × 1 g negative milk powder samples (accurate to 0.01 g) and add 8 mL water into 50-mL brown centrifuge tubes. Add standard solutions of the four metabolites of nitrofuran. Form 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL, 1.0 ng/mL, 2.0 ng/mL solutions of the four metabolites of nitrofuran. Add internal standard as 2 ng/mL of the four metabolites of nitrofuran. This should be the same the above-mentioned extraction and cleanup steps.

7.4.6 DETERMINATION

(1) Operating conditions

LC column: Atlantis-C_{18}, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent;
Column temperature: 40°C;
Injection volumes: 30 μL.

TABLE 7.7 Mobile Phase and Flow Rate

Time (min)	Flow Rate (μL/min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	200	80	20
3.00	200	50	50
8.00	200	50	50
8.01	200	80	20
16.00	200	80	20

Mobile phase: mobile phase A is 0.1% formic acid solution and mobile phase B is acetonitrile+0.1% formic acid; see in Table 7.7.
Ion source: ESI source;
Scan mode: Positive scan;
Monitor mode: Multiple reaction monitor;
Ion spray voltage: 5000 V;
Turbo ion spray gas rate: 7 L/min;
Source temperature: 480°C;
Focusing Potential: 150 V;
Collision cell exit potential: 11 V;
Declustering potential (DP): 45 V;
Precursor/product ion combinations, collection time (dwell) and collision energy: see Table 7.3.

(2) Identification

Under the same experimental conditions, if the ratio between the retention times of analytes and internal standards, called relative retention times, of the sample chromatogram peaks are consistent with the standards and the differences are within ±2.5%,; and if after background compensation, all the diagnostic ions are present, the relative ion intensities correspond to those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 7.4, then the corresponding analyte must be present in the sample.

(3) Quantification

Internal standard method: Use the software from the instrument.

External standard method: Inject the different concentrations of mixed matrix calibration standard solution of 3-amino-5-morpholinomethyl-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone, respectively, in duplication under LC and MS conditions. Draw the standard curves of the four metabolites of nitrofuran (concentration vs. peak area). Calculate the concentrations of the corresponding content from

the working curve. The responses of the four metabolites of nitrofuran in the sample solutions should be in the linear range of the instrumental detection.

7.4.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379.1 and GB/T6379.2. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); repeatability and content range of this method are listed in Table 7.8.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations shall be re-conducted, and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for four metabolites of nitrofuran are shown in Table 7.8.

7.4.8 RECOVERY

Under optimized condition, the recoveries of four metabolites of nitrofuran in milk and milk powder using this method are listed in Table 7.9.

TABLE 7.8 Content Range and Limits of Repeatability and Reproducibility

Derivatized Metabolite of Nitrofuran	Content Range (μg/kg)	Limit of Repeatability (r)	Limit of Reproducibility (R)
2-NP-AMOZ	0.2–5.0	$\lg r = 0.8950 \lg m - 1.0874$	$\lg R = 0.9448 \lg m - 0.711\ 6$
2-NP-SEM	0.2–5.0	$\lg r = 1.1209 \lg m - 1.0110$	$\lg R = 0.9787 \lg m - 0.731\ 7$
2-NP-AHD	0.2–5.0	$\lg r = 0.8502 \lg m - 1.0206$	$\lg R = 1.0293 \lg m - 0.709\ 5$
2-NP-AOZ	0.2–5.0	$\lg r = 1.0710 \lg m - 0.8852$	$\lg R = 0.9933 \lg m - 0.700\ 8$

Note: m *is the average values obtained from two independent determination results.*

TABLE 7.9 Test Data of Fortification Concentration and Average Recovery for Four Metabolites of Nitrofuran ($n = 10$)

Sample	Fortifying Concentration (μg/kg)	Average Recovery (%) in Milk and Milk Powder			
		2-NP-AMOZ	2-NP-SEM	2-NP-AHD	2-NP-AOZ
Milk	0.2	96.4	92.8	91.8	96.1
	0.5	91.2	94.7	92.1	93.5
	1.0	98.2	97.4	93.2	92.1
	2.0	95.3	95.6	98.7	93.2
Milk powder	1.6	96.1	91.2	92.4	94.7
	4.0	93.5	94.3	98.4	97.4
	8.0	97.4	92.5	94.3	91.2
	16.0	91.4	93.8	92.8	94.3

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7.5

Determination of Residues of the Metabolites of Furaltadone, Nitrofurazone, Nitrofurantoin, and Furazolidone in Honey—LC-MS-MS Method (GB/T 18932.24-2005)

7.5.1 SCOPE

This method is applicable to the determination of 5-morpholinomethyl-3-amino-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone residues in honey.

The limit of determination of this method for 5-morpholinomethyl-3-amino-2-oxazolidinone and 3-amino-2-oxazolidinone is 0.2 μg/kg; for semicarbazide and 1-aminohydantoin it is 0.5 μg/kg.

7.5.2 PRINCIPLE

The metabolites of nitrofuran antibacterials in honey are hydrolyzed and derivatized with 2-nitrobenzaldehyde. The honey solution is cleaned up with Oasis HLB or equivalent extraction and analyzed by LC-MS-MS using an external standard.

7.5.3 APPARATUS

LC-MS-MS: Equipped with ESI source; Analytical balance: Capable of weighing to 0.1 mg, 0.01 g; Vortex mixer; Solid phase extraction vacuum apparatus; Nitrogen evaporator; Incubator; Vacuum pump: Vacuum to 80 kPa; Microsyringes: 25 μL, 100 μL; Brown centrifuge tubes: 25 mL, 50 mL; pH meter: Capable of measuring ±0.02 unit; Reservoirs and adapters to fit SPE columns: 50 mL.

7.5.4 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be analytically pure; "water" is deionized water. Methanol: HPLC grade; Acetonitrile: HPLC grade; Ethyl acetate: HPLC grade; Dipotassium hydrogen phosphate: G.R.; Acetate acid: G.R.; Dimethyl sulfoxide: G.R.; Hydrochloric acid: G.R.; Sodium hydroxide: G.R.; 2-Nitrobenzaldehyde: purity ≥90%; 5-morpholinomethyl-3-amino-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone standards: purity ≥99%; Dilute solution: Acetonitrile + 0.4% acetate acid solution (1+9).

Dipotassium hydrogen phosphate solution: 0.1 M. Place 17.4 g dipotassium hydrogen phosphate into 1000-mL volumetric flask and dissolve in H_2O. Dilute solution to volume with H_2O.

Hydrochloric acid solution: 0.2 M. Combine 17 mL hydrochloric acid into 1000-mL volumetric flask. Dilute solution to volume with H_2O.

Sodium hydroxide: 1 M. Place 40 g sodium hydroxide into 1000-mL volumetric flask and dissolve in H_2O. Dilute solution to volume with H_2O.

Derivatized solution: 0.05 M. Place 0.075 g 2-Nitrobenzaldehyde in 10 mL dimethyl sulfoxide. Freshly prepare every day.

Standard stock solutions of 5-morpholinomethyl-3-amino-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone: 1.0 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the standard stock solutions of 1.0 mg/mL in concentration. Keep at −18°C.

Standard working solutions of 5-morpholinomethyl-3-amino-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone: 1.0 μg/mL. Accurately pipette appropriate amount of each standard stock solution. Mix and dilute with methanol separately to prepare the standard working solutions of 1.0 μg/mL in concentration. Keep at −18°C.

Oasis HLB solid extraction columns: 60 mg, 3 mL. Condition each column with 5 mL methanol followed by 10 mL water before use; 0.45-μm syringe filter.

7.5.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath, warm at ≤60°C with occasional shaking until liquefied, mix thoroughly and promptly cool to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label. The test samples should be stored at ambient temperature.

(2) Extraction and clean-up

Weigh 5 g test sample (accurate to 0.01 g) into a 50-mL centrifuge tube with cap, and add 5 mL 0.2 M HCl and 0.15 mL derivatizing solution to each sample. Mix vigorously for 1 min on vortex mixer and allow to react for 16 h at 37°C. Remove and allow to cool to room temperature. Adjust pH to about 7.4 by addition of 3 mL of 0.1 M dipotassium hydrogen phosphate solution and 3 mL of 1.0 M NaOH, and load onto a preconditioned Oasis HLB solid extraction column with reservoir at a flow rate ≤2 mL/min. Wait till solution has thoroughly drained and then rinse centrifuge tube with 6 mL water and load the rinse onto the Oasis HLB solid extraction column. Discard all the effluents. Dry the cartridge by drawing air through it for 10 min under 65 kPa vacuum. Finally, elute derivatives with 4 mL ethyl acetate into a 25-mL brown centrifuge tube. Evaporate the eluted sample to dryness on a nitrogen evaporator at 40°C. Accurately add 1 mL dilute solution. Filter with 0.45-μm syringe filter and analyze by LC-MS-MS.

7.5.6 DETERMINATION

(1) Operation conditions

LC column: ZORBAX SB-C_{18}, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent; Column temperature: 30°C; Injection volume: 40 μL; Mobile phase, flow rate and gradient elution procedure: see Table 7.10.

Ion source: ESI source; Scan mode: Positive scan; Monitor mode: Multiple reaction monitor; Ionspray voltage: 5500 V; Nebulizer gas: 0.076 MPa; Curtain gas: 0.069 MPa; Collision gas: 0.030; Turbo ionspray gas rate: 7 L/min; Source temperature: 500°C; Focusing potential: 200 V; Collision cell

TABLE 7.10 Mobile Phase Composition, Flow Rate and Gradient Elution Parameters

Time (min)	Flow Rate (μL/min)	0.4% Acetate Acid Solution (%)	Acetonitrile (%)
0.00	200	70	30
3.00	200	70	30
3.01	200	20	80
8.00	200	20	80
8.01	200	70	30
15.00	200	70	30

exit potential: 11 V; Resolution Q1 and Q3: Unit; Precursor/product ion combinations, collision energy and declustering potential: see Table 7.11.

(2) Qualitative determination

Under the same conditions, if the metabolite of nitrofuran in the honey and standard solutions have the same reference retention times and relative ion intensities, this sample has the nitrofuran antibiotics. For reference retention times and relative ion intensities of metabolites of four nitrofuran antibiotics, see Table 7.12.

(3) Quantitative analysis

The standard working solutions of different concentrations for 5-morpholinomethyl-3-amino-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone are prepared with honey control sample extract on base 3.5.4.5. Inject 40 μL of the different concentration working standard solutions, respectively, in duplication under LC and MS conditions. Draw the standard curves of 5-morpholinomethyl-3-amino-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone (peak area vs. sulfonamide concentration). Calculate the concentrations of the corresponding content from the working curve. The responses of 5-morpholinomethyl-3-amino-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone in the standard working solution and sample solution should be in the linear range of the instrumental detection. For total ion chromatograms of the standard working solution of 5-morpholinomethyl-3-amino-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone, see Fig. 7.3.

TABLE 7.11 Precursor/product Ion Combinations, Collision Energy and Declustering Potential

Metabolite of Nitrofuran	Qualifying Parent/ production (m/z)	Quantifying Parent/ Production (m/z)	Time (ms)	Declustering Potential (V)	Collision Energy (V)
5-Morpholinomethyl-3-amino-2-oxazolidinone	335/291;335/128	335/291	100	60	32;18
Semicarbazide	209/192;209/166	209/166	200	55	17;15
1-Aminohydantoin	249/134;249/178	249/134	200	70	22;19
3-Amino-2-oxazolidinone	236/134;236/192	236/134	100	65	19;20

TABLE 7.12 Reference Retention Times and Relative Ion Intensity of Metabolites of Four Nitrofuran Antibiotics

Metabolite of Nitrofuran	Qualifying Parent/ Production (m/z)	Relative Ion Intensity (%)	Reference Retention Times (min)
5-Morpholinomethyl-3-amino-2-oxazolidinone	335/291;335/128	100; 53	2.84
Semicarbazide	209/192;209/166	93; 100	4.40
1-Aminohydantoin	249/134;249/178	100; 40	4.92
3-Amino-2-oxazolidinone	236/134;236/192	100; 12	6.82

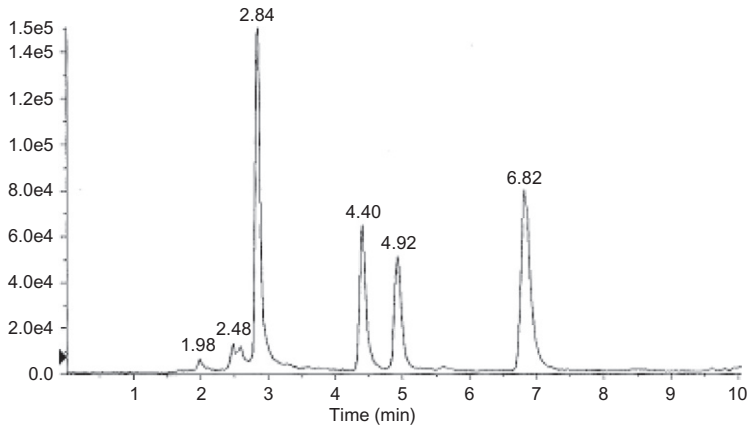

FIG. 7.3 Total ion chromatogram of the 5-morpholinomethyl-3-amino-2-oxazolidinone, semicarbazide, 1-aminohydantoin, and 3-amino-2-oxazolidinone standard.

7.5.7 PRECISION

The precision data of the method for this standard have been determined in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not

TABLE 7.13 Analytical Range, Repeatability, and Reproducibility

Metabolite of Nitrofuran	Analytical Range Range (μg/kg)	Repeatability (r)	Reproducibility (R)
5-Morpholinomethyl-3-amino-2-oxazolidinone	0.2–4	$\lg r = 0.9656 \lg m - 1.0897$	$\lg R = 0.9625 \lg m - 0.7128$
Semicarbazide	0.5–10	$\lg r = 0.9660 \lg m - 1.1445$	$\lg R = 0.9152 \lg m - 0.6513$
1-Aminohydantoin	0.5–10	$\lg r = 0.9844 \lg m - 1.0935$	$R = 0.2058\, m + 0.0119$
3-Amino-2-oxazolidinone	0.2–4	$r = 0.0735\, m + 0.0016$	$\lg R = 1.0813 \lg m - 0.7982$

Note: m *is the average value obtained from two independent determinations.*

exceed the limit of repeatability(r); for repeatability and content range of this method, see Table 7.13.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); for reproducibility and content range of this method, see Table 7.13.

If the difference of values exceeds the limit of reproducibility, the testing results should be discarded and two individual testing determinations reconducted and completed.

7.5.8 RECOVERY

Under optimized conditions, the recoveries of nitrofurans in honey using this method are listed in Table 7.14.

TABLE 7.14 The Recoveries of Nitrofurans in Honey

Metabolite of Nitrofuran	Fortifying Concentration (μg/kg)	Average Recovery (%)
5-Morpholinomethyl-3-amino-2-oxazolidinone	0.2 0.4 2 4	96.7 93.0 90.9 89.5
Semicarbazide	0.5 1.0 5.0 10	96.4 93.3 88.7 88.9
1-Aminohydantoin	0.5 1.0 5.0 10	97.5 93.8 91.9 87.3
3-Amino-2-oxazolidinone	0.2 0.4 2 4	92.9 92.6 89.6 88.1

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Chapter 8

Anabolic Steroids

8.1

Curative Effects and Side Effects of Trenbolone

Trenbolone acetate (17β-acetoxyestra-4,9,11-trien-3-one) is a synthetic anabolic steroid used for increasing cattle weight in veterinary practice. Administration of anabolic steroids for fattening livestock has been prohibited in the European Union since 1988 as they were identified as being possibly carcinogenic. While the WHO/FAO has established a maximum residue limit (MRL) of 2 ng/g in muscle and 10 ng/g in liver, the EU has banned the use of trenbolone in food-producing animals.

8.2

Metabolites of Trenbolone

This compound has been known to be metabolized to β-trenbolone (17β-hydroxyestra-4,9,11-trien-3-one) in muscle and α-trenbolone (17α-hydroxyestra-4,9,11-trien-3-one) in liver. Chemical structures for the metabolites of trenbolone are shown in Fig. 8.1.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00008-7

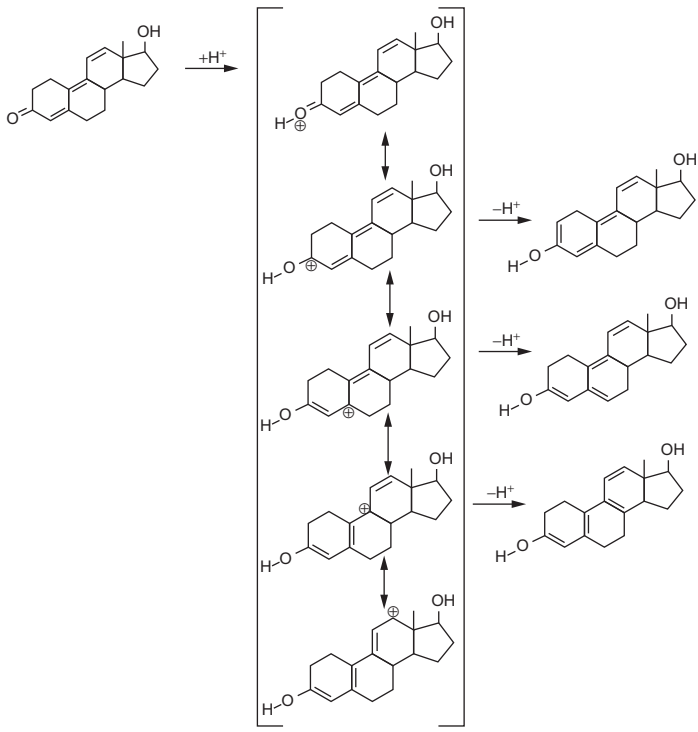


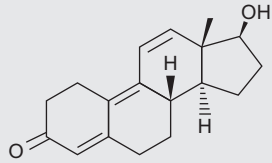
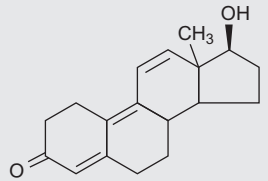
FIG. 8.1 Chemical structures of the metabolites of trenbolone.

8.3

Chemical Structures and Maximum Residue Limits for α-Trenbolone and β-Trenbolone

See Table 8.1.

TABLE 8.1

Compound Name	Molecular Structure	Molecular Weight	Cas. No.	MRL (μg/kg)
α-Trenbolone		270.37	10161-33-8	Codex: 10 (liver) JP: 2 (Cattle, muscle, fat); 10 (Cattle, kidney, liver)
β-Trenbolone		270.37	10161-33-8	Codex: 2 (muscle) JP: 2 (cattle muscle), 10 (Cattle, kidney, liver)

8.4

Determination of α-Trenbolone, β-Trenbolone Residues in Bovine Muscle, Liver, and Kidney—LC-UV and LC-MS-MS Method (GB/T 20760-2006)

8.4.1 SCOPE

This method is applicable to the determination of α-trenbolone, β-trenbolone residues in bovine muscle, liver, and kidney.

The limit of determination of this method for α-trenbolone and β-trenbolone residues by HPLC or LC-MS/MS is 2 μg/kg.

8.4.2 PRINCIPLE

The sample is adjusted to pH 4.9–5.1 and incubated overnight at 37°C with β-glucuronidase/aryl sulfatase. The analytes are extracted with ethyl acetate and cleaned up by gel permeation chromatography (GPC) and silica gel SPE columns. Finally, the extract is analyzed by HPLC-UV or LC-MS-MS, using an external standard.

8.4.3 REAGENTS AND MATERIALS

All reagents used are of analytical grade and "water" is deionized unless specifically noted. Cyclohexane; ethyl acetate; methanol; acetonitrile; acetic acid are of HPLC grade; acetone; hexane; ethyl acetate-cyclohexane (50+50, v/v); acetone-hexane (10+90, v/v). Acetone-hexane (30+70, v/v); β-glucuronidase/aryl sulfatase: 100,000 unit/mL; 0.02 mol/L sodium acetate buffer (pH 5.0): weigh 0.82 g sodium acetate and dissolve in 500 mL water. Adjust the pH to pH 5.0; Silica gel SPE column: 3 mL, 0.5 g. Condition the columns with 5 mL acetone-hexane (10+90) before using. Standards of α-trenbolone and β-trenbolone: purity ≥98.0%.

Stock standard solution: Accurately weigh 10 mg (accurate to 0.1 mg) standard; dissolve in acetonitrile to mark in a 100-mL amber volumetric flask. The concentration of each solution will be 100 μg/mL. The solutions should be stored at –18°C in the dark.

Mixed medium standard solution: pipette 1.00 mL stock standard solution of α-trenbolone, β-trenbolone, nortestosterone and epi-nortestosterone respectively into a 100-mL amber volumetric flask, dilute with acetonitrile to the mark, and mix well. The concentration of the solution is 10.0 μg/mL. The solutions should be stored at –18°C.

Working standard solution: *prepare from mixed medium standard solution* according to the concentration required with HPLC mobile phase. Use immediately after preparation.

8.4.4 APPARATUS

High-performance chromatography: equipped with UV detector; High performance liquid chromatography–tandem mass spectrometry system; Homogenizer; Rotary vacuum evaporator; Vortex shaker; Centrifuge: the max rotate speed is 5000 rpm; Nitrogen evaporator; Constant temperature shaking water bath; SPE vacuum manifold; pH meter.

8.4.5 SAMPLE PRETREATMENT

(1) Extraction

Weigh ca. 5.0 g of test sample (accurate to 0.01 g) into a 50-mL centrifuge tube with cap, add 5 mL 0.02 M sodium acetate buffer and 40 μL

β-glucuronidase, cap it, and incubate overnight at 37°C. Remove and allow to cool to room temperature, add 20 mL ethyl acetate, and homogenize 1 min at 10,000 rpm. Repeat centrifugation for 5 min at 3000 rpm. Transfer the extraction into a heart-shaped flask, extract once more with another 20 mL ethyl acetate, and combine the extracts into one flask.

(2) Clean-up

(A) Clean-up by GPC

Conditions of GPC: Column: 200 mm × 25 mm (i.d.) Packed with 22 g S-X3 Bio-Beads, or equivalent; Mobile phase: Cyclohexane-ethyl acetate (50+50), flow rate: 5.0 mL/min; Injection volume: 5 mL.

The first fraction of 0–10.5 min is discarded; the second fraction of 10.5–15.5 min is collected, and the third fraction of 15.5–18 min is discarded.

(B) Clean-up by SPE

Evaporate the extracts to dryness using a rotary vacuum evaporator at 40°C. Dissolve the residues in 10 mL cyclohexane-ethyl acetate (50+50), and then pass the solution through a GPC to clean it up. Evaporate the collected eluate to dryness and dissolve the residue with 3 mL acetone-hexane (10+90). Transfer the solution into the reservoir above a conditioned silca gel cartridge. Dissolve the residues again with 3 mL acetone-hexane (10+90) and combine into the same reservoir. Then let the solution pass through the cartridge at the speed of ≤2 mL/min. Wash the cartridge with 3 mL acetone-hexane (10+90) and finally elute the cartridge with 5 mL acetone-hexane (30+70) into a 10-mL glass flask. Evaporate to dryness with nitrogen evaporator at 40°C. Residues are dissolved in 1.0 mL mobile phase and vortex-mixed. The solution is filtered through a 0.45-μm filter for HPLC or HPLC-MS/MS analysis.

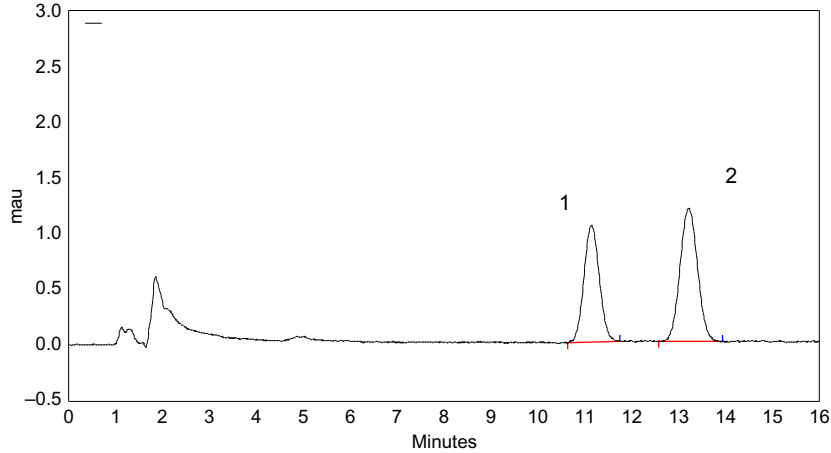
8.4.6 DETERMINATION

(1) HPLC operation conditions

HPLC operating conditions: Column: Dikma C18 150 mm × 4.6 mm (i.d.) 5-μm particle size, or equivalent; Mobile phase: deionized water-acetonitrile-methanol (50+20+30, v/v/v); Flow rate: 1.0 mL/min; UV detector: 345 nm; Column temperature: 35°C; Sample volume: 50 μL.

(2) HPLC determination

According to the approximate concentration of analyte residues in the sample, select the standard working solution with similar responses to that of the sample solution. The responses of analytes in the working standard and the sample solution should be within the linear range of the instrument. The working standard solution and the sample solution should be injected alternatively. Under the preceding HPLC operating conditions, the retention times of β-trenbolone and α-trenbolone are 11.2 min and 13.4 min. The chromatogram of the standard solution is shown in Fig. 8.2.



1. β-trenbolone, 2. α-trenbolone.

FIG. 8.2 LC chromatogram of α-trenbolone and β-trenbolone standards (50 ng/mL).

(3) LC-MS-MS operating conditions

HPLC operating conditions: Column: Inertsil C18 150 mm × 2.1 mm (i.d.), 5-μm particle size, or equivalent; Mobile phase: 0.1% acetic acid-acetonitrile (62+38, v/v); Flow rate: 0.3 mL/min; Column temperature: 35°C; Injection volume: 20 μL.

MS conditions: Ion source type: ESI, negative mode; Nebulizer: 35.0 psi; Dry gas: 9.0 L/min; Dry temp: 350°C; The voltages of capillary, skim, fragmentation and so on have been optimized; The ions of determination are shown in Table 8.2.

TABLE 8.2 The Retention Time, Parent Ions, and Product Ions for the Analytes

Analytes	Retention Time (min)	Parent Ions (m/z)	Product Ions (m/z)	
β-Trenbolone	6.5	271	253[a]	199
α-Trenbolone	7.2	271	253[a]	199

[a]*Quantitative ions.*

TABLE 8.3 Maximum Permitted Tolerances for Relative Ion Intensities While Confirmation

Relative intensity (k)	$k > 50$	$20 < k \leq 50$	$10 < k \leq 20$	$k \leq 10$
Permitted tolerance	±20	±25	±30	±50

(4) Determination by LC-MS-MS

(a) Qualitative analysis

If the retention time of an analyte in a sample chromatogram matches that of the standards, and if after background compensation, the relative ion intensities corresponds to those of the calibration standard, at comparable concentrations under similar conditions to within the tolerances listed in Table 8.3, then the corresponding analyte is present in the sample.

(b) Quantitative analysis

According to the approximate concentration of analyte in the sample solution, select the standard working solution with similar responses to that of the sample solution. The responses of the analyte in the standard working solution and the sample solution should be within the linear range of the instrument detection. The mixed standard working solution and the sample solution should be injected with equal volume alternatively. Under the preceding operating conditions, for the chromatogram of the standard, see Fig. 8.3.

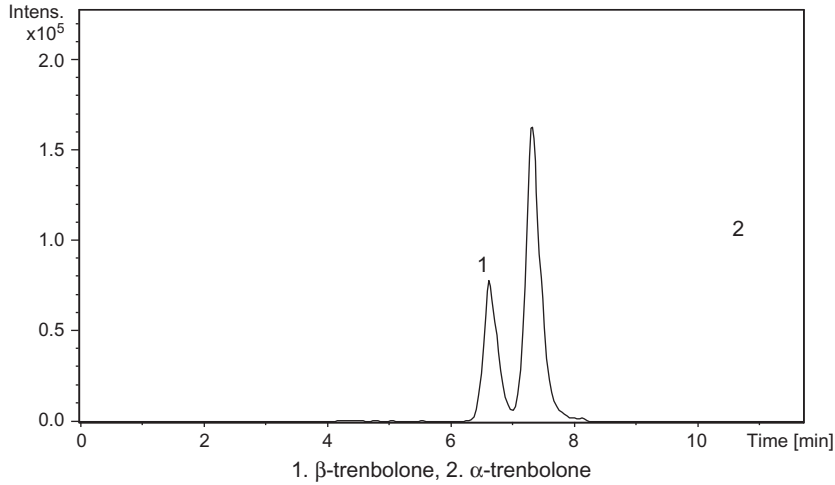


FIG. 8.3 Extracted ion chromatogram of α-trenbolone and β-trenbolone standards (20 ng/mL).

TABLE 8.4 Analytical Range, Repeatability, and Reproducibility (μg/kg)

Analytes	Fortified Levels Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
β-Trenbolone	2–40	$\lg r = 0.4376 \lg m - 0.7122$	$\lg R = 0.6430 \lg m - 0.5267$
α-Trenbolone	2–40	$\lg r = 0.4990 \lg m - 0.7543$	$\lg R = 0.8253 \lg m - 0.7207$

Note: m *is average value of parallel test results.*

8.4.7 PRECISION

The precision data of the method have been determined in accordance with the stipulations of GB/T6379.1 and GB/T 6379.2. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for α-trenbolone, β-trenbolone residues in bovine muscle, liver, and kidney are shown in Table 8.4.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations shall be reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for α-trenbolone, β-trenbolone residues in bovine muscle, liver, and kidney are shown in Table 8.4.

8.4.8 RECOVERY

Under optimized conditions, the recoveries of α-trenbolone and β-trenbolone at varying concentrations in fortified tissues are listed in Table 8.5.

TABLE 8.5 The Fortifying Concentrations and Corresponding Recoveries

	Fortifying Concentrations (μg/kg) and Corresponding Average Recoveries (%)								
	Bovine Muscle			*Bovine Liver*			*Bovine Kidney*		
Analytes	***2***	***5***	***10***	***2***	***5***	***10***	***2***	***5***	***10***
β-Trenbolone	80.7	82.1	81.3	79.5	84.0	80.3	80.8	83.2	80.3
α-Trenbolone	78.5	82.4	83.2	81.0	81.2	80.3	82.7	81.5	80.8

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8.5

Curative Effects and Side Effects of α-Trenbolone, β-Trenbolone, Nortestosterone and Epi-Nortestosterone

Hormonal growth promotants (HGPs) are substances with anabolic properties which are sometimes used to increase feed efficiency, increase formation of lean muscle mass and accelerate attainment of market weight in cattle. Those HGPs with estrogenic or androgenic activity are also used to control the reproductive cycle and lactation and treat malignant neoplasms. The side effects of these substances, which include increased risk of coronary heart disease and hepatic carcinogenicity, are related to their androgenic and/or anabolic properties. The use of HGPs is banned in the European Union (EU) and no residues of these substances are allowed in meat products. Therefore, any cattle raised for export to the EU must be shown to be free of these substances.

The recognition of the growth-promoting properties of these hormones such as nortestosterone led to their introduction as a tool to increase meat production. The effect of these anabolic steroids is to increase lean tissue growth. Nortestosterone (17α-hydroxyestr-4-en-3-one, 17β-19-nortestosterone, also named nandrolone), used as a growth promoter in meat production. is a C18 anabolic steroid hormone, which differs from testosterone in that it does not possess a C-19 methyl group.

8.6

Chemical Structures and Maximum Residue Limits for α-Trenbolone, β-Trenbolone, Nortestosterone, and Epi-Nortestosterone

See Table 8.6.

8.7

Determination of α-Trenbolone, β-Trenbolone, Nortestosterone and Epi-Nortestosterone Residues in Bovine Urine—LC-MS-MS Method (GB/T 20761-2006)

8.7.1 SCOPE

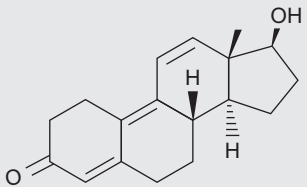
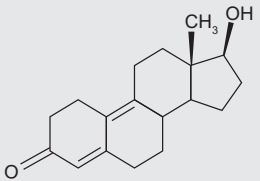
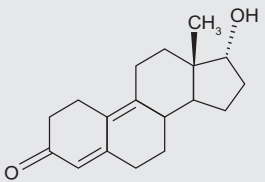
This method is applicable to the determination of α-trenbolone, β-trenbolone, nortestosterone, and epi-nortestosterone residues in bovine urine.

The limit of detection of this method for α-trenbolone, β-trenbolone, nortestosterone, and epi-nortestosterone is 2 μg/kg.

8.7.2 PRINCIPLE

The urine sample is adjusted to pH 4.9–5.1 and incubated for 2 h at 37°C with β-glucuronidase/aryl sulfatase and then the prepared sample is extracted and cleaned up by immunoaffinity extraction column (IAC). The analytes of interest are determined by LC-MS-MS, using an external standard method.

TABLE 8.6 Chemical Structures and Maximum Residue Limits for α-Trenbolone, β-Trenbolone, Nortestosterone, and Epi-Nortestosterone

Compound Names	Chemistry Structure	Molecular Weight	Cas. No.	MRL (μg/kg)
α-Trenbolone		270.37	10161-33-8	Codex: 10 (liver) JP: 2 (Cattle, muscle, fat); 10 (Cattle, kidney, liver)
β-Trenbolone		270.37	10161-33-8	Codex: 2 (muscle) JP: 2 (cattle muscle), 10 (Cattle, kidney, liver)
Nortestosterone		406.56	62-90-8	–
Epi-Nortestosterone		406.56	62-90-8	–

8.7.3 REAGENTS AND MATERIALS

Standards of α-trenbolone, β-trenbolone, nortestosterone, and epi-nortestosterone: purity ≥98.0%.

All reagents used should be A.R. and "water" is deionized, unless specifically noted. Methanol, acetonitrile, and acetic acid are HPLC grade; β-glucuronidase/aryl sulfatase:100,000 unit/mL; 1 M sodium hydroxide: Dissolve 10 g of sodium hydroxide in 250 mL of water; 1 M hydrochloric acid: Put 24 mL acid in water and dilute to 250 mL; Ethanol-water (70+30): Combine 70 volumes of methanol with 30 volumes of water; Stock column wash buffer and column storage buffer: (in immunoaffinity column kit).

Working column wash buffer: 1 volume stock buffer diluted with 19 volumes of water. Freshly prepare enough diluted buffer sufficient to process the day's batch of standards and samples.

Working column storage buffer: 1 volume stock buffer diluted with 4 volumes of water. Freshly prepare enough diluted buffer sufficient to process the day's batch of standards and samples.

Trenbolone/19-nortestosterone immunoaffinity column: kit with columns and buffers, column and concentrated buffers are stable up to expiration date when stored at 2–8°C.

Stock standard solution: Accurately weigh 10 mg (accurate to 0.1 mg) standard, dissolve with acetonitrile to mark in a 100-mL amber volumetric flask individually. The concentrations of the solutions are 100 μg/mL. The solutions should be stored at –18°C in the dark.

Mixed medium standard solution: pipette 1.00 mL stock standard solution of α-trenbolone, β-trenbolone, nortestosterone and epi-nortestosterone respectively into a 100-mL amber volumetric flask, dilute with acetonitrile to the mark, and mix well. The concentration of the solution is 10.0 μg/mL. The solutions should be stored at –18°C.

Working standard solution: prepare using the mixed medium standard solution with HPLC mobile phase. Use immediately after preparation.

8.7.4 APPARATUS

High performance liquid chromatography tandem mass spectrometer; Centrifuge: max rotation speed 5000 rpm; Constant temperature shaking waterbath; Vortex shaker; Nitrogen evaporator; SPE vacuum manifold; pH meter.

8.7.5 SAMPLE PRETREATMENT

(1) Hydrolysis

Centrifuge urine sample for 10 min at 4000 rpm. Accurately transfer 5.0 mL sample into 15 mL polypropylene centrifuge tubes, and adjust the pH of each urine sample to pH 4.9–5.1 with 1 M HCl. Add 40 μL

β-glucuronidase/aryl sulfatase and incubate for 2 h at 37°C. Add 4 mL of diluted column wash buffer to each urine sample after the temperatures of samples reach room temperature. Then adjust the pH to 7–9 with 1 M NaOH.

(2) Extraction and clean-up

Allow the column storage buffer to run through each IAC column and discard the eluate. Equilibrate each column with 15 mL diluted column wash buffer. Then load the pretreated urine sample onto separate columns with gravity flow. Wash each column with 8 mL of dilute column wash buffer and 5 mL water. Elute each column with 4 mL methanol-water (70+30) and collect eluates into 10 mL graduated tubes. Dry the eluate to 1 mL at 60°C under a gentle nitrogen flow. Finally, dilute the solutions to 2 mL with the mobile phase, and filter each sample into vials through 0.45-μm filters after vortexing for 30 s.

Note: To ensure there is no remaining interference, wash the column with an additional 10 mL of methanol-water (70+30). If further samples are to be processed, then repeat preceding steps. If no further samples are to be processed, then the columns must be washed with 15 mL diluted column storage buffer, before storing the columns in the buffer at 2–8°C.

8.7.6 DETERMINATION

(1) Operation conditions

Column: Inertsil C18 150 mm × 2.1 mm (i.d.), 5-μm particle size,or equivalent; Mobile phase: 0.1 % acetic acid- acetontrile (62+38, v/v); Flow rate: 0.3 mL/min; Column temperature: 35°C; Injection volume: 20 μL.

Ion Source Type: ESI, negative mode; Nebulizer: 35.0 psi; Dry gas: 9.0 L/min; Dry temp: 350°C; The voltages of capillary, skim, fragmentation, and so on have to be optimized; The ions for determination are shown in Table 8.7.

TABLE 8.7 The Retention Time, Precursor, and Product Ions of Analytes

Analytes	Retention Time (min)	Precursor Ions (m/z)	Product Ions (m/z)	
β-Trenbolone	6.5	271	253[a]	199
α-Trenbolone	7.2	271	253[a]	199
Nortestosterone	8.4	275	257[a]	239
Epi-nortestosterone	11.4	275	257[a]	239

[a]*Quantitative ions.*

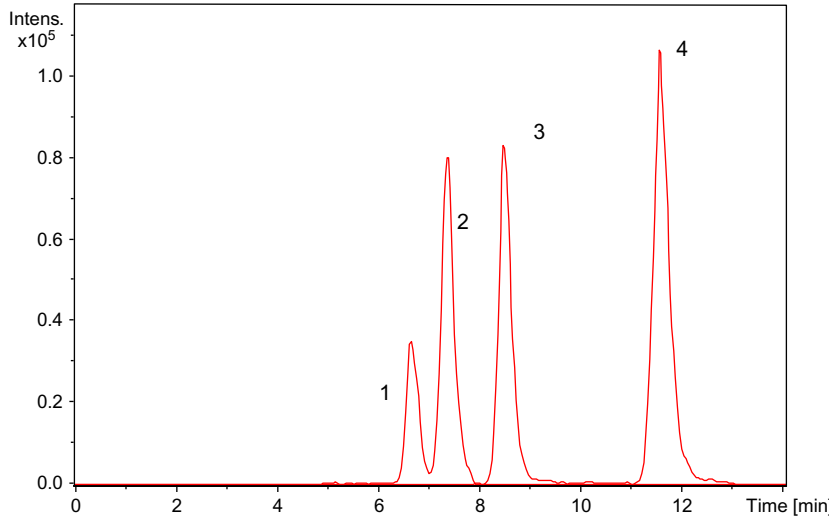


FIG. 8.4 Extracted ion chromatogram of α-trenbolone, β-trenbolone, nortestosterone and epi-nortestosterone standards (1. β-trenbolone, 2. α-trenbolone, 3. nortestosterone, 4. epi-nortestosterone).

(2) Qualitative analysis

If the retention times of sample chromatogram peaks match those of the standards and if, after background compensation, the relative ion intensities correspond to those of the calibration standard, at comparable concentrations, measured under similar conditions within the tolerances listed in Table 8.3, then the corresponding analyte is present in the sample.

(3) Quantitative analysis

According to the approximate concentration of analyte in sample solution, select the standard working solution with similar responses to that of the sample solution. The responses of the analyte in the standard working solution and the sample solution should be within the linear range of the instrument detection. The mixed standard working solution and the sample solution should be injected with equal volume alternatively. Under these operating conditions, a typical chromatogram of the standard is shown in Fig. 8.4.

8.7.7 PRECISION

The precision data of the method have been determined in accordance with the stipulations of GB/T6379 and GB/T 6379.2. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability

TABLE 8.8 Analytical Range, Repeatability, and Reproducibility

Analytes	Fortified Levels Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
β-Trenbolone	2–40	$\lg r = 0.923 \lg m - 0.879$	$\lg R = 0.871 \lg m - 0.546$
α-Trenbolone	2–40	$\lg r = 0.828 \lg m - 0.820$	$\lg R = 0.863 \lg m - 0.571$
Nortestosterone	2–40	$\lg r = 0.711 \lg m - 0.831$	$\lg R = 0.458 \lg m - 0.606$
Epi-nortestosterone	2–40	$\lg r = 0.708 \lg m - 0.716$	$\lg R = 0.984 \lg m - 0.881$

Note: m *is average value of parallel test results.*

equations for α-trenbolone, β-trenbolone, nortestosterone, and epi-nortestosterone residues in bovine urine are shown in Table 8.8.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for α-trenbolone, β-trenbolone, nortestosterone, and epi-nortestosterone residues in bovine urine are shown in Table 8.8.

8.7.8 RECOVERY

Under optimized conditions, the recoveries of α-trenbolone, β-trenbolone, nortestosterone, and epi-nortestosterone at varying concentrations in urine are listed in Table 8.9.

TABLE 8.9 The Fortifying Concentrations and Corresponding Recoveries

Added Level (μg/L)	Recovery (%)			
	β-Trenbolone	*α-Trenbolone*	*Nortestosterone*	*Epi-Nortestosterone*
2	77.5	84.0	74.0	82.5
5	85.6	79.0	80.8	79.6
10	77.9	77.5	72.8	77.8

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8.8

Determination of Melengestrol Acetate, Chlormadinone Acetate, and Megestrol Acetate Residues in Milk and Milk Powder—HPLC-MS-MS Method (GB/T 22973-2008)

8.8.1 SCOPE

This method is applicable for determination of melengestrol acetate, chlormadinone acetate, and megestrol acetate in milk and milk powder.

The limit of determination of melengestrol acetate, chlormadinone acetate, and megestrol acetate in milk is 2 μg/kg.

The limit of determination of melengestrol acetate, chlormadinone acetate, and megestrol acetate in milk powder is 20 μg/kg.

8.8.2 PRINCIPLE

The analyte is extracted from milk and then partitioned into acetonitrile. After saponification, the progestogens are cleaned up on a cyanopropyl solid-phase

extraction column. The progestogen residue concentration is measured by reverse-phase HPLC with mass spectrometric detection, using an external standard method to quantify.

8.8.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents are of analytical-reagent grade.

Water: GB/T 6682.
Acetonitrile: HPLC grade.
Methanol: HPLC grade.
Ethyl acetate: HPLC grade.
Hexane: HPLC grade.
Magnesium chloride ($MgCl_2 \cdot 6H_2O$).
Sodium hydroxide.
Formic acid.
Ammonium solution: 25%.
95% ethanol.
Petroleum ether.
Anhydro sodium sulfate.
Ethyl acetate-hexane (1+19): Combine 10 mL ethyl acetate with 190 mL hexane.
Ethyl acetate-hexane (1+4): Combine 40 mL ethyl acetate with 160 mL hexane.
Magnesium chloride solution: 1.0 mol/L. Add 20.3 g magnesium chloride ($MgCl_2 \cdot 6H_2O$) to a 100-mL volumetric flask. Bring to volume with water.
Sodium hydroxide solution: 0.1 mol/L. Add 1.00 g sodium hydroxide to a 250-mL volumetric flask. Bring to volume with water.
Acetonitrile-water (70+30).
Standards: melengestrol acetate ($C_{25}H_{32}O_4$, FW = 396.52, minimum ≥97%), chlormadinone acetate ($C_{23}H_{29}ClO_4$, FW = 404.93, minimum ≥98%), megestrol acetate ($C_{24}H_{32}O_4$, FW = 384.51, minimum ≥99%).
Stock standard solutions: 1000 μg/mL. Accurately weigh 0.0500 g of melengestrol acetate, chlormadinone acetate, and megestrol acetate into individual 50-mL volumetric flasks. Dilute with methanol. Prepare yearly. Store in glass at 4°C.
Intermediate standards solution I: 100 μg/mL. Pipette 10 mL each of melengestrol acetate, chlormadinone acetate, and megestrol acetate stock standard solution into a 100-mL volumetric flask to volume with methanol. Prepare yearly. Store in glass at 4°C.
Intermediate standards solution II: 1.00 μg/mL. Pipette 1.00 mL of 100 μg/mL intermediate standards solution I into a 100-mL volumetric flask to volume with methanol. Prepare every 6 months. Store in glass at 4°C.

Working standard solutions with blank matrix solution: Pipette proper volume of intermediate standards solution II to prepare working standard solutions with blank matrix solution, depending on the concentration requirement. Prepare daily.
Cyanopropyl (CN) SPE column: 3 mL/500 mg. Prior to use, condition with 5 mL ethyl acetate, followed by 6 mL hexane.
Filter membrane: 0.20 μm.

8.8.4 APPARATUS

Liquid chromatograph mass spectrometry, equipped with ESI source.
Balance: 0.1 mg and 0.01 g sensitivity.
Nitrogen evaporator.
Vacuum manifold for solid-phase extraction.
Centrifuge: refrigeration to −5°C, ≤5000 rpm.
Vortex mixer.
Water bath boiler.
Microwave oven.
Pipettors: 10 μL to 100 μL and 100 μL to 1000 μL.

8.8.5 SAMPLE PRETREATMENT

(1) Sample preparation

Homogenize 500 g sample. Seal and label.

Transfer sample into scintillation vial and store at 4°C until analysis.

Accurately weigh 10 g milk, to nearest 0.01 g, into a 50-mL centrifuge tube with cap.

Accurately weigh 1.0 g milk powder, to nearest 0.01 g, and reconstitute powdered milk by adding 8 parts water.

(2) Extraction

Add 1.0 mL ammonium hydroxide and mix. Add 10 mL 95% ethanol and 10 mL ethyl acetate and then shake the capped tube for 1 min. Add 10 mL petroleum ether and shake for 1 min; centrifuge tube at 4000 rpm for 5 min. Decant supernatant into another 50-mL centrifuge tube through 15 g anhydro sodium sulfate column. Rewash sample solution with another 8 mL ethyl acetate and 8 mL petroleum ether. Centrifuge tube at 4000 rpm for 5 min. Combine supernatant through the same anhydro sodium sulfate column. Wash sodium sulfate column with 5 mL ethyl acetate. Evaporate the combined supernatants to dryness at 60°C on the nitrogen evaporator.

Add 5 mL acetonitrile and heat in 60°C water bath at for 3 min or until the fat is liquid. Shake capped tube at high speed on a Vortex mixer for 1 min. Centrifuge tube at 4000 rpm for 7 min at −5°C. Decant supernatant into a 15-mL centrifuge tube. Reextract the pellet, repeating acetonitrile extraction of fat. Combine supernatants. Add 2 mL hexane to the combined

acetonitrile extracts and then shake the capped tube at high speed for 1 min. Rewash the acetonitrile extraction with another 2 mL hexane. Centrifuge tube at 4000 rpm for 5 minutes at −5°C. Discard the hexane. Evaporate the acetonitrile extracts to dryness at 60°C on the nitrogen evaporator.

(3) Saponification

Add 4 mL hexane, 1 mL 0.1 mol/L sodium hydroxide solution and 500 µL 1.0 mol/L magnesium chloride solution in that order and vortex mix capped tube for 10 s. Heat tube in 60°C waterbath for 15 min and then centrifuge at 4000 rpm for 5 min at −5°C. Decant supernatant into a 15-mL centrifuge tube. Add 4 mL hexane to the pellet, vortex, and repeat previous steps, combining supernatants. Evaporate the combined supernatants to dryness at 60°C on the nitrogen evaporator in 1.0 mL hexane.

(4) Clean-up

Load the sample on the CN SPE column. Rinse centrifuge tube with two 1-mL portions of hexane and add to the column. Wash the column with 5 mL hexane follwed by 6 mL ethyl acetate-hexane (1+19). Dry column for 2 min. Elute with 3.5 mL ethyl acetate-hexane (1+4). Evaporate the eluate to dryness at 60°C on the nitrogen evaporator. Reconstitute with 1 mL acetonitrile-water (70+30) and filter with 0.20-µm filter membrane. Sample is ready for HPLC-MS-MS.

8.8.6 DETERMINATION

(1) HPLC Conditions

Column: C_{18} column, 50 mm × 2.0 mm (i.d.), 5 µm, or equivalent;
Column temperature: 30°C;
Flow rate: 0.3 mL/min;
Injection volume: 10 µL;
gradient program of elution: see Table 8.10.
Ion source: positive ESI;
Monitor mode: MRM mode;
Capillary voltage: 4000 V;

TABLE 8.10 Gradient Program of Elution

Time (min)	0.1% Formic Acid in Water (%)	0.1% Formic Acid in Acetonitrile (%)
0	90	10
1.0	20	80
7.0	20	80
8.0	90	10

TABLE 8.11 Qualitation Ions, Quantitation Ions, Collision Potential, and Declustering Potential

Compound	Qualitation Ions (m/z)	Quantitation Ions (m/z)	Declustering Potential (V)	Collision Potential (V)
Melengestrol acetate	397/337	397/337	120	5
	397/279		120	28
Chlormadinone acetate	405/345	405/345	80	6
	405/309		80	13
Megestrol acetate	385/325	385/325	130	6
	385/267		130	16

Ion source temperature: 350°C;
Ion source gas, curtain gas, auxiliary gas: high purity nitrogen;
Qualitation ions, quantitation ions, collision potential, and declustering potential: refer to Table 8.11.

(2) Qualitative Analysis

One father ion and more than two daughter ions are selected to confirm analyte. Under the same experimental conditions, the ratio of retention time between analyte and standard shall be identical to that of standard solution at comparable concentration. The deviation of the ratio of retention time should be in the range of ±2.5%. The relative intensities of the qualitative ion in the sample chromatogram shall correspond to those of standard solutions with blank matrix solution at comparable concentrations; tolerances for relative ion intensities shall be within those specified in Table 8.3; then the corresponding analyte is confirmed to exist.

(3) Quantitative Analysis

Standard solutions and sample extract are injected for analysis under optimum conditions; an external method is used for quantitative analysis. Under these conditions, the MRM chromatograms of melengestrol acetate, chlormadinone acetate, and megestrol acetate standards are shown in Figs. 8.5–8.7.

8.8.7 PRECISION

The precision data of this part are confirmed according to GB/T 6379. The values of repeatability and reproducibility are calculated at a 95% confidence level.

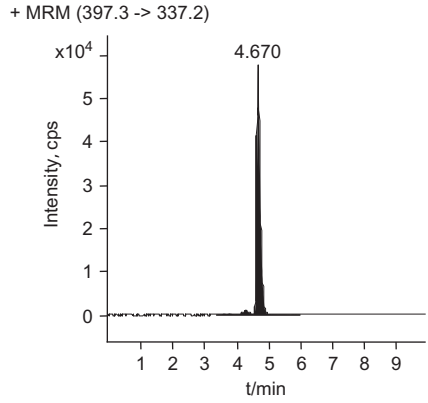

FIG. 8.5 MRM chromatogram of melengestrol acetate standard.

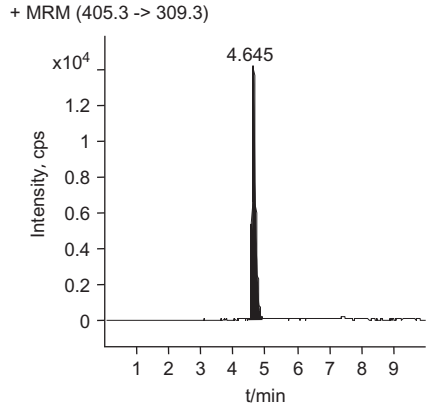

FIG. 8.6 MRM chromatogram of chlormadinone acetate standard.

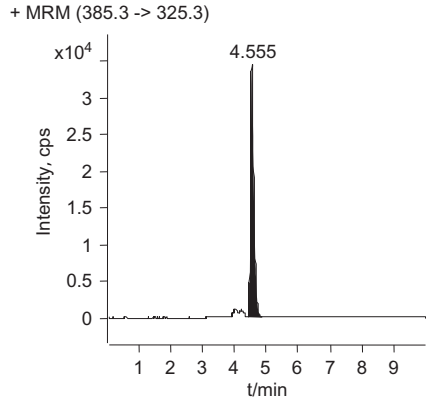

FIG. 8.7 MRM chromatogram of megestrol acetate standard.

(1) Repeatability

Under the repeatability condition, the difference of absolute value of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equations of the three progestogens in milk are shown in Table 8.12. The content ranges and the repeatability equations of the three progestogens in milk powder are shown in Table 8.13.

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned and two independent experiments should be redone.

TABLE 8.12 The Content Ranges and the Reproducibility Equations of the Three Progesterones in Milk (Unit: mg/kg)

Analyte	Content Range	Repeatability Limit (r)	Reproducibility Limit (R)
Melengestrol acetate	0.002–0.020	$\lg r = 0.7576 \lg m - 0.9527$	$\lg R = 0.9725 \lg m - 0.5187$
Chlormadinone acetate	0.002–0.020	$\lg r = 0.8868 \lg m - 0.9084$	$\lg R = 0.9314 \lg m - 0.4942$
Megestrol acetate	0.002–0.020	$\lg r = 0.8718 \lg m - 0.9059$	$\lg R = 0.8964 \lg m - 0.4976$

Note: m *equals to the average of two results.*

TABLE 8.13 The Content Ranges and the Reproducibility Equations of the Three Progesterones in Milk Powder (Unit: mg/kg)

Analyte	Content Range	Repeatability Limit (r)	Reproducibility Limit (R)
Melengestrol acetate	0.020–0.200	$\lg r = 0.9336 \lg m - 0.9724$	$\lg R = 0.9876 \lg m - 0.5460$
Chlormadinone acetate	0.020–0.200	$\lg r = 0.6814 \lg m - 0.4461$	$\lg R = 0.9166 \lg m - 0.4235$
Megestrol acetate	0.020–0.200	$\lg r = 0.9876 \lg m - 0.9798$	$\lg R = 0.9636 \lg m - 0.5525$

Note: m *equals to the average of two results.*

(2) Reproducibility

Under the reproducibility condition, the difference of absolute value of two independent results is not above the reproducibility limit (R). The content ranges and the reproducibility equations of the three progesterones in milk are shown in Table 8.12. The content ranges and the reproducibility equations of the three progesterones in milk powder are shown in Table 8.13.

8.8.8 RECOVERY

Under optimized condition, the recoveries of melengestrol acetate, chlormadinone acetate, and megestrol acetate in milk and milk powder using this method are listed in Table 8.14.

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8.9

Determination of α-Trenbolone, β-Trenbolone, Nortestosterone, and Epi-Nortestosterone Residues in Milk and Milk Powder—LC-MS-MS Method (GB/T 22976-2008)

8.9.1 SCOPE

This method is applicable to the determination of α-trenbolone, β-trenbolone, nortestosterone, and epi-nortestosterone residues in milk and milk powder.

The limit of quantification for α-trenbolone, β-trenbolone, nortestosterone, and epi-nortestosterone in milk is 1 μg/kg and in milk powder is 5 μg/kg.

TABLE 8.14 Spiked Level and Recovery Data

Matrix	Compound	Spiked Level (µg/kg)	Average Recovery (%) ($n = 10$)
Milk	Melengestrol acetate	2.0	86.7
		4.0	89.6
		10	90.2
		20	89.7
	Chlormadinone acetate	2.0	86.5
		4.0	87.8
		10	91.0
		20	90.8
	Megestrol acetate	2.0	86.9
		4.0	87.1
		10	89.3
		20	89.7
Milk powder	Melengestrol acetate	20	88.3
		40	89.7
		100	90.3
		200	90.3
	Chlormadinone acetate	20	88.7
		40	87.8
		100	90.3
		200	89.9
	Megestrol acetate	20	87.2
		40	87.6
		100	90.1
		200	91.3

8.9.2 PRINCIPLE

The test sample is first enzymolyzed under the conditions of a pH 5.0 sodium acetate buffer. Then residues are extracted with acetonitrile-ethyl acetate and cleaned up using gel permeation chromatography (GPC). Determination is made by LC-MS-MS using an external standard method.

8.9.3 REAGENTS AND MATERIALS

Unless specifically noted, all reagents used should be of HPLC grade; "water" is deionized water.

Acetonitrile.
Ethyl acetate.
Methanol.
Cyclohexane.
Acetic acid.
Anhydrous sodium sulfate: analytical grade, ignite at 650°C for 4 h and keep in a tightly closed container after cooling.
Sodium acetate: analytical grade.
β-glucuronidase/aryl sulfatase: 100,000 unit/mL.
0.02 mol/L sodium acetate buffer (pH 5.0): weigh 0.82 g sodium acetate to dissolve in 500 mL water and adjust the pH of the solution to 5.0 with acetic acid.
Methanol-water (70+30, v/v): mix 70 volume units methanol and 30 volume units water.
Cyclohexane-ethyl acetate (50+50): Mix the same volume ethyl acetate and cyclohexane.
Standards of α-trenbolone (CAS, 80657-17-6), β-trenbolone (CAS, 10161-33-8), nortestosterone (CAS, 434-22-0), and epi-nortestosterone (CAS, 4409-34-1): purity $\geq$98.0%.
Stock standard solution: Respectively, accurately weigh appropriate amount of standards and dissolve in 100 mL methanol to make a solution concentration of approximately 100 mg/mL.
Mixed standard solution: Accurately measure 1.00 mL stock standard solution respectively into a 100-mL umber volumetric flask, dilute with methanol to 100 mL, and mix to homogeneity. The concentration of the solution is 1 μg/mL.
0.45-μm filter.

8.9.4 APPARATUS

High performance liquid chromatography tandem mass spectrograph.
Gel permeation chromatography with UV detector (fixed wavelength: 254 nm).
Analytical balance: Sensibility reciprocal is 0.1 mg and 0.01 g.
Centrifuge.
Water bath shaker.
Vortex shaker.
Rotary vacuum evaporator.

8.9.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Milk

About 250 g representative samples should be taken from all samples, placed in clean sample containers, sealed, and labeled. The test samples should be stored at −4°C and kept away from light.

Milk powder

About 250 g representative samples should be taken from all samples, placed in clean sample containers, sealed, and labeled. The test samples should be stored at room temperature and kept away from light.

(2) Extraction

For the milk sample, weigh approximate 5.00 g (accurate to 0.01 g) of the test sample into a 50-mL centrifuge tube. For the milk powder sample, weigh approximate 1 g of the test sample, add 5 mL water, and mix for 1 min by vortex shaker. Then add 5 mL sodium acetate buffer and 20 μL β-glucuronidase/aryl sulfatase. Put the tubs into a water bath shaker and shake for one night at 37°C. Afer enzymolysis, add 5 mL acetonitrile into the tubes and shake for 2 min to deposit protein. Then add 20 mL ethyl acetate and shake the tubes for 2 min. Afterwards, centrifuge for 10 min at 3000 rpm and filter the extraction through an anhydrous sodium sulfate layer into a heart-shaped flask. Then use 20 ethyl acetate extract again and mix the extraction into the same flask.

(3) Clean-up

Conditions of GPC

- **(a)** Column: Packed with 22 g S-X3 Bio-Beads, 200 mm × 20 mm (i.d.), or equivalent;
- **(b)** Mobile phase: Cyclohexane-ethyl acetate; flow rate: 5.0 mL/min;
- **(c)** Injection volume: 5 mL;
- **(d)** Clean-up fraction: The first fraction of 0–10 min is discarded; the second fraction of 10–15.5 min is collected.

Clean-up Procedure

Evaporate the extractions to dryness at 45°C using a rotary vacuum evaporator. Dissolve the residues with 10 mL cyclohexane-ethyl acetate and the solution then passes GPC to clean-up. Evaporate the collection to dryness and dissolve the residues with 1.0 mL methanol-water; vortex to homogeneity. After being filtrated with a 0.45-μm filter, the final solution is ready for analysis by HPLC-MS-MS.

(4) Preparation of blank matrix solution

For milk, accurately weigh a 5-g negative sample, and for milk powder accurately weigh a 1-g negative sample (accurate to 0.01 g) to prepare the blank matrix solution according to the above-mentioned extraction and cleanup steps.

8.9.6 DETERMINATION

(1) HPLC Operating Conditions

Column: C_{18} 150 mm × 2.1 mm (i.d.), 5-μm particle size, or equivalent;
Column temperature: 30°C;
Injection volume: 20 μL.
Mobile phase: The elution gradient and flow rate are listed in Table 8.15.
Ionization mode: ESI+;
Scan mode: MRM;
Sheath gas: 15 unit;
Auxiliary gas: 20 unit;
Ion spray voltage: 4000 V;
Capillary temperature: 320°C;
Source CID: 10 V;
Width of Q1 and Q3: 0.4 and 0.7;
Collision gas: Argon;
Collision gas pressure: 1.5 mTorr.
Other mass operating conditions are listed in Table 8.16.

(2) Qualitative Analysis

The qualification ions include at least one precursor and two daughter ions. In the same experimental conditions, the variation range of the retention time for the peak of the analyte in the unknown sample and in the matrix standard working solution should be within a tolerance of ±0.25%; and for the same analysis batch and the same compound, the variation of the ion intensities between the two daughter ions of the unknown sample and the matrice standard working solution at a similar concentration cannot be out of the range given in Table 8.3; then the corresponding analyte must be present in the sample.

(3) Quantitative Analysis

Under the best conditions of the apparatus, inject a series of mix matrix standard working solutions separately. The mix matrix standard working

TABLE 8.15 Elution Gradient of LC

Time (min)	Flow Rate (μL/min)	0.1 % Acetic Acid Solution (%)	Acetonitrile (%)
0.00	200	80	20
6.00	200	65	35
8.00	200	65	35
8.10	200	80	20
10.0	200	80	20

TABLE 8.16 Scan Segment, Ion Pairs, and Collision Energy of the Analytes

Analyte	Retention Time (min)	Ion Pairs (m/z)	Collision Energy (eV)
β-Trenbolone	8.93	271.16/253.08[a]	21
		271.16/199.08	21
α-Trenbolone	9.47	271.16/253.08[a]	21
		271.16/199.08	21
Nortestosterone	10.28	275.18/239.10[a]	15
		275.18/257.12	25
Epi-nortestosterone	11.64	275.18/239.10[a]	15
		275.18/257.12	25

[a]*Quantitative ion pair.*

curves are made by plotting the responses vs. the concentration of standards. Use the curve to quantify each analyte in the unknown sample. The responses of the analyte in the standard working solution and the sample solution should be within the linear range of the instrument's detection. Under these operating conditions, the chromatograms of the standard can be found in Fig. 8.8.

8.9.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the repeatability condition, the difference of absolute value of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equations of the five analytes in milk and milk powder are shown in Tables 8.17 and 8.18.

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned and two independent experiments should be redone.

(2) Reproducibility

Under the reproducibility condition, the difference of absolute value of two independent results is not above the reproducibility limit (R). The content ranges and the reproducibility equations of the five analytes in milk and milk powder are shown in Tables 8.17 and 8.18.

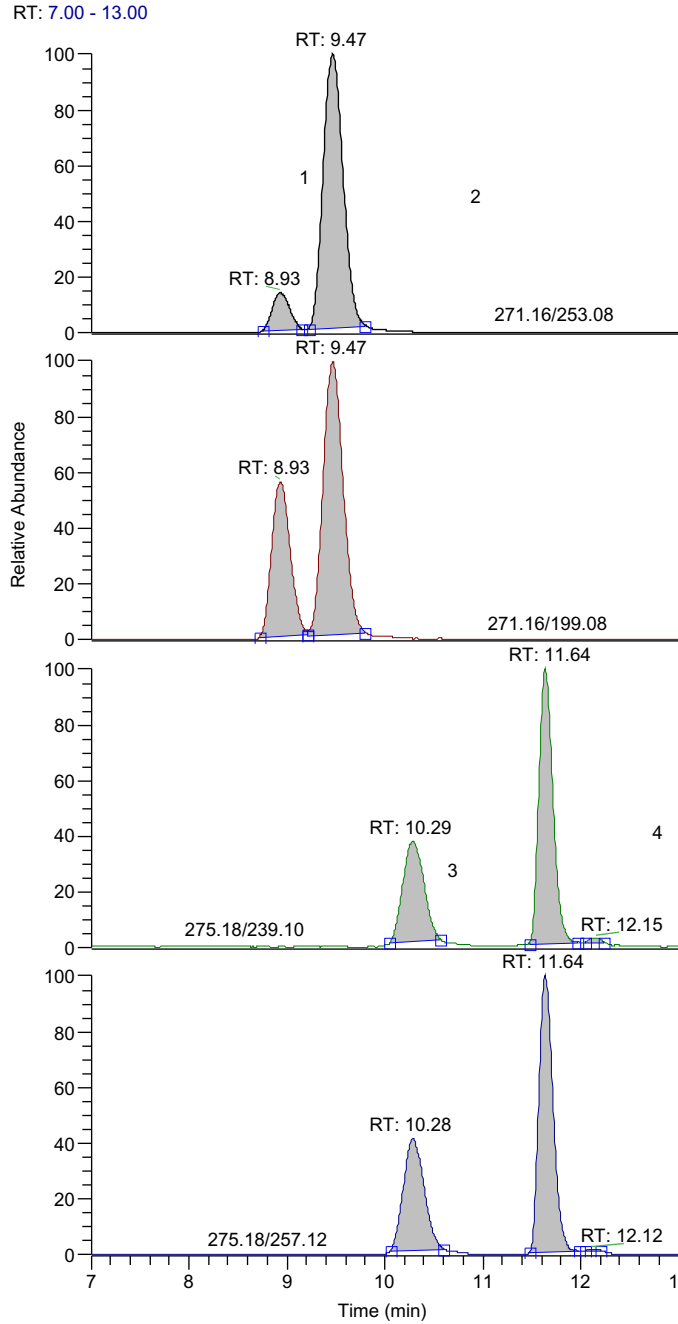

FIG. 8.8 MRM chromatograms of standard working solution. 1, β-trenbolone, 2, α-trenbolone, 3, nortestosteron, 4, epi-nortestosterone.

TABLE 8.17 Content Ranges and the Repeatability and Reproducibility Equations for Milk Sample

Analyte	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
β-Trenbolone	1–10	$\lg r = 1.3046 \lg m - 1.0259$	$\lg R = 1.005 \lg m - 0.6902$
α-Trenbolone	1–10	$\lg r = 0.8278 \lg m - 0.7811$	$\lg R = 0.9415 \lg m - 0.6367$
Nortestosterone	1–10	$\lg r = 0.9121 \lg m - 0.8603$	$\lg R = 0.9049 \lg m - 0.5791$
Epi-nortestosterone	1–10	$\lg r = 1.1868 \lg m - 0.9711$	$\lg R = 0.8888 \lg m - 0.5975$

Note: m *equals to the average of two results.*

TABLE 8.18 Content Ranges and the Repeatability and Reproducibility Equations for Milk Powder Sample

Analyte	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
β-Trenbolone	5–50	$\lg r = 1.1785 \lg m - 1.2094$	$\lg R = 1.2993 \lg m - 0.9517$
α-Trenbolone	5–50	$\lg r = 0.7749 \lg m - 0.5326$	$\lg R = 0.9135 \lg m - 0.6682$
Nortestosterone	5–50	$\lg r = 0.8205 \lg m - 0.5518$	$\lg R = 0.8513 \lg m - 0.7618$
Epi-nortestosterone	5–50	$\lg r = 0.8629 \lg m - 0.7944$	$\lg R = 0.7029 \lg m - 0.4504$

Note: m *equals to the average of two results.*

For different MS equipment, the parameters may be different, and the MS parameters should be optimized to the best possible before analysis.

Noncommercial statement: the reference mass parameters listed are accomplished by Thermo TSQ Quantum Ultra AM LC-MS/MS. The type of equipment involved in the standard method is only for reference and is not related to any commercial aim; analysts are encouraged to use equipment of different types or from different companies.

8.9.8 RECOVERY

Under optimized condition, the recoveries of α-trenbolone, β-trenbolone, nortestosterone, and epi-nortestosterone residues in milk and milk powder using this method are listed in Table 8.19.

TABLE 8.19 Recovery Range of the Analytes in Milk and Milk Powder at Four Fortifying Levels

	Milk		Milk powder	
Compound	*Spiked Level (μg/kg)*	*Recovery Range (%)*	*Spiked Level (μg/kg)*	*Recovery Range (%)*
β-Trenbolone	1	76.0 ~91.0	5	75.2 ~95.6
	2	74.5–91.0	10	75.6–93.0
	5	73.6–90.6	25	70.5–89.4
	10	77.4–94.2	50	74.0–97.0
α-Trenbolone	1	73.0–94.0	5	75.0–95.8
	2	73.5–96.0	10	75.5–88.6
	5	74.4–96.0	25	76.7–98.2
	10	79.9–92.5	50	77.0–92.8
Nortestosterone	1	75.0–90.0	5	78.2–96.4
	2	74.0–94.0	10	73.8–89.0
	5	78.0–97.8	25	82.3–99.0
	10	80.7–88.2	50	74.4–96.2
Epi-nortestosterone	1	76.0–96.0	5	81.4–96.0
	2	76.5–92.5	10	71.8–94.6
	5	76.8–95.0	25	80.8–98.1
	10	76.1–96.7	50	81.0–93.5

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8.10

Determination of Testosterone, Epi-Testosterone and Progesterone Residues in Bovine Liver and Muscle—LC-MS-MS Method (GB/T 20758-2006)

8.10.1 SCOPE

This method is applicable to the determination of testosterone, epi-testosterone, and progesterone in bovine liver and muscle tissues.

The limits of quantitation of the method: 0.5 μg/kg for testosterone, epi-testosterone, and progesterone in bovine liver, 0.1 μg/kg for testosterone and epi-testosterone, and 0.5 μg/kg for progesterone, in bovine muscle.

8.10.2 PRINCIPLE

Testosterone, epi-testosterone, and progesterone residues in bovine liver and muscle tissues are extracted with methanol/methyl t-butyl ether. The extracts are cleaned up on a C_{18} SPE column. For liver, this is followed by subsequent clean-up on an LC-Si SPE column. The eluate is evaporated to dryness and the residue is reconstituted and determined by LC-MS-MS.

8.10.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents are analytically pure and "water" is grade deionized, specified in GB/T 6682. Acetonitrile, methanol, methyl t-butyl ether, and n-hexane are all HPLC grade; Trichloromethane; Ammonium formate; Sodium chloride saturated water; Water saturated ethyl acetate; β-glucuronidase/sulfatase: type H-2, Helix pomatia. 0.20-μm membrane filter.

Standards: testosterone, epi-testosterone, and progesterone: purity ≥99%; testosterone-D_5 and progesterone-D_9: purity ≥98%;

1.0-M phosphate buffer: Weigh 13.6 g potassium dihydrogen phosphate and dissolve with 100 mL of water. Weigh 8.7 g dipotassium hydrogen phosphate and dissolve with 50 mL of water. Separately add 70 mL of potassium dihydrogen phosphate solution and 28 mL of dipotassium hydrogen phosphate, mix well, and adjust the PH of the solution to 5.0. The buffer is stable for 1 week at 4°C.

Stock standard solutions (100 μg/mL): accurately weigh an adequate amount of testosterone, epi-testosterone, and progesterone standard into individual volumetric flasks. Dissolve in methanol to prepare a solution of 100 μg/mL as the standard stock solution. Solution is stable for 6 months at −18°C.

Standard working solution (5 μg/mL): Pipette 5.00 mL of testosterone, epi-testosterone, and progesterone stock solution into three separate 100-mL volumetric flasks and dilute to volume with methanol. Solution is stable for 6 months at −18°C.

Mixed standard working solution (50 μg/L): Pipette 1.00 mL of testosterone, epi-testosterone. and progesterone standard working solution into a 100-mL volumetric flask and dilute to volume with methanol. Solution is stable for 3 months at −18°C.

Internal stock standard solution (100 μg/mL): Separately, accurately weigh an adequate amount of testosterone-D_5 and progesterone-D9 standard, dissolve in methanol, and prepare a solution of 100 mg/L as the standard stock solution. Solution is stable for 6 months at −18°C.

Internal standard working solution (5 μg/mL): Into separate 100-mL volumetric flasks, pipette 5.00 mL of testosterone-D6 and progesterone-D9 stock solution and dilute to volume with methanol. Solution is stable for 6 months at −18°C.

Mixed internal working solution (25 μg/L): Pipette 0.50 mL of testosterone-D6 and progesterone-D9 internal standard working solution into a 100-mL volumetric flask and dilute to volume with methanol. Solution is stable for 3 months at −18°C.

C_{18} SPE column, 500 mg, 6 mL: Condition the column with 6 mL of methanol and 6 mL of water before use. LC-Si SPE column, 200 mg, 6 mL. Condition the column with 6 mL of hexane before use.

8.10.4 APPARATUS

Liquid chromatography tandem mass spectrometer equipped with electrospray ionization; Balance: 0.1 mg and 0.01 g sensitivity; Homogenizer; High-speed blender; Low-temperature centrifuge: ≥4000 rpm; High-speed centrifuge: ≥12,000 rpm; Ultrasonic water bath; Vortex mixer; Shaking water bath; Nitrogen evaporator; Vacuum manifold for solid phase extraction; Rotary vacuum evaporator; KD container.

8.10.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Sample, about 500 g, is blended and homogenized thoroughly in a blender and placed in clean containers as the test sample, which is sealed and labeled. The test sample should be stored at −18°C.

(2) Enzymatic hydrolysis

Weigh ca. 5 g of the test sample (accurate to 0.01 g) into a 50-mL centrifuge tube; add 200 μL of internal mixed working solution and

10 mL phosphate buffer. Homogenize for 30 s at 14,000 rpm. Add 60 μL of glucuronidase and vortex mix for 30 s. Place tube in a shaking water bath at 37°C for 16 h.

(3) Extraction

Add 10 mL methanol to the test sample solution after enzymic digestion and vortex mix for 2 min. Place in ice bath for 5 min. Centrifuge for 10 min at 4000 rpm (5°C). Transfer the supernatant into another 50-mL centrifuge tube. Add 5 mL of methanol to the residue and extract once more. Centrifuge for 10 min at 4000 rpm and combine the supernatants. Add 15 mL of methyl t-butyl ether into the second centrifuge tube and shake for 5 min. Centrifuge for 5 min at 4000 rpm. Transfer the supernatant into a 125-mL funnel. Extract the lower layer twice with 15 mL methyl t-butyl ether and 10 mL of methyl t-butyl ether. Combine the supernatants, add 10 mL of NaCl saturated water and shake for 30 s; let stand to separate. Transfer the supernatant into a pear-shaped flask and evaporate to dryness with a rotary evaporator at 40°C. Dissolve the residue in 1.5 mL of methanol and vortex mix for 1 min. Sonicate for 5 min and add 20 mL of water; mix well for clean up.

(4) Clean-up

Pass the sample extract solution through the preconditioned C_{18} column; rinse the pear-shaped flask with 5 mL of water to C_{18} column at a rate less than 2 mL/min. After loading, rinse the column with 5 mL of water and discard the effluents. Elute the column with 10 mL of methanol and collect eluate into a pear-shaped flask. Evaporate to dryness with rotary evaporator at 40°C. For the muscle sample, add 0.5 mL of methanol to dissolve the residue and vortex mix for 1 min. Add 0.5 mL of water, mix well and filter with 0.20-μm membrane for LC-MS-MS determination.

For the liver sample, add 0.5 mL of trichloromethane to dissolve the residue described; vortex mix for 1 min and then add 5 mL of *n*-hexane and sonicate for 1 min. Pass the sample extract solution through the preconditioned LC-Si column at a flow rate of 1 mL/min. After loading, rinse the column with 5 mL of *n*-hexane and discard the effluents. Elute the column with 6 mL of water-saturated ethyl acetate and transfer the eluate to a KD container. Evaporate to dryness with rotary evaporator at 40°C. Add 0.5 mL of methanol to dissolve the residue and vortex mix for 1 min. Add 0.5 mL of water and mix well; filter with 0.20-μm membrane for LC-MS-MS determination.

8.10.6 DETERMINATION

(1) Operating conditions

Column: C_{18}: 150 mm × 2.1 mm (i.d.), 5-μm particle size; Column temperature: 40°C; Injection volume: 20 μL; Mobile phase, flow rate, and the chromatographic gradient are shown in Table 8.20.

TABLE 8.20 Mobile Phase, Flow Rate, and Elution Program

Time (min)	Flow Rate (mL/min)	Acetonitrile (%)	Ammonium Formate (%)
0.00	0.200	30	70
10.00	0.200	85	15
15.00	0.200	85	15
15.01	0.200	30	70
23.01	0.200	30	70

Ion source: ESI; Scan mode: Positive mode; Monitor mode: multiple reaction monitoring; Nebulizer gas, curtain gas, heater gas, and collision gas are high purity nitrogen or equivalent, optimize the flow rate of each gas to reach the requirement of the senitivity of mass spectrometer; Ionspray voltage (IS), declustering potential (DP) and collision energy (CE) should be optimized to reach the requirement of the senitivity of the mass spectrometer; Quality ions, quantity ions, declustering potential (DP) and collision energy (CE) are shown in Table 8.21.

TABLE 8.21 Mass Spectrometric Acquisition Parameters for Testosterone, Epi-Testosterone, and Progesterone

Analytes	Quality Ions (m/z)	Quantity Ions (m/z)	Quantity Internal Standard	Dwell Time (ms)	DP (V)	CE (V)
Testosterone	289/97	289/97	Testosterone-D5	100	65	35
	289/109					39
Epi-testosterone	289/97	289/97	Testosterone-D5	100	65	35
	289/109					39
Progesterone	315/109	315/109	Progesterone-D9	100	54	38
	315/97					37
Testosterone-D5	294/100	294/100	–	100	57	35
Progesterone-D9	324/100	324/100	–	100	50	34

(2) Qualitative analysis

The qualitative ions for each analyst include one precursor ion and two product ions at least. Under the same determination conditions, the retention time of the analyte in the sample shall match that of the calibration standard within the tolerance of 2.5%. A compound is present in the sample if, in addition, the relative intensities of the detected ions correspond to those of the calibration standard at comparable concentrations, within the tolerances shown in Table 8.3.

(3) Quantitative analysis

Under optimum conditions of LC-MS/MS, inject mixed standard working solutions and generate a linear regression curve based on the ratio of the chromatographic peak area of the analyte to that of the internal standard vs. the ratio of the concentration of the analyte to that of the internal standard. Quantify the concentration of analyte in the unknown or test sample from the regression curve. The responses of the analyte in the standard working solution and the sample solution should be within the linear range of the instrument detection. The MRM chromatograms of testosterone, epitestosterone, and progesterone standards are shown in Figs. 8.9 and 8.10.

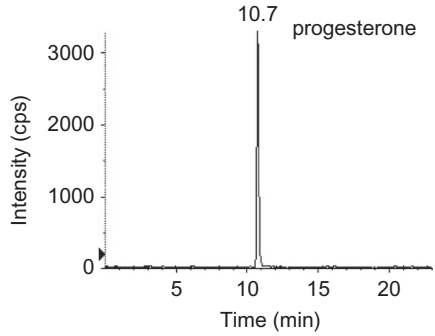

FIG. 8.9 MRM chromatogram of progesterone.

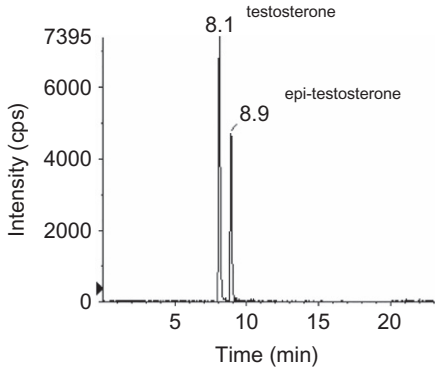

FIG. 8.10 MRM chromatogram of testosterone and epi-testosterone

8.10.7 PRECISION

The method for the determination of the precision of this standard is according to GB/T6379. The repeatability and reproducibility are calculated to a 95% confidence level.

(1) Repeatability

For two independent test results carried out under repeatability conditions, the absolute difference value would be $\leq r$ (repeatability limit). The fortified levels range and repeatability equations are shown in Tables 8.22 and 8.23.

If the absolute difference value exceeds r (repeatability limit), the two independent test results would be rejected and retested.

TABLE 8.22 The Analytical Range and Repeatability and Reproducibility Equations (Muscle)

Analytes	Fortified Levels Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Testosterone	0.05–0.5	$r=0.0732m+0.0229$	$R=0.072m+0.0268$
Epi-testosterone	0.05–0.5	$r=0.143m+0.0287$	$R=0.0149+0.0168$
Progesterone	0.25–2.5	$r=0.0739m+0.0716$	$R=0.218m+0.0178$

Note: m *is the average of two test results.*

TABLE 8.23 The Analytical Range and Repeatability and Reproducibility Equations (Liver)

Analytes	Fortified Levels Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Testosterone	0.25–2.5	$r=0.102m+0.0216$	$R=0.153m+0.0008$
Epi-testosterone	0.25–2.5	$r=0.207m$	$R=0.183m+0.0321$
Progesterone	0.25–2.5	$r=0.0805+0.0811$	$R=0.0926m+0.0677$

Note: m *is the average of two test results.*

(2) Reproducibility

For two independent test results carried out under reproducibility conditions, the absolute difference value would be $\leq R$ (reproducibility limit). The analytical range and reproducibility equations are shown in Tables 8.22 and 8.23.

8.10.8 RECOVERY

Under optimized conditions, the recoveries for testosterone, epi-testosterone, and progesterone at varying concentrations are listed in Table 8.24.

TABLE 8.24 The Recoveries of Testosterone, Epi-Testosterone, and Progesterone

Matrix	Analytes	Fortified Level (μg/kg)	Average Recovery (%)
Muscle	Testosterone	0.1	101
		0.2	97.3
		0.5	106
	Epi-testosterone	0.1	95.9
		0.2	86.4
		0.5	105
	Progesterone	0.5	86.8
		1.0	86.1
		2.5	99.4
Liver	Testosterone	0.5	106
		1.0	103
		2.5	102
	Epi-testosterone	0.5	114
		1.0	102
		2.5	118
	Progesterone	0.5	93.4
		1.0	86.5
		2.5	91.8

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FURTHER READING

[1] Xu CL, Chu XG, Peng CF. J Pharm Biomed Anal 2006;41:616.

8.11

Method for the Determination of Melengestrol Acetate, Chlormadinone Acetate, and Megestrol Acetate Residues in Bovine and Porcine Fat—LC-UV (GB/T 20753-2006)

8.11.1 SCOPE

This method is applicable for determination of melengestrol acetate, chlormadinone acetate, and megestrol acetate in fat of beef and pork.

The limit of determination of melengestrol acetate, chlormadinone acetate, and megestrol acetate is 0.010 mg/kg.

8.11.2 PRINCIPLE

The fat sample is thawed in a microwave. Rendered fat is extracted with acetonitrile. After saponification, the progestogens are cleaned up on a cyanopropyl solid-phase extraction column. Chromatography is performed on a C18 column and with UV detection, using an external standard method to quantify.

8.11.3 REAGENTS AND MATERIALS

Unless other specified, all reagents are of analytical-reagent grade.

Acetonitrile: HPLC grade.
Methanol: HPLC grade.

Ethyl acetate: HPLC grade.
Hexane: HPLC grade.
Magnesium chloride ($MgCl_2 \cdot 6H_2O$).
Sodium hydroxide.
Hydrochloric acid.
Ethyl acetate-hexane (1+19): Combine 10 mL ethyl acetate with 190 mL hexane.
Ethyl acetate-hexane (1+4): Combine 40 mL ethyl acetate with 160 mL hexane.
Hydrochloric acid solution: 0.2 % (*v*/*v*). Combine 2.0 mL hydrochloric acid in 1 L of water.
Magnesium chloride solution: 1.0 mol/L. Add 20.3 g magnesium chloride ($MgCl_2 \cdot 6H_2O$) to a 100-mL volumetric flask. Bring to volume with water.
Sodium hydroxide solution: 0.1 mol/L. Add 1.00 g sodium hydroxide to a 250-mL volumetric flask. Bring to volume with water.
Acetonitrile-water (65+35).
Standards: melengestrol acetate ($C_{25}H_{32}O_4$, FW = 396.52, minimum ≥97%), chlormadinone acetate ($C_{23}H_{29}ClO_4$, FW = 404.93, minimum ≥98%), megestrol acetate ($C_{24}H_{32}O_4$, FW = 384.51, minimum ≥99%).
Stock standard solutions: 1000 µg/mL. Accurately weigh 0.0500 g of melengestrol acetate, chlormadinone acetate, and megestrol acetate into individual 50-mL volumetric flasks. Dilute with methanol. Prepare yearly. Store in glass at 4°C.
Intermediate standards solution I: 100 µg/mL. Pipette 10 mL each of melengestrol acetate, chlormadinone acetate, and megestrol acetate stock standard solution into a 100-mL volumetric flask and bring to volume with methanol. Prepare yearly. Store in glass at 4°C.
Intermediate standards solution II: 1.00 µg/mL. Pipette 1.00 mL of 100 µg/mL intermediate standards solution I into a 100-mL volumetric flask and bring to volume with methanol. Prepare every 6 months. Store in glass at 4°C.
Working standard solutions: Prepare working standard solutions by adding 10, 20, 40, 80, and 100 µL intermediate standards solution II into 980, 970, 950, 910, and 890 µL, respectively, of acetonitrile-water (65+35). Add 10 µL hydrochloric acid solution to the solutions and vortex mix, obtaining 0.010, 0.020, 0.040, 0.080, and 0.10 µg/mL. Prepare daily.
SPE columns: Cyanopropyl (CN) end-capped 3 mL, 500 mg packing. Prior to use, condition with 5 mL ethyl acetate, followed by 6 mL hexane.

8.11.4 APPARATUS

High-performance liquid chromatograph equipped with UV-Vis detector.
Balance: 0.1 mg and 0.01 g sensitivity.
Nitrogen evaporator.
SPE vacuum manifold.
Centrifuge: refrigeration to −5°C, ≤5000 rpm.

Vortex mixer.
Water bath boiler.
Microwave oven.
Pipettors: 10 μL to 100 μL and 100 μL to 1000 μL.

8.11.5 SAMPLE PRETREATMENT

(1) Sample Preparation

Into a glass funnel, packed at the bottom with glass wool, place approximately 5 g adipose tissue cut into 1-cm cubes. Place funnel over a 150-mL beaker. Place beaker into the microwave. Turn on at high power for 30–60 s, depending on the amount of tissue to be rendered. If fat is not melted, repeat in 30–60 s intervals, until fat melts from tissue and drips through the funnel. Avoid overheating the tissue cubes. Seal and label.

Transfer rendered fat into scintillation vial and store at −18°C until analysis.

(2) Extraction

Accurately weigh 2 g (nearest 0.01 g) of the rendered fat sample into a 50-mL centrifuge tube with cap. Add 5 mL acetonitrile and heat in 60°C water bath at for 3 min or until the fat is liquid. Shake capped tube at high speed on a vortex mixer for 1 min. Centrifuge tube at 3500 rpm for 7 min at −5°C. Decant supernatant into a 15-mL centrifuge tube. Reextract the pellet, repeating acetonitrile extraction of fat. Combine supernatants. Add 2 mL hexane to the combined acetonitrile extracts and then shake the capped tube at high speed for 1 min. Rewash the acetonitrile extraction with another 2 mL hexane. Centrifuge tube at 3500 rpm for 5 minutes at −5°C. Discard the hexane. Evaporate the acetonitrile extracts to dryness at 60°C on the nitrogen evaporator.

(3) Saponification

Add 4 mL hexane, 1 mL 0.1 mol/L sodium hydroxide solution, and 500 μL 1.0 mol/L magnesium chloride solution in that order, and vortex mix capped tube for 10 s. Heat tube in 60°C water bath for 15 min and then centrifuge at 3500 rpm for 5 min at −5°C. Decant supernatant into a 15-mL centrifuge tube. Add 4 mL hexane to the pellet, vortex, and repeat steps above, combining supernatants. Evaporate the combined supernatants to dryness at 60°C on the nitrogen evaporator. Reconstitute in 1.0 mL hexane.

(4) Clean-up

Load the sample on the CN SPE column. Rinse centrifuge tube with two 1-mL portions of hexane and add to the column. Wash the column with 5 mL hexane follwed by 6 mL ethyl acetate-hexane (1 + 19). Dry column for 2 min. Elute with 3.5 mL ethyl acetate-hexane (1 + 4). Evaporate the eluate to dryness at 60°C on the nitrogen evaporator. Reconstitute with 990 μL acetonitrile-water (65 + 35) and allow sample to sit at least 15 min. Add 10 μL 0.2% hydrochloric acid solution to samples and vortex mix.

8.11.6 DETERMINATION

(1) HPLC Conditions

Column: C18 (4.6 mm × 250 mm, 5 μm), or equivalent;
Safeguard column: C18 (4.6 mm × 12.5 mm, 5 μm);
Mobile phase: Acetonitrile-water (65 + 35);
Flow rate: 1.0 mL/min;
Column temperature: 35°C;
Detector wavelength: 292 nm;
Injection volume: 40 μL.

(2) HPLC Determination

According to the approximate concentration of progestogens in the test sample, select the standard working solution with similar peak area to that of the sample solution. The responses of the analyte in the standard working solution and sample solution should be within the linear range of the instrumental detection. The standard working solution should be injected randomly in-between the injection of sample solution of equal volume. Under these chromatographic conditions, the chromatogram of the standard is shown as Fig. 8.11.

8.11.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the repeatability condition, the difference of absolute value of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equations of the three progestogens in bovine and porcine fat are shown in Tables 8.25 and 8.26.

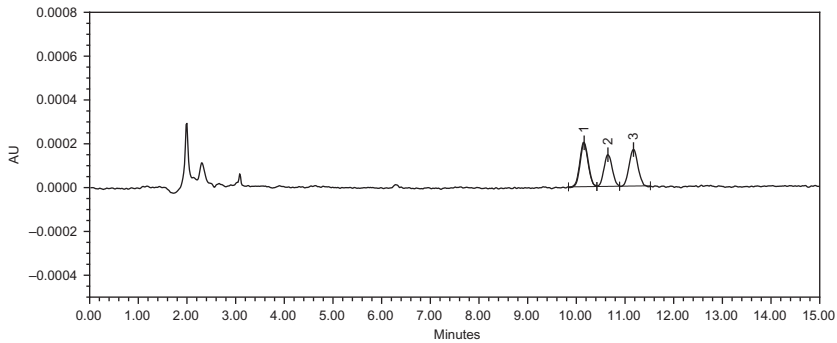

FIG. 8.11 Chromatogram of seven derivatives of melengestrol acetate, chlormadinone acetate, and megestrol acetate (0.020 μg/mL). 1. Megestrol acetate, 2. chlormadinone acetate, 3. melengestrol acetate.

TABLE 8.25 Content Ranges in Bovine Fat and the Repeatability and Reproducibility Equations

Analyte	Content Range (mg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Melengestrol acetate	0.010–0.050	$r=0.1.38m+0.2532$	$R=0.2093m$
Chlormadinone acetate	0.010–0.050	$r=0.1246m$	$R=0.1341m$
Megestrol acetate	0.010–0.050	$r=0.0857m$	$R=0.1228m$

Note: m *equals to the average of two results.*

TABLE 8.26 Content Ranges in Porcine Fat and the Repeatability and Reproducibility Equations

Analyte	Content Range (mg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Melengestrol acetate	0.010–0.050	$r=0.6518m$	$R=0.6771m$
Chlormadinone acetate	0.010–0.050	$r=0.0458m+1.0587$	$R=0.0617m+1.2235$
Megestrol acetate	0.010–0.050	$r=0.059m+0.7958$	$R=0.0763m+1.0742$

Note: m *equals to the average of two results.*

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned and two independent experiments should be redone.

(2) Reproducibility

Under the reproducibility condition, the difference of absolute value of two independent results is not above the reproducibility limit (R), The content ranges and the reproducibility equations of the three progesterones in bovine and porcine fat are shown in Tables 8.25 and 8.26.

TABLE 8.27 Mean Recoveries of Sample Spiked With Three Progesterones

Compound	Spiked Level (mg/kg)	Mean Recovery (%)
Melengestrol acetate	0.010	91.8
	0.020	87.4
	0.050	88.8
Chlormadinone acetate	0.010	89.1
	0.020	86.7
	0.050	89.0
Megestrol acetate	0.010	88.1
	0.020	89.6
	0.050	90.2

8.11.8 RECOVERY

Under optimized condition, the recoveries of melengestrol acetate, chlormadinone acetate, and megestrol acetate in fat of beef and pork using this method are listed in Table 8.27.

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Chapter 9

Inanabolic Steroids

9.1

Curative Effects and Side Effects of Zeranol, Zearalanone, Diethylstilbestrol, Hexestrol, and Dienoestrol

α-Zearalanol (zeranol) is a growth promotant made commercially by reduction of zearalenone, an estrogenic β-resorcylic acid lactone produced by *Fusarium* species. Although a normal product of *Fusarium* metabolism, zeranol is deemed an industrial anabolic by European Union (EU) legislation, and its use was banned in 1989. The use of zeranol as a growth promotant is, however, permitted in many countries, such as New Zealand.

Zearalenone (ZON) [6-(10-hydroxy-6-oxo-trans-1-undecenyl)-resorcyclic acid lactone] is a secondary fungal metabolite produced by *Fusarium culmorum* and *Fusarium graminearum*. Fusarium species colonize several grains such as maize, barley, oats, wheat, and sorghum. The chemical conformation of ZON resembles that of 17α-estradiol and can bind to the estrogen receptor to disrupt the endocrine system. Occurrence of ZON in animal feed causes hyperestrogenism with severe reproductive and infertility problems, especially in swine. Concentration ratios of ZON and its metabolites and the susceptibility to the adverse effects of ZON vary considerably with animal species.

Diethylstilbestrol (DES) contains two hydroxyl groups on its phenyl ring. In human medicine, it has been widely used to cure some diseases. For example, some studies have shown that DES can be used to cure prostate cancer. To date, it has been found that DES also affects the intracellular Ca^{2+} levels in renal tubular cells.

The nonsteroidal synthetic estrogen hexestrol (HES), which is diethylstilbestrol hydrogenated at the C-3–C-4 double bond, is carcinogenic. The chemical structures and activity of dienestrol (DEN) and hexestrol (HES) are very similar to those of DES. However, because of their potential carcinogenic properties and other adverse effects to humans and animals, their use poses significant human health issues. As a result, the use of these estrogens in food-producing animals

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and other products has been prohibited in many countries, and suitable analytical methods are needed to regulate and control the illegal use of estrogens.

9.2

The Metabolite of Zeranol, Zearalanone, Diethylstilbestrol, Hexestrol, and Dienoestrol

Feeding grains contaminated with fusarium mycotoxin including ZON has been shown to cause hyperestrogenic and adverse metabolic effects in livestock and poultry. In mammals, ZON is mainly metabolized into α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) with α-ZOL being dominant in pigs and β-ZOL being the predominant metabolite in cows. α-ZOL is the only ZON derivative found to be generated by *Fusarium* fungi. It has been shown that the reduced forms exhibit a higher estrogenic activity than ZON. Because of its anabolic effect, α-ZAL is used as a growth promoter. Although its use has been banned in the EU, there is still a need to monitor its abuse. Zearalanone (ZAN) was identified in a few samples of pig and cattle urine. At this point the mechanism of ZON formation is still unclear.

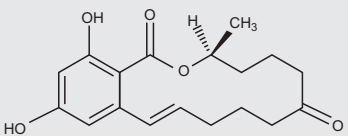
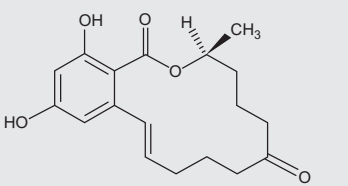
The major metabolite of hexestrol (HEX) is the catechol, 3-OH-HES, which can be metabolically converted to the catechol quinone, HES-3, 4-Q. Study of HES was undertaken with the scope of substantiating evidence that natural catechol estrogen-3,4-quinones are endogenous and carcinogenic. HES-3,4-Q was previously shown to react with deoxyguanosine to form the depurinating adduct 3-OH-HES-6-N7Gua by 1,4-Michael addition.

9.3

Chemical Structures and Maximum Residue Limits for Zeranol, Zearalanone, Diethylstilbestrol, Hexestrol, and Dienoestrol

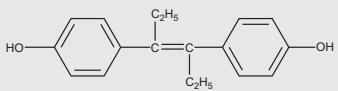
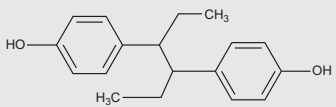
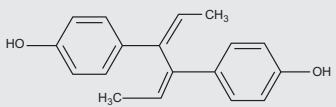
See Table 9.1.

TABLE 9.1 Chemical Structures and Maximum Residue Limits for Zeranol, Zearalanone, Diethylstilbestrol, Hexestrol, and Dienoestrol

Compound Names	Molecular Structure	Molecular Weight	Cas. No	MRLs (μg/kg)	
Zearalanone		318.36	17924-92-4	American	Total zeranol residues: 1. 150 (in muscle); 2. 450 (in kidney); 3. 300 (in liver)
				China	
				EU	
				Japan	1. 2 (in muscle) 2. 10 (in liver)
Zeranol		322.40	26538-44-3	American	Banned
				Codex	10 (bovine liver), 2.0 (bovine muscle)
				Austrilia	5.0 (bovine muscle)
				China	Banned

Continued

TABLE 9.1 Chemical Structures and Maximum Residue Limits for Zeranol, Zearalanone, Diethylstilbestrol, Hexestrol, and Dienoestrol—cont'd

Compound Names	Molecular Structure	Molecular Weight	Cas. No	MRLs (μg/kg)	
Diethylstilbestrol		268.35	56-53-1	–	–
Hexestrol		270.37	84-16-2	–	–
Dienoestrol				–	–

9.4

Determination of Zearalanol, Zearalanone, Diethylstilbestrol, Hexestrol, and Dienoestrol Multiresidues in Bovine and Equine Liver, Kidney, and Muscle—LC-MS-MS Method (GB/T 20766-2006)

9.4.1 SCOPE

This method is applicable to the determination of zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol in bovine and equine liver, kidney, and muscle.

The limit of determination of zeranol, zearalanone, and hexestrol is 0.5 μg/kg. The limit of determination of diethylstilbestrol and dienoestrol is 1.0 μg/kg.

9.4.2 PRINCIPLE

Zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol residues in the test sample are extracted with methyl *tert*-butyl ether and acetate buffer solution. The extract is cleaned up with a silica gel column. Determination is by LC-MS-MS using an internal standard.

9.4.3 REAGENTS AND MATERIALS

Methyl *tert*-butyl ether (MTBE), methanol, acetonitrile, ethyl acetate, n-Hexane, acetic acid, and dichloromethane are all of HPLC grade; Sodium hydroxide is guaranteed reagent; Sodium acetic acid (CH3COONa·3H2O) is of analytical grade; Acetate buffer solution (0.2 mol/L, pH 5.2): Weigh 2.52 g acetic acid and 12.95 g sodium acetic acid and dissolve in 800 mL water. Adjust the pH to 5.2 ± 0.1 with sodium hydroxide solution and make up to 1000 mL with water; Sodium hydroxide solution (3 mol/L): Weigh 100 g sodium hydroxide and dissolve in 1000 mL water; Silica gel Sep-Pak column: 500 mg, 3 mL. Condition by washing with 3 mL n-hexane twice and the flow rate is 4 mL/min;

Filter: 0.45 μm; Dissolving solution: *n*-Hexane-Dichloromethane (60+40); Washing solution: Ethyl acetate-*n*-Hexane (6+94); Eluant: Ethyl acetate-Hexane (25+75); β-Glucuronidase: Type H-2, 124,400 β-glucuronidase units/mL, 3610 sulfatase units/mL;

Standards: Zeranol (ZER, including zeranol and taleranol, 50%, respectively); Purity ≥97%; Zearalanone (ZEAR) Purity ≥97%; Diethylstilbestrol (DES) Purity ≥99%; Hexestrol (HEX) Purity ≥98%; Dienoestrol (DEN) Purity ≥98%.

Standard solutions: Accurately weigh an adequate amount of each standard (ZER, ZEN, DES, DEN, and HEX) and separately dissolve in methanol to prepare a solution of 1 mg/mL in concentration as the standard stock solution. According to the requirement, prepare a mixed standard working solution of appropriate concentrations by mixing and diluting the stock solutions with methanol. Store at 4°C in a refrigerator; the solution can be stored for 3 months.

Internal standard: Zeranol (ZER-d4, including zeranol-d4 and taleranol-d4, 50% respectively) Purity ≥99%; Diethylstilbestrol (DES-d8) Purity ≥98%.

Internal standard solution: Accurately weigh an adequate amount of ZER-d4 and DES-d8 and dissolve in methanol to prepare a solution of 1 mg/mL in concentration as the standard stock solution. According to the requirement, prepare a standard working solution of appropriate concentrations by mixing and diluting the stock solutions with methanol. Store at 4°C in a refrigerator; the solution can be stored for 3 months.

9.4.4 APPARATUS

LC-MS-MS: Equipped with atmospheric pressure chemical ion source; Analytical balance: 0.1 mg and 0.01 g; Automatic solid phase extraction or device of SPE; Homogenizer: rotate speed > 10,000 rpm; Nitrogen evaporator; Vortex mixer; Centrifuge: rotate speed >3000 rpm.

9.4.5 SAMPLE PRETREATMENT

(1) Extraction

Weigh ca. 5 g of the test sample (accurate to 0.01 g) into a 50-mL centrifuge tube and add the internal standard solution, making 2 μg/kg of internal standard concentration. Add 20 mL MTBE into the tube and homogenize for 1 min at 10,000 rpm speed. Centrifuge at 3000 rpm for 5 min; decant supernatant liquid into another 50-mL tube with stopper. Place the residue of the centrifuge in the ventilated cabinet and volatilize for 30 min. Add 15 mL acetate buffer solution and homogenize for 1 min at high speed. Centrifuge at 3000 rpm for 5 min, decant supernatant liquid into a 25-mL tube with topper and evaporate the residue MTBE at 40°C in a

nitrogen evaporator. Then add 80 μL β-Glucuronidase and keep overnight at 52°C. Add sodium hydroxide solution to adjust the pH of the solution to 7, add 10 mL MTBE, and mix well. Centrifuge at 3000 rpm for 2 min. Transfer the MTBE layer, combine with the previous MTBE extraction, and evaporate to dryness at 40°C under nitrogen flow. Add 1 mL dissolving solution, vortex 30 s, and prepare for clean-up.

(2) Clean-up

Add the extract with 2 mL/min flow rate onto silica gel Sep-Pak column. Add 3 mL washing solution into the test tube and mix and load with 2 mL/min flow rate. Wash with 3 mL washing solution and a flow rate of 3 mL/min; add 2 mL air push with 4 mL/min for the dryness of the column. Elute with 6 mL eluant and a flow rate of 2 mL/min; add 2 mL air push with 6 mL/min for the dryness of the column. Collect the eluant. Evaporate the eluant to dryness at 40°C under nitrogen flow. Dissolve dry residue in 1 mL mobile phase, vortex 30 s, and put through the filter. Collect the filtrate and prepare for injection.

9.4.6 DETERMINATION

(1) Operation condition

LC column: ZORBAX Eclipse SC-C8 (150 mm × 4.6 mm, 3.5 μm) or equivalent; column temp: 25°C; Mobile phase: Acetonitrile-H2O (70 +30, V/V); Flow rate: 1.0 mL/min; Injection volume: 50 μL.

Ionization mode: Atmospheric pressure chemical ionization; Scan mode: Negative ion scan; Determination mode: Multiple reaction monitoring (MRM); Ion source temperature: 325°C; Collision activated dissociation gas pressure: 10 psi; Curtain gas pressure: 15 psi; GAS 1: 35 psi; Nebulizer current: −5 μA; Ionspray voltage: −4500 V; Diagnostic ions, quantitative ions, collision energy, and declustering potential: see Table 9.2.

(2) Qualitative analysis

Under the same experimental conditions, the ratios between the retention times of analytes and internal standards, called relative retention times, of the sample chromatogram peaks are consistent with the standards and the differences are within ±2.5%, and if after background compensation, all the diagnostic ions are present, the relative ion intensities shall correspond to those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 9.3, then the corresponding analyte must be present in the sample.

(3) Quantitative analysis

Under optimum working conditions, inject a series of matrix standard working solutions separately and make the standard working curve. Check the capability of the instrument and determine the linear range.

TABLE 9.2 Diagnostic Ions, Quantitative Ions, Collision Energy, and Declustering Potential of Five Hormones

Analytes	Diagnostic Ions (m/z)	Quantitative Ions (m/z)	Collision Energy (V)	Declustering Potential (V)
ZER	321.1/277.2	321.1/277.2	−35	−100
	321.1/303.2		−35	−100
ZEAR	319.3/205.1	319.3/205.1	−42	−126
	319.3/205.1		−34	−126
ZER-d4	325.1/208.9	325.1/263.1	−36	−100
	325.1/263.1		−38	−100
DES	267.0/222.1	267.0/222.1	−40	−90
	267.0/237.1		−40	−90
DEN	265.0/221.1	265.0/235.1	−35	−90
	265.0/235.1		−32	−90
HEX	269.0/134.0	269.0/134.0	−22	−75
	269.0/119.0		−46	−75
DES-d8	275.1/245.0	275.1/245.0	−40	−73
	275.1/227.9		−40	−74

TABLE 9.3 The Relative Abundance of Ion Maximum Allowable Deviation for Qualitative Identification

Relative abundance	>50%	>20% to 50%	>10% to 20%	≤10%
Maximum allowable deviation	±20%	±25%	±30%	±50%

Quantitatively determine the samples through the instrument workstation or through the curve by emendation of the internal standard. The responses of hormones in the sample solutions should be within the linear range of the instrumental detection.

9.4.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under repeatability conditions, the difference of absolute value of two independent results is not above the repeatability limit (r). The analytical ranges and the repeatability equations of the four hormones in bovine urine are shown in Table 9.4.

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned and two independent experiments should be repeated.

(2) Reproducibility

Under reproducibility conditions, the difference of the absolute value of two independent results is not above the reproducibility limit (R). The analytical range and the reproducibility equations of the four hormones in bovine urine are shown in Table 9.4.

9.4.8 RECOVERY

Under optimized conditions, the recoveries of zearalanol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol using this method are listed in Table 9.5.

TABLE 9.4 Analytical Range, Repeatability, and Reproducibility Equations

Analytes	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
ZER	0.25–5	$\lg r = 0.9695 \lg m - 0.7326$	$\lg R = 0.8152 \lg m - 0.6343$
ZEAR	0.25–5	$\lg r = 1.1888 \lg m - 0.5939$	$\lg R = 1.2064 \lg m - 0.5195$
DES	0.5–10	$\lg r = 1.0198 \lg m - 0.8763$	$\lg R = 1.0846 \lg m - 0.5621$
DEN	0.5–10	$\lg r = 0.5401 \lg m - 0.2908$	$\lg R = 0.8781 \lg m - 0.2471$
HEX	0.25–5	$\lg r = 0.9546 \lg m - 0.7379$	$\lg R = 0.9088 \lg m - 0.4473$

Note: m *equals the average of two results.*

TABLE 9.5 The Recoveries of Zearalanol, Zearalanone, Diethylstilbestrol, Hexestrol, and Dienoestrol

Analytes	Added Level (μg/kg)	Average Recovery (%)
ZER	0.5	84.93
	2.5	88.58
	5.0	91.35
ZEAR	0.5	76.10
	2.5	79.18
	5.0	101.0
DES	1.0	80.12
	5.0	78.67
	10	102.7
DEN	1.0	74.83
	5.0	93.00
	10	99.53
HEX	0.5	91.33
	2.5	72.67
	5.0	92.47

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9.5

Determination of Zearalanol, Diethylstilbestrol, Hexestrol and Dienoestrol Multiresidues in Bovine Urine—LC-MS-MS Method (GB/T 20767-2006)

9.5.1 SCOPE

This method is applicable to the determination of zeranol, diethylstilbestrol, hexestrol, and dienoestrol in bovine urine.

The limit of determination of zeranol and hexestrol is 0.5 μg/L. The limit of determination of diethylstilbestrol and dienoestrol is 1.0 μg/L.

9.5.2 PRINCIPLE

The bovine urine is cleaned up directly by immunoaffinity columns. Each immunoaffinity column contains gel with immobilized antibodies cross-reactive for diethylstilbestrol, hexestrol, and dienoestrol or zeranol. The analytes are combined with the antibodies on the immunoaffinity columns. Wash the columns with water to remove the impurities, elute with eluant, and collect the eluant. Determination is by LC-MS-MS using an internal standard.

9.5.3 REAGENTS AND MATERIALS

Methanol, ethanol, and acetonitrile are all of HPLC grade; Wash solution: Dilute 1 part column wash buffer with 19 parts double deionized water; Eluant: Ethanol-water (70+30); Storage solution: Dilute 1 part column storage buffer with 4 parts double deionized water; Immunoaffinity column: Randox or equivalent; Zeranol immunoaffinity columns: binding capacity = 100 ng zeranol; Stilbene immunoaffinity columns: binding capacity = 50 ng hexestrol, 50 ng diethylstilbestrol, 50 ng dienestrol.

Standards: Zeranol (ZER, including zeranol and taleranol, 50% respectively) Purity ≥97%; Diethylstilbestrol (DES) Purity ≥99%; Hexestrol (HEX) Purity ≥98%; Dienoestrol (DEN) Purity ≥98%.

Standard solutions: Accurately weigh an adequate amount of each standard (ZER, DES, DEN and HEX) and separately dissolve in methanol to

prepare a solution of 1 mg/mL in concentration as the standard stock solution. According to the requirement, prepare a mixed standard working solution of appropriate concentrations by mixing and diluting the stock solutions with methanol. Store at 4°C in a refrigerator; the solution can be stored for 3 months.

Internal standard: Zeranol (ZER-d4, including zeranol-d4 and taleranol-d4, 50% respectively) Purity ≥99%; Diethylstilbestrol (DES-d8) Purity ≥98%.

Internal standard solution: Accurately weigh an adequate amount of ZER-d4 and DES-d8 and dissolve in methanol to prepare a solution of 1 mg/mL in concentration as the standard stock solution. According to the requirement, prepare a standard working solution of appropriate concentrations by mixing and diluting the stock solutions with methanol. Store at 4°C in a refrigerator; the solution can be stored for 3 months.

9.5.4 APPARATUS

LC-MS-MS: Equipped with atmospheric pressure chemical ion source; Centrifuge: rotate speed > 3000 rpm; Nitrogen evaporator; Vortex mixer.

9.5.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Part of representative sample is taken from the primary sample and divided into two equal portions; each portion is placed in a clean vessel as the test sample, which is sealed and labeled. The test samples should be stored at 2–8°C.

(2) Extraction

After centrifuging at 3000 rpm for 15 min, put 3 mL of the test sample into a 50-mL centrifuge tube and add the internal standard solution, making 2 μg/kg of internal standard concentration. Decant 3 mL supernatant liquid into the tube. Vortex 30 s and prepare for clean-up.

(3) Clean-up

The immunoaffinity columns are conditioned twice by washing with 15 mL wash solution and the flow rate is under 3 mL/min. Add the extract into columns with natural flow rate. Wash the columns twice with the same 5-mL washing solution with the flow rate under 2 mL/min. Wash the columns again with 5 mL water with the flow rate under 2 mL/min. Elute with 4 mL eluant with the flow rate under 2 mL/min. Collect the eluant. Evaporate the eluant to dryness at 40°C in nitrogen evaporator. Dissolve dry residue in 1 mL mobile phase, vortex 30 s, and put through the filter. Collect the filtrate and prepare for injection.

9.5.6 DETERMINATION

(1) Operation conditions

LC column: ZORBAX Eclipse XDB-C8 (150 mm × 4.6 mm, 3.5 μm) or equivalent; column temp: 25°C; Mobile phase: Acetonitrile-H_2O (70+30); Flow rate: 1.0 mL/min; Injection volume: 50 μL.

Ionization mode: Atmospheric pressure chemical ionization; Scan mode: Negative ion scan; Determination mode: Multiple reaction monitoring (MRM); Ion source temperature: 325°C; Collision activated dissociation gas pressure: 15 psi; Curtain gas pressure: 10 psi; GAS 1: 35 psi; Nebulizer current: −5 μA; Ionspray voltage: −4500 V; Diagnostic ions, quantitative ions, collision energy, and declustering potential: see Table 9.6.

(2) Qualitative determination

Under the same experimental conditions, the ratios between the retention times of analytes and internal standards, called relative retention times, of the sample chromatogram peaks are consistent with the standards and the differences are within ±2.5%; and if after background

TABLE 9.6 Diagnostic Ions, Quantitative Ions, Collision Energy, and Declustering Potential of Four Hormones and Two Internal Standards

Analytes	Diagnostic Ions (m/z)	Quantitative Ions (m/z)	Collision Energy (V)	Declustering Potential (V)
ZER	321.1/277.2	321.1/277.2	−35	−100
	321.1/303.2		−35	−100
ZER-d4	325.1/208.9	325.1/263.1	−36	−100
	325.1/263.1		−38	−100
DES	267.0/222.1	267.0/222.1	−40	−90
	267.0/237.1		−40	−90
DEN	265.0/221.1	265.0/235.1	−35	−90
	265.0/235.1		−32	−90
HEX	269.0/134.0	269.0/134.0	−22	−75
	269.0/119.0		−46	−75
DES-d8	275.1/245.0	275.1/245.0	−40	−73
	275.1/227.9		−40	−74

compensation, all the diagnostic ions are present, the relative ion intensities correspond to those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 9.3, then the corresponding analyte must be present in the sample.

(3) Quantitative determination

Under optimum working conditions, inject the series of matrix standard working solutions separately, to make the standard working curve. Check the capability of the instrument and determine the linear range. Quantitatively determine the samples through the instrument workstation or through the curve by emendation of the internal standard. The responses of hormones in the sample solutions should be within the linear range of the instrumental detection.

9.5.7 PRECISION

The precision data of this part are confirmed according to GB/T 6379. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the repeatability conditions, the difference of the absolute value of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equations of the four hormones in bovine urine are shown in Table 9.7.

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned and two independent experiments should be redone.

TABLE 9.7 Content Ranges and the Repeatability and Reproducibility Equations

Analytes	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
ZER	0.25–5	$\lg r = 0.9656 \lg m - 1.0897$	$\lg R = 0.9625 \lg m - 0.7128$
DES	0.5–10	$\lg r = 0.7192 \lg m - 0.2685$	$\lg R = 0.7883 \lg m - 0.2926$
DEN	0.5–10	$\lg r = 0.5312 \lg m - 0.3127$	$\lg R = 0.7507 \lg m - 0.2487$
HEX	0.25–5	$\lg r = 0.7728 \lg m - 0.4185$	$\lg R = 0.8698 \lg m - 0.2830$

Note: m *equals the average of two results.*

TABLE 9.8 The Recoveries of Zeranol, Diethylstilbestrol, Hexestrol, and Dienoestrol

Analytes	Added Level (μg/kg)	Average Recovery (%)
ZER	0.5	85.30
	2.5	99.29
	5.0	96.99
DES	1.0	80.81
	5.0	101.4
	10	100.2
DEN	1.0	105.2
	5.0	93.30
	10	97.50
HEX	0.5	83.74
	2.5	88.95
	5.0	103.2

(2) Reproducibility

Under the reproducibility conditions, the difference of absolute value of two independent results is not above the reproducibility limit (R). The content ranges and the reproducibility equations of the four hormones in bovine urine are shown in Table 9.7.

9.5.8 RECOVERY

Under optimized conditions, the recoveries of zeranol, diethylstilbestrol, hexestrol, and dienoestrol in bovine urine using this method are listed in Table 9.8.

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9.6

Determination of Zearalanol, Zearalanone, Diethylstilbestrol, Hexestrol, and Dienoestrol Multiresidues in Milk and Milk Powder—LC-MS-MS Method (GB/T 22992-2008)

9.6.1 SCOPE

This method is applicable to the determination of zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol in milk and milk powder.

The limit of determination of zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol in milk is 1.0 μg/L, and the limit of determination of zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol in milk powder is 10 μg/kg.

9.6.2 PRINCIPLE

Acetonitrile is used as a precipitant and protein extraction agent for extraction; the extract is cleaned up with an anion solid phase extraction column. Determination is made by LC-MS-MS, using the internal standard method.

9.6.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be analytically pure. "Water" is the first-degree water as described in GB/T 6682.

Methanol: HPLC grade.
Acetonitrile: HPLC grade.

Ammonia.
Formic acid.
Sodium hydroxide.
Sodium hydroxide solution (5 mol/L): Weigh 200 g sodium hydroxide and dissolve in 1000 mL water.
Washing solution: Ammonia-water (5+95, v/v).
Eluant: Formic acid–Methanol (5+95, v/v).
Standards: Zeranol (ZER, including zeranol and taleranol, 50% respectively) Purity ≥97%; Zearalanone (ZEAR) Purity ≥97%; Diethylstilbestrol (DES) Purity ≥99%; Hexestrol (HEX) Purity ≥98%; Dienoestrol (DEN) Purity ≥98%.
Standard solutions: Accurately weigh an adequate amount of each standard (ZER, ZEN, DES, DEN, and HEX). Separately dissolve them in methanol to prepare a solution of 1 mg/mL in concentration as the standard stock solution. According to the requirement, prepare a mixed standard working solution of appropriate concentrations by mixing and diluting the stock solutions with methanol. Store at 4°C in a refrigerator. The solution can be stored for 3 months.
Internal standard: α-Zearalenol-d4, Purity ≥99%; Diethylstilbestrol (DES-d8) Purity ≥98%.
Internal standard solution: Accurately weigh an adequate amount of α-ZEAR-d4 and DES-d8 and dissolve in methanol to prepare a solution of 1 mg/mL in concentration as the standard stock solution. According to the requirement, prepare a standard working solution of appropriate concentrations by mixing and diluting the stock solutions with methanol. Store at 4°C in a refrigerator. The solution can be stored for 3 months.
Matrix standard working solutions: Mix the standard working solution and the internal solution, and evaporate the combined organic phase to dryness under nitrogen flow. Add matrix extract to dissolve and vortex for 30 s.
Anion solid phase extraction column Oasis MAX: 60 mg, 3 mL.
Filter: 0.22 μm, organic phase.

9.6.4 APPARATUS

LC-MS-MS: Equipped with atmospheric pressure chemical ion source.
Automatic solid phase extraction or device of SPE.
Nitrogen evaporator.
Vortex mixer.
Centrifuge: rotate speed >3000 rpm.
Homogenizer: rotate speed >10,000 rpm.

9.6.5 SAMPLE PRETREATMENT

(1) Preparation of Test Sample

Milk

Take 50 mL fresh or thawed milk and mix well; centrifuge at 3500 rpm for 5 min, get lower.

Milk powder

Weigh 10 g milk powder in the beaker and add 35–50°C water appropriately; it will slowly dissolve. Transfer to 100-mL volumetric flask and cool to room temperature. Add water to 100 mL and mix well. Take 50 mL and centrifuge at 3500 rpm for 5 min; get lower.

(2) Extraction

In 50-mL centrifuge tubes, add moderate standard solutions respectively; the corresponding concentrations of zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol are 2.5 ng/mL, 5.0 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL. Then add moderate internal standard solutions respectively; the corresponding concentrations of α-Zearalenol-d4, DES-d8 are both 10 ng/mL. Evaporate to dryness at 40°C under nitrogen flow. Take 5 negative samples, each 5 mL, and put the samples into the centrifuge tubes and vortex; add 10 mL acetonitrile and vortex 3 min and then centrifuge at 3500 rpm for 5 min. Decant supernatant liquid into a centrifuge tube and add 5 mL acetonitrile. Operate as previously: combine the extraction and evaporate to 0.1 mL at 50°C under nitrogen flow. Add 10 mL water and adjust the pH of the solution to 11.0 with 5 mol/L sodium hydroxide solution, at 4°C. Centrifuge at 9000 rpm for 5 min.

(3) Clean-up

The anion SPE columns are conditioned by washing with 2 mL methanol and 2 mL water with 4 mL/min flow rate. Add the extract with 1 mL/min flow rate. Wash with 1 mL washing solution and 0.5 mL methanol with 3 mL/min flow rate. Add 20 mL air push with 4 mL/min for drying of the Oasis MAX column. Elute with 4 mL eluant with 1 mL/min flow rate. Add 30 mL air push with 6 mL/min for drying the Oasis MAX column. Collect the eluant. Evaporate the eluant to dryness at 40°C under nitrogen flow. Dissolve dry residue in 1 mL mobile phase, vortex 30 s, and put through the filter. Collect the filtrate and prepare for injection.

(4) Preparation of the measured sample solution

Put the sample (5 mL) into a 50-mL centrifuge tube. Add the internal standard solution to ensure the final concentration is 10 ng/mL. Carry out the procedures according to the above-mentioned extraction and cleanup steps.

(5) Preparation of the blank matrix solution

Take the negative sample (5 mL) into a 50-mL centrifuge tube. Carry out the procedures according to the above-mentioned extraction and cleanup steps.

9.6.6 DETERMINATION

(1) Operating Conditions
LC column: ZORBAX Eclipse SC-C8 (150 mm × 4.6 mm, 3.5 μm) or equivalent;
column temp: 25°C;
Mobile phase: Acetonitrile-H_2O (70+30, V/V);
Flow rate: 0.5 mL/min;
Injection volume: 50 μL.
Ionization mode: Electronic spray ionization;
Scan mode: Negative ion scan;
Determination mode: Multiple reaction monitoring (MRM);
Ion source temperature: 350°C;
Collision activated dissociation gas pressure: 0.083 MPa;
Curtain gas pressure: 0.1240 MPa;
GAS 1: 0.2756 MPa;
GAS 2: 0.2412 MPa;
Nebulizer current: −5 μA;
Ionspray voltage: −4500 V;
Diagnostic ions, quantitative ions, collision energy, and declustering potential: see Table 9.9.

(2) Confirmation

Choose one mother ion and more than two ions in each sample. Under the same experimental conditions, the deviation of the target analyte and internal standard ratio of retention time, that is, the relative retention time, compared with the relative retention time of the matrix calibration standard solution, should be within ±2.5%, and the deviation of the relative abundance of the diagnostic ions of the sample chromatogram compared with the relative abundance of the matrix calibration standard solution should be no more than the range in Table 9.3; if this is the case, then the corresponding analyte must be present in the sample.

(3) Quantitative Determination

Inject the series of matrix standard working solutions (7.1) separately, to make the standard working curve. Check the capability of the instrument and determine the linear range. Quantitatively determine the samples through the instrument workstation or through the curve by emendation of the internal standard. The responses of hormones in the sample solutions should be within the linear range of the instrumental detection. LC-MS-MS chromatograms of the standards are shown in Fig. 9.1.

9.6.7 PRECISION

The precision data of this part are confirmed according to GB/T 6379. The values of repeatability and reproducibility are calculated at a 95% confidence level.

TABLE 9.9 Diagnostic Ions, Quantitative Ions, Collision Energy, and Declustering Potential of Five Hormones

Analyte	Diagnostic Ions (m/z)	Quantitative Ions (m/z)	Collision Energy (V)	Declustering Potential (V)
ZER	321.1/277.2	321.1/277.2	−35	−100
	321.1/303.2		−35	−100
ZEAR	319.3/205.1	319.3/205.1	−42	−126
	319.3/205.1		−34	−126
α-Zearalenol -d4	325.1/208.9	325.1/263.1	−36	−100
	325.1/263.1		−38	−100
DES	267.0/222.1	267.0/222.1	−40	−90
	267.0/237.1		−40	−90
DEN	265.0/221.1	265.0/235.1	−35	−90
	265.0/235.1		−32	−90
HEX	269.0/134.0	269.0/134.0	−22	−75
	269.0/119.0		−46	−75
DES-d8	275.1/245.0	275.1/245.0	−40	−73
	275.1/227.9		−40	−74

(1) Repeatability

Under the repeatability condition, the difference of the absolute value of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equations of the four hormones in milk are shown in Table 9.10. The content ranges and the repeatability equations of the four hormones in milk powder are shown in Table 9.11.

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned and two independent experiments should be redone.

(2) Reproducibility

Under the reproducibility condition, the difference of absolute value of two independent results is not above the reproducibility limit (R). The content ranges and the repeatability equations of the four hormones in milk are shown in Table 9.10. The content ranges and the repeatability equations of the four hormones in milk powder are shown in Table 9.11.

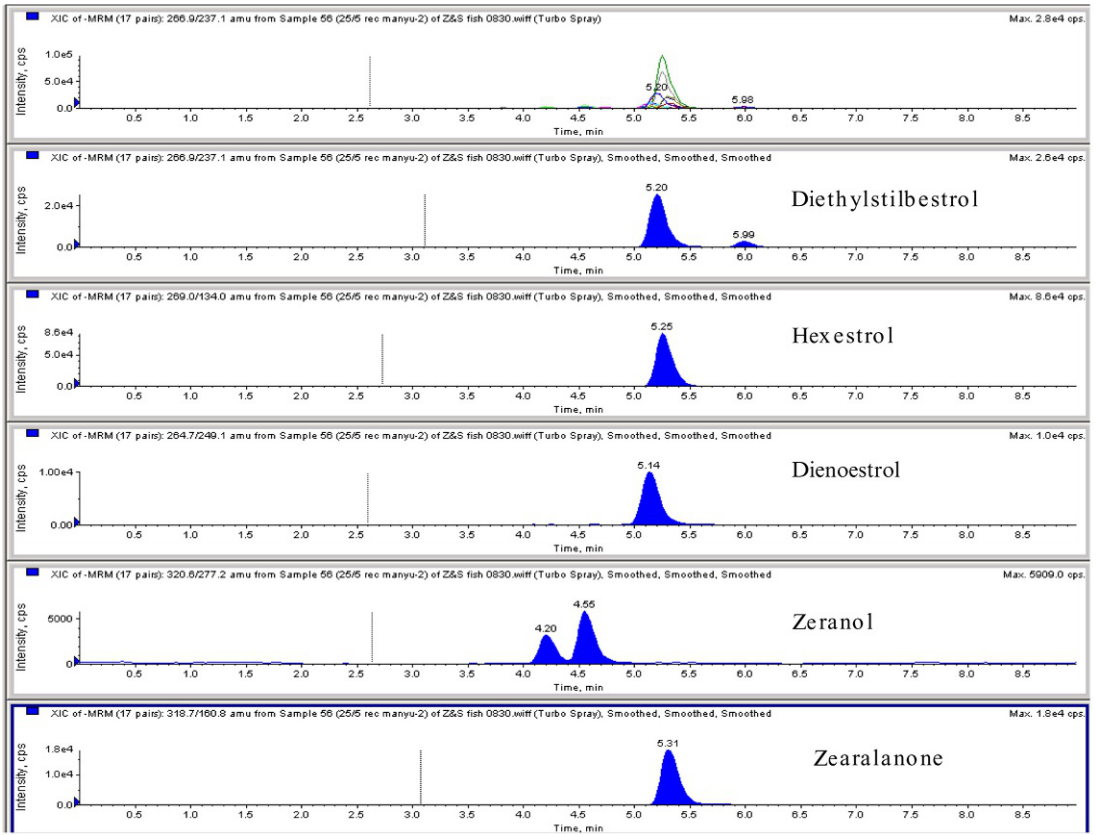

FIG. 9.1 Standard chromatography.

TABLE 9.10 Content Ranges and the Repeatability and Reproducibility Equations in Milk

Analyte	Content Range (μg/L)	Repeatability Limit (r)	Reproducibility Limit (R)
ZER	1.0–10	$\lg r = 0.9656 \lg m - 1.0897$	$\lg R = 0.9625 \lg m - 0.7128$
ZEAR	1.0–10	$\lg r = 0.9660 \lg m - 1.1445$	$\lg R = 0.9152 \lg m - 0.6513$
DES	1.0–10	$\lg r = 0.9844 \lg m - 1.0935$	$\lg R = 0.2058 \lg m + 0.0119$
DEN	1.0–10	$\lg r = 0.0735 \lg m + 0.0016$	$\lg R = 1.0813 \lg m - 0.7982$
HEX	1.0–10	$\lg r = 0.9760 \lg m - 1.0445$	$\lg R = 0.9252 \lg m - 0.6613$

Note: m *equals the average of two results.*

TABLE 9.11 Content Ranges and the Repeatability and Reproducibility Equations in Milk Powder

Analyte	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
ZER	10–100	$\lg r = 0.9656 \lg m - 1.0897$	$\lg R = 0.9625 \lg m - 0.7128$
ZEAR	10–100	$\lg r = 0.9660 \lg m - 1.1445$	$\lg R = 0.9152 \lg m - 0.6513$
DES	10–100	$\lg r = 0.9844 \lg m - 1.0935$	$\lg R = 0.2058 \lg m + 0.0119$
DEN	10–100	$\lg r = 0.0735 \lg m + 0.0016$	$\lg R = 1.0813 \lg m - 0.7982$
HEX	10–100	$\lg r = 0.9760 \lg m - 1.0445$	$\lg R = 0.9252 \lg m - 0.6613$

Note: m *equals to the average of two results.*

9.6.8 RECOVERY

Under optimized condition, the recoveries of zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol in milk using this method are listed in Table 9.12.

TABLE 9.12 Recovery Data

Analyte	Added Concentration (μg/L)	Average Recovery (%)
ZER	1.0	84.93
	2.0	88.58
	5.0	91.35
	10.0	97.80
ZEAR	1.0	76.10
	2.0	79.18
	5.0	101.0
	10.0	102.4
DES	1.0	80.12
	2.0	78.67
	5.0	102.7
	10.0	96.35
DEN	1.0	74.83
	2.0	93.00
	5.0	99.53
	10.0	95.32
HEX	1.0	91.33
	2.0	72.67
	5.0	92.47
	10.0	100.6

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9.7

Determination of Zearalanol, Zearalanone, Diethylstilbestrol, Hexestrol, and Dienoestrol Residues in Fugu, Eel, and Baked Eel—LC-MS-MS Method (GB/T 22963-2008)

9.7.1 SCOPE

This method is applicable to the determination of zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol in globe fish, eel, and eel products.

The limit of determination of zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol is 1.0 μg/kg.

9.7.2 PRINCIPLE

The zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol residues in the test sample are extracted with methyl *tert*-butyl ether and acetate buffer solution. The extract is cleaned up with a silica gel column. Determination is made by LC-MS-MS using the internal standard method.

9.7.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be analytically pure; "water" is the first-degree water as described by GB/T 6682.

Methyl *tert*-butyl ether (MTBE): HPLC grade.
Acetic acid: HPLC grade.
Sodium acetic acid ($CH_3COONa \cdot 3H_2O$).
Acetate buffer solution (0.2 mol/L, pH 5.2): Weigh 2.52 g acetic acid and 12.95 g sodium acetic acid and dissolve in 800 mL water. Adjust the pH to 5.2 ± 0.1 with sodium hydroxide solution and make up to 1000 mL with water.
Sodium hydroxide.
Sodium hydroxide solution (3 mol/L): Weigh 100 g sodium hydroxide and dissolve in 1000 mL water.
Methanol: HPLC grade.

Acetonitrile: HPLC grade.
Ethyl acetate: HPLC grade.
n-Hexane: HPLC grade.
Dichloromethane: HPLC grade.
Dissolving solution: *n*-Hexane-Dichloromethane (60+40).
Washing solution: Ethyl acetate-*n*-Hexane (6+94).
Eluant: Ethyl acetate-*n*-Hexane (25+75).
β-Glucuronidase: Type H-2, 124,400 β-glucuronidase units/mL, 3610 sulfatase units/mL.
Standards: Zeranol (ZER, including zeranol and taleranol, 50% respectively), Purity ≥97%; Zearalanone (ZEAR), Purity ≥97%; Diethylstilbestrol (DES), Purity ≥99%; Hexestrol (HEX), Purity ≥98%; Dienoestrol (DEN), Purity ≥98%.
Standard solutions: Accurately weigh an adequate amount of each standard (ZER, ZEN, DES, DEN, and HEX) and separately dissolve in methanol to prepare a solution of 1 mg/mL in concentration as the standard stock solution. According to the requirement, prepare a mixed standard working solution of appropriate concentrations by mixing and diluting the stock solutions with methanol. Store at 4°C in a refrigerator; the solution can be stored for 3 months.
Internal standard: α-Zearalenol-d4, Purity ≥99%; Diethylstilbestrol (DES-d8), Purity ≥98%.
Internal standard solution: Accurately weigh an adequate amount of α-ZEAR-d4 and DES-d8 and dissolve in methanol to prepare a solution of 1 mg/mL in concentration as the standard stock solution. According to the requirement, prepare a standard working solution of appropriate concentrations by mixing and diluting the stock solutions with methanol. Store at 4°C in a refrigerator; the solution can be stored for 3 months.
Matrix standard working solutions: Mix the standard working solution and the internal solution; evaporate the combined organic phase to dryness under nitrogen flow. Add matrix extract to dissolve and vortex for 30 s.
Silica gel Sep-Pak column: 500 mg, 3 mL.
Filter: 0.45 μm.

9.7.4 APPARATUS

LC-MS-MS: Equipped with atmospheric pressure chemical ion source.
Automatic solid phase extraction (SPE) device.
Homogenizer: rotate speed > 10,000 rpm.
Nitrogen evaporator.
Vortex mixer.
Centrifuge: rotate speed >3000 rpm.

9.7.5 SAMPLE PRETREATMENT

(1) Preparation of test sample.

500 g of representative sample is taken from the primary sample, divided into two equal portions; each portion is placed in a clean vessel as the test sample, which is sealed and labeled.

The test samples should be stored at 2–8°C.

(2) Weighing

Weigh five negative samples of 5 g each (accurate to 0.1 g). Put the sample into a 50-mL centrifuge tube. Add moderate standard solutions, respectively; the corresponding concentrations of zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol are 2.5 ng/mL, 5.0 ng/mL, 10 ng/mL, 25 ng/mL, and 50 ng/mL. Then add moderate internal standard solutions respectively; the corresponding concentrations of α-Zearalenol-d4, DES-d8 are both 10 ng/mL.

(3) Extraction

Add 20 mL MTBE and homogenize for 1 min at 10,000 rpm. Centrifuge at 3000 rpm for 5 min; decant supernatant liquid into another 50-mL tube. Place the residue of the centrifuge in the ventilated cabinet and volatilize for 30 min. Add 15 mL acetate buffer solution and homogenize for 1 min at high speed. Centrifuge at 3000 rpm for 5 min; decant supernatant liquid into a 25-mL tube with stopper and evaporate the residue MTBE at 40°C under nitrogen flow. Then add 80 μL β-glucuronidase and keep overnight at 52°C. Add sodium hydroxide solution to adjust the pH of the solution to 7; add 10 mL MTBE and mix well. Centrifuge at 3000 rpm for 2 min. Transfer the MTBE layer and combine with the previous MTBE extraction and evaporate to dryness at 40°C under nitrogen flow. Add 1 mL dissolving solution, vortex 30 s, and prepare for clean-up.

(4) Clean-up

The Sep-Pak columns are conditioned by washing with 3 mL *n*-hexane twice and the flow rate is 4 mL/min. Add the extract with 2 mL/min flow rate. Add 3 mL washing solution into the test tube, mix and load with 2 mL/min flow rate. Wash with 3 mL washing solution with a flow rate of 3 mL/min; add 2 mL air push with 4 mL/min for drying the column. Elute with 6 mL eluant with a flow rate of 2 mL/min; add 2 mL air push with 6 mL/min for drying of the column and collect the eluant. Evaporate the eluant to dryness at 40°Cunder nitrogen flow. Dissolve dry residue in 1 mL mobile phase, vortex 30 s, and put through the filter. Collect the filtrate and prepare for injection.

(5) Preparation of the measured sample solution

Weigh 5 g sample (accurate to 0.1 g) and put the sample into a 50-mL centrifuge tube. Add internal standard solution to ensure the final concentration is 10 ng/mL. Carry out the procedures according to the above-mentioned extraction and cleanup steps.

(6) Preparation of the blank matrix solution

Weigh 5 g negative sample (accurate to 0.1 g) and put the sample into a 50-mL centrifuge tube. Carry out the procedures according to the above-mentioned extraction and cleanup steps.

9.7.6 DETERMINATION

(1) Operating Condition

LC column: ZORBAX Eclipse SC-C8 (150 mm × 4.6 mm, 3.5 μm) or equivalent;
Column temp: 25°C;
Mobile phase: Acetonitrile-H_2O (70+30, V/V);
Flow rate: 0.5 mL/min;
Injection volume: 50 μL.
Ionization mode: Electronic spray ionization;
Scan mode: Negative ion scan;
Determination mode: Multiple reaction monitoring (MRM);
Ion source temperature: 350°C;
Collision activated dissociation gas pressure: 0.083 MPa;
Curtain gas pressure: 0.1240 MPa;
GAS 1: 0.2756 MPa;
GAS 2: 0.2412 MPa;
Nebulizer current: −5 μA;
Ionspray voltage: −4500 V;
Diagnostic ions, quantitative ions, collision energy, and declustering potential: see Table 9.9.

(2) Confirmation

Choose one mother ion and more than two ions in each sample. In the same experimental conditions, the deviation of target analyte and internal standard ratio of retention time, that is, the relative retention time, compared with the relative retention time of the matrix calibration standard solution should be within ±2.5%; and the deviation of the relative abundance of the diagnostic ions of the sample chromatogram compared with the relative abundance of the matrix calibration standard solution should be no more than the range in Table 9.3. If these conditions are true, then the corresponding analyte must be present in the sample.

(3) Quantitative Determination

Inject the series of matrix standard working solutions separately, to make the standard working curve. Check the capability of the instrument and determine the linear range. Quantitatively determine the samples through the instrument workstation or through the curve by emendation of the internal standard. The responses of hormones in the sample solutions should be within the linear range of the instrumental detection. LC-MS-MS chromatograms of the standards are shown in Fig. 9.1.

9.7.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the repeatability condition, the difference of absolute values of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equations of the four hormones in globe fish, eel, and eel products are shown in Table 9.13.

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned and two independent experiments should be redone.

(2) Reproducibility

Under the reproducibility condition, the difference of absolute values of two independent results is not above the reproducibility limit (R). The content ranges and the reproducibility equations of the four hormones in globe fish, eel, and eel produets are shown in Table 9.13.

9.7.8 RECOVERY

Under optimized condition, the recoveries of zeranol, zearalanone, diethylstilbestrol, hexestrol, and dienoestrol in eel using this method are listed in Table 9.14.

TABLE 9.13 Content Ranges and the Repeatability and Reproducibility Equations

Analyte	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
ZER	1.0–10	$\lg r = 0.9656 \lg m - 1.0897$	$\lg R = 0.9625 \lg m - 0.7128$
ZEAR	1.0–10	$\lg r = 0.9660 \lg m - 1.1445$	$\lg R = 0.9152 \lg m - 0.6513$
DES	1.0–10	$\lg r = 0.9844 \lg m - 1.0935$	$\lg R = 0.2058 \lg m + 0.0119$
DEN	1.0–10	$\lg r = 0.0735 \lg m + 0.0016$	$\lg R = 1.0813 \lg m - 0.7982$
HEX	1.0–10	$\lg r = 0.9760 \lg m - 1.0445$	$\lg R = 0.9252 \lg m - 0.6613$

Note: m *equals the average of two results.*

TABLE 9.14 Recovery Data

Analyte	Added Concentration (μg/kg)	Average Recovery (%)
ZER	1.0	91.79
	2.0	108.8
	5.0	106.2
	10.0	79.36
ZEAR	1.0	105.1
	2.0	103.4
	5.0	106.4
	10.0	103.3
DES	1.0	87.44
	2.0	98.53
	5.0	86.99
	10.0	89.07
DEN	1.0	100.3
	2.0	94.79
	5.0	82.19
	10.0	95.07
HEX	1.0	70.95
	2.0	84.96
	5.0	82.17
	10.0	95.75

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Chapter 10

Glucocorticoid

10.1 DETERMINATION OF NINE GLUCOCORTICOID RESIDUES IN FUGU, EEL, AND BAKED EEL—LC-MS-MS METHOD (GB/T 22957-2008)

10.1.1 Scope

This method is applicable to the determination of nine glucocorticoid residues in fugu, eel, and baked eel.

The limit of detection of this method for prednisolone, prednisone, hydrocortisone, cortisone, methylprednisolone, betamethasone, and dexamethasone is 0.2 μg/kg; for beclomethasone and fludrocortisone acetate it is 1.0 μg/kg.

10.1.2 Principle

The fugu, eel, and baked eel samples with anhydrous sodium sulfate are extracted with ethyl acetate and the extracts are concentrated and cleaned up with a Cleanert Silica solid phase extraction cartridge; the solutions are analyzed by LC-MS-MS using an external standard method.

10.1.3 Reagents and Materials

Water: GB/T6682, first grade.

Methanol: HPLC grade.

Acetonitrile: HPLC grade.

Ethyl acetate: HPLC grade.

Formic acid: G.R.

Hexane: HPLC grade.

Acetone: HPLC grade.

Acetone + Hexane: (2 + 3), Mixed with 200 mL acetone and 300 mL hexane.

Sodium sulfate: Anhydrous, analytically pure. Ignited at 650°C for 4 h and kept in a desiccator.

Prednisolone (CAS No.: 50-24-8), prednisone (CAS No.: 53-03-2), hydrocortisone (CAS No.: 50-23-7), cortisone (CAS No.: 53-06-5), methylprednisolone (CAS No.: 83-43-2), betamethasone (CAS No.: 378-44-9), dexamethasone (CAS No.: 50-02-2), beclomethasone (CAS No.: 4419-39-0), fludrocortisone acetate (CAS No.: 514-36-3) standards: Purity ≥98%.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00010-5

Stock standard solutions: 1.0 mg/mL. Accurately weigh adequate amount of glucocorticoid standards. Separately prepare stock standard solutions of 1.0 mg/mL with methanol. The solutions should be stored in the dark below −20°C.

Working standard solutions: 5.0 μg/mL. Separately pipette adequate amount of stock standard solutions to prepare working standard solutions of 5.0 μg/mL with methanol. The solutions should be stored in the dark below 4°C.

Working standard mixed solutions I: Separately pipette adequate amount of working standard solutions of hydrocortisone and cortisone to prepare working standard solutions of 0.05 μg/mL with methanol. The solutions should be stored in the dark below 4°C. During sample determination, working standard mixed solutions I are prepared to different concentration working standard mixed solutions with 20% acetonitrile solution.

Working standard mixed solutions II: Separately pipette adequate amount of working standard solutions of prednisolone, prednisone, methylprednisolone, betamethasone, dexamethasone, beclomethasone, and fludrocortisone acetate to prepare working standard solutions of 0.05 μg/mL of prednisolone, prednisone, methylprednisolone, betamethasone, dexamethasone; and 0.25 μg/mL of beclomethasone and fludrocortisone acetate with methanol. The solutions should be stored in the dark below 4°C.

Working standard mixed solution in matrix: During sample determination, working standard mixed solutions II are prepared to different concentration matrix working standard mixed solutions with blank extract that has been taken through the method with the rest of the samples. Working standard mixed solutions in matrix must be freshly prepared.

Cleanert Silica cartridge or equivalent: 500 mg, 6 mL. Condition the cartridge with 6 mL hexane before using. Keep the cartridge wet.

Membrane filter: 0.2 μm.

10.1.4 Apparatus

LC-MS-MS: Equipped with ESI.
Analytical balances: Capable of weighing to 0.1 mg, 0.01 g.
Solid phase extraction vacuum apparatus.
Vacuum pump: Vacuum to 80 kPa.
Homogenizer.
Shaker.
Centrifuge: Speed to 11,000 rpm.
Rotary evaporator.
Nitrogen evaporator.
Stoppered plastic centrifuge tube: 50 mL.
Centrifuge tubes: 10 mL.
Pear-shaped flask: 150 mL.

10.1.5 Sample Pretreatment

(1) Preparation of Test Sample

Take approximately 1 kg of representative sample. Mash thoroughly using a chopper. Mix thoroughly. Put into clean containers, seal, and label. In the course of sampling and sample preparation, attention must be taken to avoid contamination or any factors that may cause any change of residue content.

The test samples should be stored below −18°C.

(2) Extraction

Weigh 5 g (accurate to 0.01 g) of the test sample into a 50-mL stoppered plastic centrifuge tube filled with 20 g anhydrous sodium sulfate. Add 25 mL of ethyl acetate. Homogenize for 1 min, shake for 20 min, and centrifuge for 3 min at 10,000 rpm. Collect supernatant in pear-shaped flask. Proceed twice with 25 mL ethyl acetate and combine the extracts in the pear-shaped flask. Evaporate to dryness on a rotary evaporator at 40°C; dissolve with 1 mL ethyl acetate and 5 mL hexane.

(3) Clean-Up

Transfer the extracts into the Cleanert Silica cartridge. Rinse the pear-shaped flask and cartridge with 6 mL hexane. Discard all the effluents and dry the cartridge by drawing air through it for 1 min; elute with 6 mL acetone + hexane into 10-mL centrifuge tubes. Evaporate the elute solution to dryness on a nitrogen evaporator. Dissolve with 1 mL 20% acetonitrile solution. Centrifuge 5 min at 4000 rpm; pass supernatant through the 0.2-μm membrane filter for LC-MS-MS determination.

10.1.6 Determination

(1) Operating Condition

Column: Atlantis dC18, 3 μm, 150 mm × 2.1 mm, or equivalent;
Mobile phase and flow rate: see Table 10.1.
Column temperature: 30°C;
Injection volume: 20 μL;

MS Operating Conditions

Ion source: ESI source;
Scan mode: Negative scan;
Monitor mode: Multiple reaction monitor;
Curtain gas: 0.138 MPa
Ion source gas 1: 0.276 MPa;
Ion source gas 2: 0.138 MPa;
Source temperature: 400°C;
MRM transitions for precursor/product ion, quantifying for precursor/product ion, declustering potential, collision energy: see Table 10.2.

TABLE 10.1 Mobile Phase and Flow Rate

Time (min)	Flow Rate (µL/min)	Acetonitrile (%)	0.1% Formic Acid Solution (%)
0	200	20	80
10.00	200	70	30
13.00	200	90	10
13.01	200	20	80
20.00	200	20	80

TABLE 10.2 MRM Transitions for Precursor/Product Ion, Quantifying for Precursor/Product Ion, Declustering Potential, Collision Energy

Name	MRM Transitions for Precursor/Product Ion (m/z)	Quantifying for Precursor/Product Ion (m/z)	Declustering Potential (V)	Collision Energy (V)
Prednisolone	405/329 405/359	405/329	−30 −30	−25 −15
Prednisone	403/327 403/357	403/327	−19 −19	−21 −15
Hydrocortisone	407/331 407/361	407/331	−28 −28	−25 −15
Cortisone	405/329 405/359	405/329	−22 −22	−24 −15
Methylprednisolone	419/343 419/373	419/343	−32 −32	−23 −16
Betamethasone	437/361 437/391	437/361	−25 −25	−24 −15
Dexamethasone	437/361 437/391	437/361	−25 −25	−24 −15
Beclomethasone	453/377 453/407	453/377	−22 −22	−20 −17
Fludrocortisone acetate	467/421 467/349	467/421	−28 −28	−17 −32

TABLE 10.3 Maximum Permitted Tolerances for Relative Ion Intensities During Confirmation WS %

Relative intensity (K)	$K > 50$	$20 < K < 50$	$10 < K < 20$	$K \leq 10$
Permitted tolerances	±20	±25	±30	±50

(2) Qualitative Determination

The qualification ions for every compound must be found and must at least include one precursor ion and two daughter ions. For the same analysis batch and the same compound, the variation range of the retention time for the peak of analyte in the unknown sample and in the standard working solution cannot be out of range of ±0.25%. The variation range of the ion ratio between the two daughter ions for the unknown sample and the standard matrix working solution at similar concentration cannot be out of range of Table 10.3.

(3) Quantitative Determination

External standard method for quantitative determination: Inject the working standard mixed solutions I and working standard mixed solution in matrix in sequence. Draw the standard curve with peak area vs. sample concentration. The sample is quantified with the standard curve. The responses of glucocorticoids in sample solution should be in the linear range of the instrumental detection. For total ion chromatograms of nine glucocorticoid standards, see Fig. 10.1. Under these operating conditions, for the retention times of nine glucocorticoid standards, see Table 10.4.

10.1.7 Precision

The precision data of the method for this standard have been determined according to the stipulations of GB/T 6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results should not exceed the limit of repeatability (r); the content range and repeatability equations of glucocorticoids are shown in Table 10.5.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determination shall be reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results should not

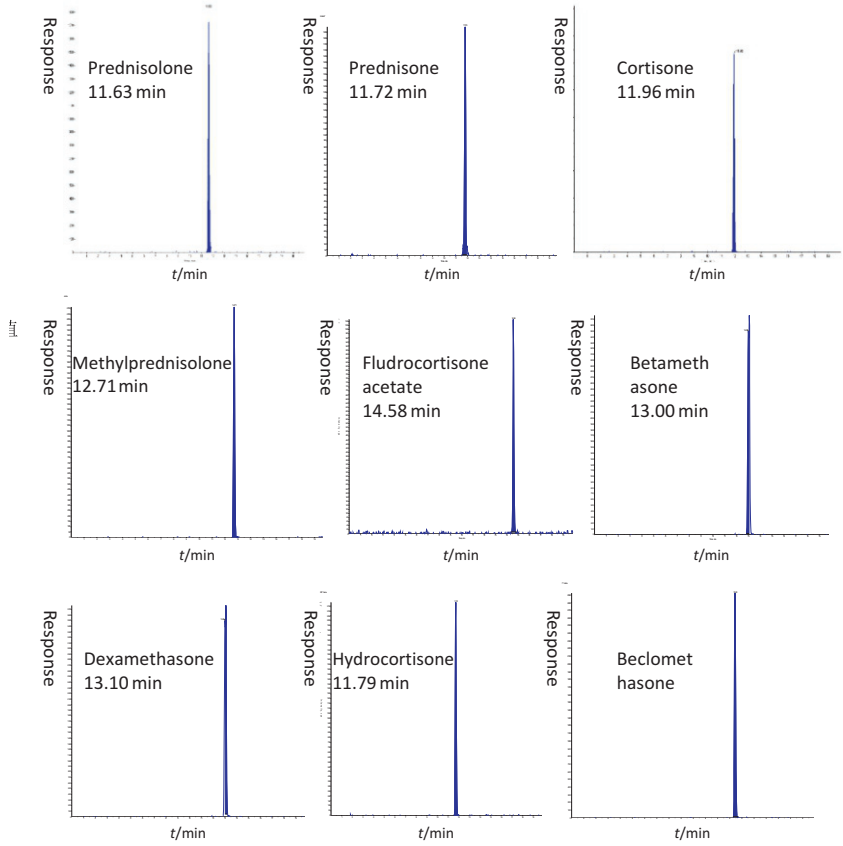

FIG. 10.1 Multiple reaction monitor (MRM) ion chromatograms of nine glucocorticoids.

TABLE 10.4 Retention Times of Nine Glucocorticoids

Name	Retention Time (min)
Prednisolone	11.63
Prednisone	11.72
Hydrocortisone	11.79
Cortisone	11.96
Methylprednisolone	12.71
Betamethasone	13.00
Dexamethasone	13.10
Beclomethasone	13.43
Fludrocortisone acetate	14.58

TABLE 10.5 The Content Range and Repeatability and Reproducibility Equations

Name	Content Range (μg/kg)	Repeatability (μg/kg)	Reproducibility (μg/kg)
Prednisolone	0.2～5.0	lg r = 0.8594 lg m − 0.7782	R = 0.1703 m + 0.0319
Prednisone	0.2～5.0	r = 0.2100 m − 0.0006	lg R = 1.0798 lg m − 0.6196
Hydrocortisone	0.2～5.0	lg r = 1.0962 lg m − 0.7226	lg R = 1.1142 lg m − 0.6614
Cortisone	0.2～5.0	lg r = 0.9552 lg m − 0.7584	R = 0.2366 m − 0.0041
Methylprednisolone	0.2～5.0	lg r = 1.0621 lg m − 0.7187	lg R = 0.9643 lg m − 0.6423
Betamethasone	0.2～5.0	lg r = 1.0686 lg m − 0.6899	lg R = 1.1585 lg m − 0.6168
Dexamethasone	0.2～5.0	lg r = 1.1217 lg m − 0.6900	lg R = 1.1882 lg m − 0.6229
Beclomethasone	1.0～25	lg r = 0.9993 lg m − 0.7222	lg R = 0.8613 lg m − 0.4783
Fludrocortisone acetate	1.0～25	r = 0.109 2 m + 0.1279	lg R = 0.9292 lg m − 0.5915

Note: m *is the average values obtained from two independent determination results.*

exceed the limit of reproducibility (R), the content range and reproducibility equations of glucocorticoids are shown in Table 10.5.

10.1.8 Recovery

Under optimized condition, the recoveries of nine glucocorticoid in fugu, eel and baked eel using this method are listed in Table 10.6.

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TABLE 10.6 The Test Data of Fortification and Average Recovery for Nine Glucocorticoids

Name	Fortifying Concentration (μg/kg)	Average Recovery (%)
Prednisolone	0.2	83.4
	0.4	87.1
	1.0	93.7
	5.0	79.4
Prednisone	0.2	92.5
	0.4	84.5
	1.0	82.0
	5.0	83.7
Hydrocortisone	0.2	89.0
	0.4	100.1
	1.0	100.4
	5.0	91.0
Cortisone	0.2	96.0
	0.4	89.4
	1.0	98.5
	5.0	84.4
Methylprednisolone	0.2	84.7
	0.4	87.3
	1.0	79.9
	5.0	79.7
Betamethasone	0.2	92.5
	0.4	94.5
	1.0	84.9
	5.0	76.6
Dexamethasone	0.2	89.1
	0.4	91.8
	1.0	82.9
	5.0	78.1

TABLE 10.6 The Test Data of Fortification and Average Recovery for Nine Glucocorticoids—cont'd

Name	Fortifying Concentration (μg/kg)	Average Recovery (%)
Beclomethasone	1.0	89.4
	2.0	86.5
	5.0	81.3
	25	82.0
Fludrocortisone acetate	1.0	78.0
	2.0	77.5
	5.0	80.5
	25	81.4

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Chapter 11

Fluoroquinolone

11.1

Curative Effects and Side Effects of Quinolones

Quinolones (QNs), one of the most powerful classes of antibiotics, were initially employed in the treatment of Gram-negative urinary tract infections in humans and animals. Their activity is based on the inhibition of the enzymes DNA gyrase or topoisomer ase II, which are responsible for the preservation of the DNA biological activity of bacteria. Fluoroquinolones (FQNs) are piperazinyl derivates of the quinolone nadixilic acid and represent the second generation of this family of antibiotics. They are nowadays broadly used in the treatment of a wide variety of diseases, since they are not only more effective against Gram-negative bacteria but also moderately active against Gram-positive bacteria and generally marginally active against streptococci and anaerobes.

This has led to a significant increase in antibiotic resistance and allergic reactions, having therefore important consequences on public health. Moreover, quinolone-induced acute arthropathy has been observed in several animal species. Although severe cases of arthropathy have been observed only rarely in humans, incidents of transient arthralgia have also been reported. These observations have precluded the use of quinolones in children and pregnant women. The European Union (EU) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) have established maximum residue limits (MRLs) for several quinolones.

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11.2

Pharmacokinetics of Quinolones

The microbiologically active compound of most metabolites of QNs decrease, except for ciprofloxacin and norfloxacin, which metabolize from enrofloxacin and pefloxacin. Metabolism studies have demonstrated that flumequine can be excreted in unchanged form and as its 7-hydroxy- and glucuronide- metabolites. The proportion of these metabolites can be different depending upon the tissue and the animal species evaluated. In general, it has been found that the unchanged drug represents the main microbiologically active compound in most tissues and it has been settled upon as the marker residue.

11.3

Chemical Structures and Maximum Residue Limits for Quinolones

Table 11.1 lists the chemical structures and the maximum residue limits for quinolones.

11.4

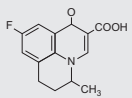
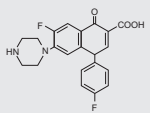
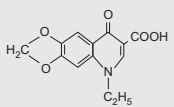
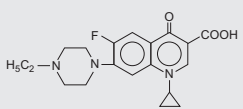
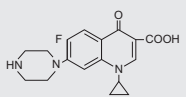
Determination of 15 Quinolone Residues in Eel and Eel Products—LC-MS-MS Method

11.4.1 SCOPE

This method is applicable to 15 quinolone residues in eel and eel products.

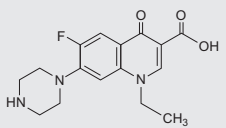
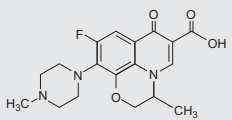
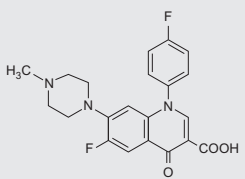
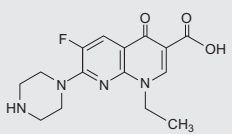
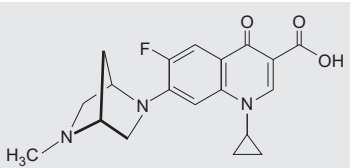
The limit of quantitation of 15 quinolone residues is 5 μg/kg.

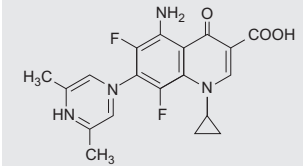
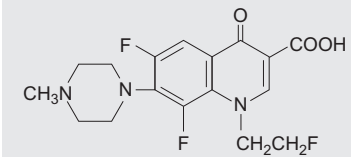
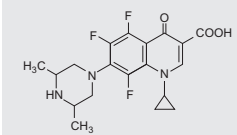
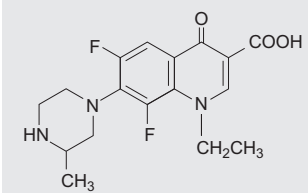
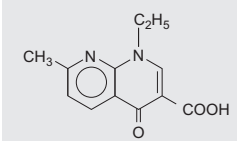
TABLE 11.1 Chemical Structures and the Maximum Residue Limits for Quinolones

Compound Names	Structure	Molecular Weight	Cas. No.	MRL (μg/kg)	
Flumequine		261.25	42835-25-6	Japan	40
				China	50
				EU	100
Sarafloxacin		385.13	91296-87-6	China Japan	10
				EU	30
Oxolinic Acid		261.23	14698-29-4	China EU	50
				Japan	20
				Australia	10
Enrofloxacin		359.16	93106-60-6	Enrofloxacin + ciprofloxacin	
				EU America China	100
Ciprofloxacin		331.33	85721-33-1	Japan	10

Continued

TABLE 11.1 Chemical Structures and the Maximum Residue Limits for Quinolones—cont'd

Compound Names	Structure	Molecular Weight	Cas. No.	MRL (μg/kg)	
Norfloxacin		319.13	70458-96-7	Australia	100
				Japan	20
Ofloxacin		361.14	82419-36-1	Japan	50
Difloxacin		399.14	91296-86-5	EU China	100
				Japan	20
Enoxacin		320.13	74011-58-8		
Danofloxacin		357.15	112398-08-0	China Japan	50
				EU	30
				America	200

Sparfloxacin		392.16	110871-86-8		
Fleroxacin		369.34	79660-72-3		
Orbifloxacin		395.38	113617-63-3	Japan	20
Lomefloxacin		351.14	98079-52-8		
Nalidixic Acid		232.24	389-08-2	Japan	30

11.4.2 PRINCIPLE

This method describes extraction of the quinolone residues in samples with acetonitrile and purification using a strong cation exchange solid phase extraction (SPE) cartridge, followed by liquid chromatography–tandem mass spectrometry analysis.

11.4.3 REAGENTS AND MATERIALS

Acetonitrile, methanol: HPLC grade; n-Hexane; Ammonium hydroxide; Formic acid; Ammonium acetate; Anhydrous sodium sulfate: Calcine at 650°C for 4 h and store in a desiccator; Ammonium hydroxide-methanol mixed solvent: 25 +75,v/v; Formic acid solution: 0.1%; Ammonium acetate buffer solution: 10 mmol/L; Strong cation exchange (SCX) SPE cartridge: 500 mg/3 mL; the extraction cartridge is conditioned using 3 mL methanol, 3 mL water, 3 mL 10 mmol/L ammonia acetate before use. Prevent the columns from running dry.

Fifteen quinolone standards: fleroxacin, ofloxacin, enoxacin, norfloxacin, ciprofloxacin, enrofloxacin, lomefloxacin, danofloxacin, orbifloxacin, difloxacin, sarafloxacin, sparfloxacin, oxolinic acid, nalidixic acid, flumequine, purity $\geq$98%.

Stock solutions of 15 quinolones: 100 mg/L. Accurately weigh 10.0 mg 15 quinolone standards; dissolve with methanol to a volume of 100 mL respectively, and store at approximately 4°C for a maximum period of 1 month.

Calibration solutions of 15 quinolones: dilute appropriate volume of stock solutions to an intended concentration with 0.1% formic acid solution + acetonitrile (9 + 1, v/v) and mix well. These solutions should be prepared just before use.

11.4.4 APPARATUS

High performance liquid chromatography–mass spectrometer equipment: equipped with electrospray (ESI) LC interface; Analytical balance: sensibility reciprocal is 0.1 mg and 0.01 g, respectively; Tissue blender; Vortex mixer; Homogenizer: rotate speed $\geqq$ 10,000 rpm; Ultrasonic water bath; Pressured gas blowing concentrator; SPE equipment; Vacuum pump: the vacuum should be 80 kPa; Centrifuge: rotate speed, 4000 rpm.

11.4.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

For eel samples, combine the muscle tissues and skins and heat in a microwave oven; mix well in the homogenizer. For eel products, combine all edible portions and mix well using the homogenizer, and then seal in a clean vessel and mark it. Precaution measures should be taken to avoid contamination or other factors that may cause a change of residue concentration in the samples. Samples may be stored at –18°C; fresh or frozen tissues may be stored at 2–6°C for 72 h.

(2) Extraction

Accurately weigh 5.00 g of the test samples into a 50-mL polypropylene centrifuge tube and then add ca. 5 g anhydrous sodium sulfate and 25 mL acetonitrile; homogenize for 30 s at 10,000 rpm, then centrifuge 5 min at 4000 rpm. Transfer the supernatant into a 50-mL colorimetric tube. Add 15 mL acetonitrile to another centrifuge tube and rinse the homogenize probe for 10 s. Transfer the solvent to the first centrifuge tube and mash into pieces using a glass rod. Then vortex to mix for 1 min and sonicate in an ultrasonic bath to extract for 5 min; then centrifuge the mixture at 4000 rpm for 5 min. Combine the supernatants in the 50-mL colorimetric tube and add acetonitrile to a volume of 50 mL and mix well. Pipe 10 mL extracts and defat with 2×5 mL n-hexane; discard the n-hexane and evaporate the acetonitrile phase to dryness at 35°C under a stream of nitrogen with a pressured gas blowing concentrator. Redissolve the residues in 3 mL ammonium acetate buffer. The dissolution is achieved using the vortex for 30 s. Then move on to clean-up.

(3) Clean-up

Pass extracts through strong cation exchange (SCX) SPE cartridges at a flow rate ca. 1 mL/min and discard the elute. Then dry the cartridges under vacuum and elute the quinolones with 1.5 mL methanol and 3 mL of a mixture of methanol and ammonia hydroxide. The elution is evaporated to dryness at 35°C under a stream of nitrogen and redissolved in 1 mL of a mixture of 0.1% formic acid solution and acetonitrile. This solution is filtered on a 0.22-μm membrane filter prior to LC-MS-MS analysis.

11.4.6 DETERMINATION

(1) Operation conditions

Column: C18 column (150 mm × 2.1 mm, 5 μm) or equivalent columns; Column temperature: 30°C; Flow rate: 200 μL/min; Injection volume: 20 μL; Gradient elution procedure: Program pump for the following gradient (Table 11.2): Ion source: ESI, positive ionization mode; Scan mode: MRM mode; Ion source temperature: 490°C.

Calibrate the mass spectrometer and electrospray interface according to the manufacturer's specifications; conditions for the MS/MS detection of quinolones are listed in Table 11.3.

(2) Qualitative analysis

The qualification ions for every compound must be found and they must at least include one precursor ion and two daughter ions. Under the same determination conditions, the variation range of the retention time for the peak of the analyte in the unknown sample and in the standard working solution cannot be out of the range of ±0.25 min. For the same analysis batch and the same compound, the variation range of the ion ratio between the two daughter ions for the unknown sample and the standard working solution at a similar concentration cannot be out of the range of Table 11.4; if this is true, then the corresponding analyte must be present in the sample.

TABLE 11.2 LC Gradient Elution Procedure

Time (min)	Flow Rate (μL/min)	Acetonitrile	Methanol	0.1% Formic Acid
0	300	2	20	78
4.00	300	5	20	75
8.00	300	10	20	70
10.00	300	40	20	40
15.00	300	40	20	40
15.50	300	2	20	78
22.00	300	2	20	78

TABLE 11.3 Conditions for the MS/MS Detection of Quinolones

Analytes	Transition (*m*/*z*)	Ions for Quantitation (*m*/*z*)	Collision Energy (V)	Defocus Pressure (V)
Fleroxacin	370.4/326.4	370.4/326.4	30	70
	370.4/269.4		40	70
Ofloxacin	362.4/318.4	362.4/318.4	30	60
	362.4/261.3		40	60
Enoxacin	321.1/303.4	321.1/303.4	35	70
	321.1/232.2		48	70
Norfloxacin	320.4/276.6	320.4/276.6	26	50
	320.4/233.2		30	50

TABLE 11.3 Conditions for the MS/MS Detection of Quinolones—cont'd

Analytes	Transition (*m*/*z*)	Ions for Quantitation (*m*/*z*)	Collision Energy (V)	Defocus Pressure (V)
Ciprofloxacin	332.4/288.3	332.4/288.3	25	60
	332.4/245.3		33	60
Enrofloxacin	360.6/316.4	360.6/316.4	30	60
	360.6/245.4		40	60
Lomefloxacin	352.3/308.4	352.3/308.4	28	60
	352.3/265.4		33	60
Danofloxacin	358.3/340.3	358.3/340.3	40	80
	358.3/283.4		40	80
Orbifloxacin	396.3/352.3	396.3/352.3	27	60
	396.3/295.4		35	60
Difloxacin	400.4/356.2	400.4/356.2	28	60
	400.4/299.3		42	60
Sarafloxacin	386.4/342.3	386.4/342.3	28	60
	386.4/299.2		43	60
Sparfloxacin	393.3/349.4	393.3/349.4	30	80
	393.3/292.4		38	80
Oxolinic acid	262.3/244.2	262.3/244.2	30	70
	262.3/216.3		42	70

Continued

TABLE 11.3 Conditions for the MS/MS Detection of Quinolones—cont'd

Analytes	Transition (*m*/*z*)	Ions for Quantitation (*m*/*z*)	Collision Energy (V)	Defocus Pressure (V)
Nalidixic acid	233.3/215.2	233.3/215.2	24	60
	233.3/187.4		36	60
Flumequine	262.3/244.3	262.3/244.3	30	70
	262.3/202.3		49	70

TABLE 11.4 Maximum Permitted Tolerances for Relative Ion Intensities During Confirmation

Relative intensity (*k*)	$k > 50$	$20 < k \leq 50$	$10 < k \leq 20$	$k \leq 10$
Permitted tolerance	±20	±25	±30	±50

(3) Quantitative analysis

According to the approximate concentration of the analyte in the sample solution, select the standard working solution with similar responses to that of the sample solution. The responses of the analytes in the standard working solutions and the sample solutions should be within the linear range of the instrument detection. The mixed standard working solutions and the sample solutions should be injected with equal volume alternatively. Under the preceding LC-MS-MS operating conditions, the retention times of 15 quinolones are listed in Table 11.5; for the multireaction monitor chromatograms of 15 quinolones, see Fig. 11.1.

11.4.7 PRECISION

The precision data are determined according to GB/T6379.1 and GB/T6379.2. The repeatability and reproducibility limits given are obtained at a 95% confidence level.

TABLE 11.5 Reference Retention Times of 15 Quinolones

Analytes	RT (min)	Analyte	RT (min)	Analyte	RT (min)
Fleroxacin	5.14	Enrofloxacin	8.41	Sarafloxacin	10.83
Ofloxacin	5.88	Lomefloxacin	8.42	Sparfloxacin	13.07
Enoxacin	6.11	Danofloxacin	8.55	Oxolinic acid	16.26
Norfloxacin	6.86	Orbifloxacin	9.46	Nalidixic Acid	17.78
Ciprofloxacin	7.67	Difloxacin	9.93	Flumequine	18.25

(1) Repeatability

Under repeatability conditions, the value of the absolute difference between two independent test results should be less than or equal to the repeatability limit (r). The concentration ranges of the analytes and the type of relationship are listed in Table 11.6.

If the difference exceeds the repeatability limit, the test results should be abandoned and the tests redone.

(2) Reproducibility

Under reproducibility conditions, the value of the absolute difference between two independent test results should be less than or equal to the reproducibility limit (R). The concentration ranges of the analytes and the type of relationship are listed in Table 11.6.

11.4.8 RECOVERY

The recoveries, under optimized conditions, of quinolones using this method are listed in Table 11.7.

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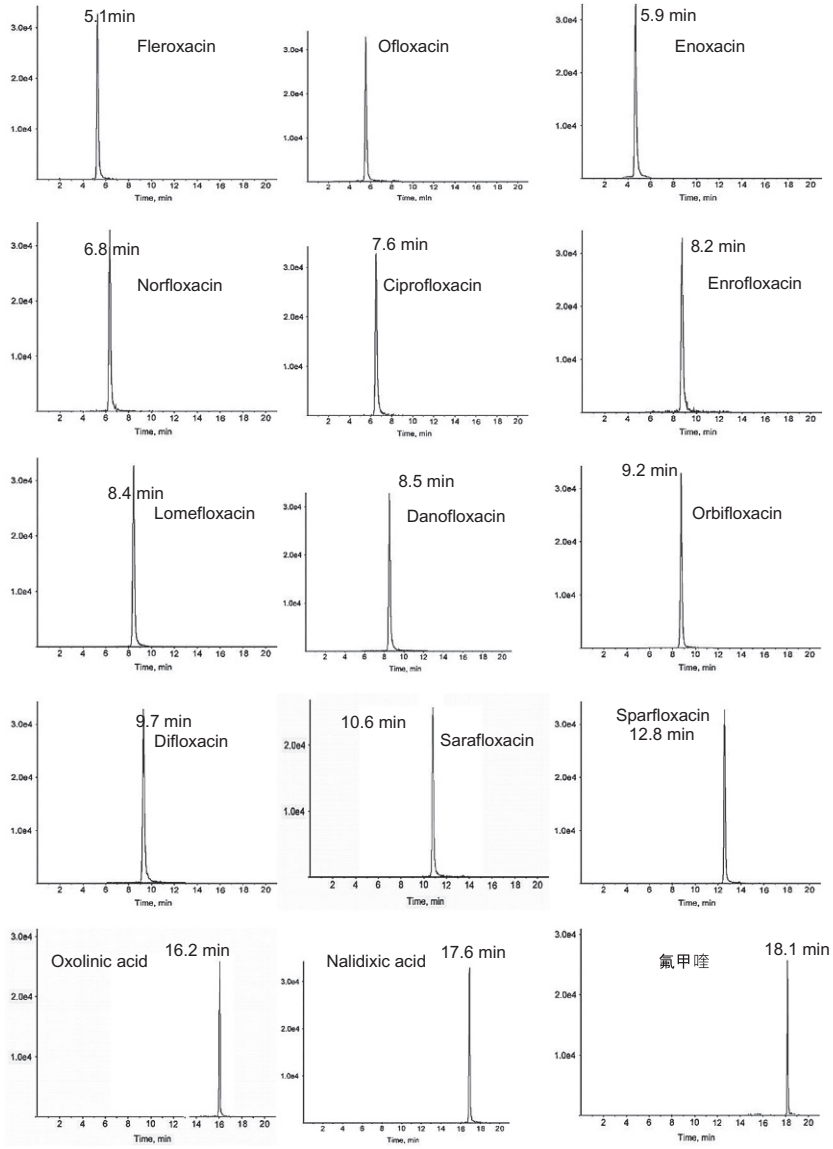

FIG. 11.1 The multireaction monitor chromatograms of 15 quinolones.

TABLE 11.6 The Repeatability and Reproducibility Equations of 15 Quinolones

Analytes	Concentration Range (μg/kg)	Matrix	Repeatability Limit	Reproducibility
Fleroxacin	5.0–40.0	Eel	$r = 0.3161m$	$R = 0.3605m$
		Eel product	$r = 0.2849m + 0.0348$	$R = 0.3615m$
Ofloxacin	5.0–40.0	Eel	$r = 0.3021m$	$R = 0.2989m + 0.1299$
		Eel product	$r = 0.2876m$	$R = 0.2769m + 0.3248$
Enoxacin	5.0–40.0	Eel	$r = 0.188m + 0.9684$	$R = 0.1599m + 1.2851$
		Eel product	$r = 0.309m$	$R = 0.3126m$
Norfloxacin	5.0–40.0	Eel	$r = 0.154m + 1.6128$	$R = 0.2026m + 1.2268$
		Eel product	$r = 0.2193m + 0.1127$	$R = 0.2648m + 0.1211$
Ciprofloxacin	5.0–40.0	Eel	$r = 0.2041m + 0.5605$	$R = 0.2825m + 0.133$
		Eel product	$r = 0.1835m + 0.6098$	$R = 0.2694m + 0.4192$
Enrofloxacin	5.0–40.0	Eel	$r = 0.2218m + 0.9717$	$R = 0.2233m + 1.0813$
		Eel product	$r = 0.3156m + 0.3862$	$R = 0.3701m$
Lomefloxacin	5.0–40.0	Eel	$r = 0.2151m + 0.6519$	$R = 0.2306m + 0.77$
		Eel product	$r = 0.196m + 0.1157$	$R = 0.219m + 0.1681$
Danofloxacin	5.0–40.0	Eel	$r = 0.4957m$	$R = 0.3672m + 0.0593$
		Eel product	$r = 0.2522m + 0.8514$	$R = 0.2765m + 0.4918$
Orbifloxacin	5.0–40.0	Eel	$r = 0.2325m$	$R = 0.2604m + 0.5997$
		Eel product	$r = 0.211m$	$R = 0.2561m + 0.2304$

Continued

TABLE 11.6 The Repeatability and Reproducibility Equations of 15 Quinolones—cont'd

Analytes	Concentration Range (μg/kg)	Matrix	Repeatability Limit	Reproducibility
Difloxacin	5.0–40.0	Eel	$r = 0.3163m + 0.3587$	$R = 0.3397m + 0.488$
		Eel product	$r = 0.2893m$	$R = 0.3672m + 0.0593$
Sarafloxacin	5.0–40.0	Eel	$r = 0.2919m$	$R = 0.3031m$
		Eel product	$r = 0.21m$	$R = 0.25m$
Sparfloxacin	5.0–40.0	Eel	$r = 0.2505m + 0.4917$	$R = 0.2588m + 0.2871$
		Eel product	$r = 0.1776m$	$R = 0.2902m$
Oxolinic acid	5.0–40.0	Eel	$r = 0.3958m$	$R = 0.385m + 0.4632$
		Eel product	$r = 0.2007m + 1.1761$	$R = 0.2693m + 1.3156$
Nalidixic acid	5.0–40.0	Eel	$r = 0.249m + 0.4497$	$R = 0.4044m + 0.4361$
		Eel product	$r = 0.196m + 0.2194$	$R = 0.3641m$
Flumequine	5.0–40.0	Eel	$r = 0.243m + 0.4749$	$R = 0.2886m + 0.6808$
		Eel product	$r = 0.453m$	$R = 0.2032m + 0.6735$

Note: m *is the arithmetic mean of two test results.*

TABLE 11.7 The Recoveries of Quinolones

Analytes	Spiked Concentration (μg/kg)	Recoveries Range (%)	
		Eel	Eel product
Fleroxacin	5	69.6–93.6	75.8–101.2
	10	68.0–105.0	75.5–93.1
	20	68.5–105.5	75.5–98.5
	40	65.3–104.0	79.8–94.5
Ofloxacin	5	75.8–98.2	73.8–90.4
	10	75.3–104.0	76.1–91.1
	20	82.5–97.0	75.5–98.5
	40	65.5–97.3	73.3–104.8
Enoxacin	5	67.4–98.0	74.0–100.4
	10	79.0–100.0	73.2–88.4
	20	73.5–103.0	73.0–92.0
	40	64.8–101.0	75.0–96.0
Norfloxacin	5	68.2–97.0	76.2–103.8
	10	74.7–102.0	74.0–93.5
	20	73.0–97.5	75.5–118.5
	40	68.8–97.5	79.5–94.8
Ciprofloxacin	5	68.2–91.0	77.8–94.4
	10	78.4–101.0	72.7–98.4
	20	79.0–97.5	75.0–93.5
	40	71.5–102.8	72.8–90.8
Enrofloxacin	5	66.8–98.2	73.2–100.4
	10	80.8–105.0	70.0–86.2
	20	70.5–98.5	64.0–96.5
	40	77.5–96.5	61.3–96.8
Lomefloxacin	5	68.8–98.0	69.4–94.4
	10	75.3–104.0	73.6–96.1
	20	74.0–96.0	75.5–108.0
	40	73.5–100.8	75.3–91.8

Continued

TABLE 11.7 The Recoveries of Quinolones—cont'd

Analytes	Spiked Concentration (μg/kg)	Recoveries Range (%)	
		Eel	Eel product
Danofloxacin	5	70.6–90.8	76.4–100.6
	10	70.9–105.0	68.9–91.7
	20	74.5–101.5	69.0–102.5
	40	65.0–102.8	75.8–94.8
Orbifloxacin	5	70.6–85.4	78.0–101.0
	10	71.9–96.9	76.9–99.0
	20	78.0–92.0	77.5–99.0
	40	68.8–92.0	76.3–100.5
Difloxacin	5	72.6–97.0	69.0–94.2
	10	77.1–94.9	72.9–94.8
	20	81.0–99.5	72.5–100.5
	40	79.5–96.3	73.3–92.0
Sarafloxacin	5	69.2–94.2	73.2–90.6
	10	74.1–95.0	76.7–102.0
	20	71.5–102.0	73.5–106.5
	40	72.0–97.3	79.0–95.0
Sparfloxacin	5	71.2–88.6	79.2–109.6
	10	74.3–98.1	73.2–96.4
	20	76.0–100.5	78.5–94.5
	40	67.5–99.0	73.3–99.5
Oxolinic acid	5	69.8–99.6	67.8–122.0
	10	75.2–107.0	64.9–104.0
	20	72.0–105.0	74.0–106.0
	40	71.0–107.0	70.3–93.8
Nalidixic acid	5	73.2–82.2	71.2–105.4
	10	78.2–98.9	76.3–107.0
	20	76.5–96.0	85.5–100.0
	40	68.0–97.8	73.5–96.3

TABLE 11.7 The Recoveries of Quinolones—cont'd

Analytes	Spiked Concentration (μg/kg)	Recoveries Range (%)	
		Eel	Eel product
Flumequine	5	66.2–103.0	75.2–95.4
	10	79.6–99.5	74.2–107.0
	20	75.5–104.0	77.0–99.5
	40	73.3–90.8	77.3–95.3

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11.5

Determination of Enrofloxacin, Danofloxacin, Ciprofloxacin, Sarafloxacin, Orbifloxacin, Difloxacin, and Marbofloxacin in Milk and Milk Powder—LC-MS-MS Method (GB/T 22985-2008)

11.5.1 SCOPE

This method is applicable to the determination of enrofloxacin, danofloxacin, ciprofloxacin, sarafloxacin, orbifloxacin, difloxacin, and marbofloxacin residues in milk and milk powder.

The limit of determination for this method is 1.0 μg/kg for enrofloxacin, danofloxacin, ciprofloxacin, sarafloxacin, orbifloxacin, difloxacin, and marbofloxacin, respectively, in milk. The limit of determination in milk powder is 4.0 μg/kg for enrofloxacin, danofloxacin, ciprofloxacin, sarafloxacin, orbifloxacin, difloxacin, and marbofloxacin, respectively.

11.5.2 PRINCIPLE

Enrofloxacin, danofloxacin, ciprofloxacin, sarafloxacin, orbifloxacin, difloxacin, and marbofloxacin residues in the test sample are extracted by an acetonitrile and phosphatic buffer solution, cleaned up by an Oasis HLB SPE column, and eluted by a 5% ammonia-methanol solution. Determination is carried out by LC-MS-MS, using an external standard method.

11.5.3 REAGENTS AND MATERIALS

Unless specifically noted, all reagents used should be of G.R grade; "water" is the first grade water prescribed by GB/T 6682.

Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Phosphate acid: HPLC grade.
Formic acid: HPLC grade.
Sodium phosphate dibasic with $12H_2O$.
Potassium dihydrogen phosphate dihydrate.
Ammonia
Phosphatic buffer solution (0.05 mol/L): 5.68 g sodium phosphate dibasic anhydrous with $12H_2O$ and 1.36 g potassium dihydrogen phosphate dihydrate dissolved in 800 mL water; adjust pH to 3.0 with phosphate acid: HPLC grade, make up to 1000 mL with water.
5% ammonia-methanol solution: Pipette 50 mL ammonia to 950 mL methanol and mix thoroughly.
Formic acid solution (pH = 3.0): Take 500 mL water and adjust pH to 3.0 with formic acid.
25% methanol solution: Take 250 mL methanol and dilute to 1000 mL with water.
Methanol-formic acid (15 + 85): Take 30 mL methanol and 170 mL formic acid solution and mix thoroughly.
Enrofloxacin, danofloxacin, ciprofloxacin, sarafloxacin, orbifloxacin, difloxacin, and marbofloxacin standard substance, purity ≥95%.
0.1 mg/mL enrofloxacin, danofloxacin, ciprofloxacin, sarafloxacin, orbifloxacin, difloxacin, and marbofloxacin stock standard solution: Accurately weigh a suitable amount of sulfonamide standards respectively; dissolve in methanol to make 0.1 mg/mL single stock standard solution. The solutions should be stored at 4°C.

1 μg/mL mixed standard medium working solution: Pipette 1 mL stock standard solution respectively into 100-mL volumetric flask, dilute with methanol to 100 mL, and mix to homogeneity. The solutions should be stored at 4°C.
Substrate standard working solution: According to the content of the analyzed matter, accurately measure an adequate volume of mixed standard medium working solution, and dilute with blank sample extraction. The solutions should be stored at 4°C.
Column of HLB or equivalent, 500 mg, 6 mL: Rinse the column of HLB with 5 mL methanol and 5 mL phosphatic buffer solution; keep the column wet.

11.5.4 APPARATUS

Liquid chromatography–mass spectrograph, equipped with electrospray ion source.
Analytic balance: sensibility is 0.1 mg and 0.01 g.
Vortex shaker.
SPE equipment.
Nitrogen concentration equipment.
Vacuum pump.
pH meter: sensibility is ±0.02 pH.
Reservoir tube: attach to the Oasis HLB column.
Centrifuge.
Rotary vacuum evaporator.
10-mL volumetric tube.
0.22-μm filter.

11.5.5 SAMPLE PRETREATMENT

(1) Sample preparation

Take representative test sample of milk or milk powder, mix thoroughly to get a homogeneous sample, and label.

Milk should be stored at −18°C in dark. Milk powder should be sealed and stored at room temperature.

(2) Extraction

Milk

Weigh ca. 2 g of the test sample, accurate to 0.01 g, into a 50-mL tube. Add 10 mL acetonitrile, shake 1 min on a vortex shaker, and centrifuge for 5 min at 5000 rpm; filter the extraction into the heart-shaped flask. Add 5 mL phosphatic buffer solution and 10 mL acetonitrile to the residue sample. Repeat the previous procedure, combining the extraction into the same heart-shaped flask. Evaporate the extraction until no acetonitrile remains, using a rotary vacuum evaporator at 50°C. Add 5 mL phosphatic buffer solution and mix well.

Milk powder

Weigh ca. 0.5 g of the test sample, accurate to 0.01 g, into a 50-mL tube. Add 6 mL phosphatic buffer solution and shake well on a vortex shaker; add 10 mL acetonitrile, shake 1 min on a vortex shaker in sequence, and centrifuge for 5 min at 5000 rpm. Filter the extraction into the heart-shaped flask. Add 5 mL phosphatic buffer solution and 10 mL acetonitrile to the residue sample. Repeat the previous procedure, combining the extraction into the same heart-shaped flask. Evaporate the extraction until no acetonitrile remains, using a rotary vacuum evaporator at 50°C. Add 5 mL phosphatic buffer solution and mix well.

(3) Clean-up

Transfer all the solution into the reservoir tube attached to the Oasis HLB column. Add 5 mL phosphatic buffer solution (4.8) to rinse the heart-shaped flask and combine into the reservoir tube. Adjust the pressure in such a way as to make the extraction pass through the column at a flow rate of ca. 1 mL/min. Then rinse the column with 4 mL water, 4 mL 25% methanol solution and discard all the effluence; dry the column. Elute the column with 4 mL 5% ammonia-methanol solution. Collect all the elution in a 10-mL volumetric tube. Evaporate the elution to no less than 0.2 mL under a stream of nitrogen at 40°C. Make up to 1.0 mL with methanol-formic acid and vortex to homogenize. Then centrifuge for 5 min at 5000 rpm. After being filtrated with 0.22-μm filter, the final solution is ready for analysis by HPLC-MS-MS.

(4) Blank sample solution preparation

Weigh ca. 2 g (accurate to 0.01 g) negative milk, and weigh ca. 0.5 g (accurate to 0.01 g) negative milk powder; the following procedure is the same as the above-mentioned extraction and cleanup steps.

11.5.6 DETERMINATION

(1) HPLC operating conditions:
Column: C_{18} 150 mm × 2.1 mm (i.d.), 5-μm particle size or equivalent;
Column temperature: 30°C;
Injection volume: 15 μL.
Mobile phase: elution gradient of LC: see Table 11.8.
Ionization mode: Electron Spray Ion Source (ESI+);
Scan mode: MRM;
Sheath gas: 15 unit;
Auxiliary gas: 20 unit;
Ion spray voltage (IS): 4000V;
Capillary temperature: 320°C;
Source CID: 10V;
Q1=0.4, Q3=0.7;

TABLE 11.8 Elution Gradient of LC

Time (min)	Flow Rate (μL/min)	0.1% Acetic Acid Solution (%)	Methanol (%)
0.00	200	80	20
5.00	200	40	60
9.00	200	40	60
9.10	200	80	20
11.00	200	80	20

Impact gas: high pure Ar;
Impact gas pressure: 1.5mTorr;
For other MS conditions, see Table 11.9.

(2) Qualitative analysis

Under the preceding determination, for the same analysis batch and the same compound, the variation of the ionization between the two daughter ions for the unknown sample and the standard working solution at similar concentration cannot be out of the range of Table 11.4; then the corresponding analyte must be present in the sample.

(3) Quantitative analysis

According to the approximate concentration of analyte in the test sample solution, select the standard working solution with similar responses to those of the sample solution. The responses of the analyte in the standard working solution and the sample solution should be within the linear range of the instrument detection. The mixed standard working solution should be injected randomly between the injections of the sample solution of equal volume. Under these operating conditions, the chromatograms of the standard can be found in Fig. 11.2.

11.5.7 PRECISION

The precision data in this part are determined according to GB/T 6379.1 and GB/T 6379.2. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under repeatability conditions, the difference of absolute value of two independent results is not above the repeatability limit (r). The content

TABLE 11.9 CAS No., Qualitative Ion, Quantitative Ion, and Collision Energy of Seven Kinds of Fluoroquinolones

Compound	Retention Time (min)	CAS No.	Ion Pairs (*m*/*z*)	Quantitative ion Pair (*m*/*z*)	Collision Energy (eV)
Enrofloxacin	5.66	93106-60-6	360.10/315.97	360.14/316.08	19
			360.10/244.86		24
Danofloxacin	6.18	119478-55-6	358.14/339.83	358.14/340.00	22
			358.14/313.80		32
Ciprofloxacin	6.51	93107-08-5	332.30/288.14	332.30/288.14	15
			332.30/244.88		23
Sarafloxacin	6.17	98105-99-8	386.11/341.77	386.10/341.77	19
			386.11/298.83		25
Orbifloxacin	6.71	113617-63-3	396.05/352.01	396.05/352.01	20
			396.05/394.98		25
Difloxacin	7.06	124858-35-1	400.12/355.71	400.12/355.71	18
			400.12/298.85		30
Marbofloxacin	7.05	115550-35-1	363.15/319.91	363.15/319.91	17
			363.15/344.95		20

Note*: For different MS equipment, the parameters may be different, and the MS parameters should be optimized before analysis.*
Noncommercial statement*: the reference mass parameters listed are accomplished by Thermo TSQ Quantum Ultra AM LC-MS-MS. The types of equipment involved in the standard method are only for reference and are not related to any commercial aim, and analysts are encouraged to use equipment from different companies or of different type.*

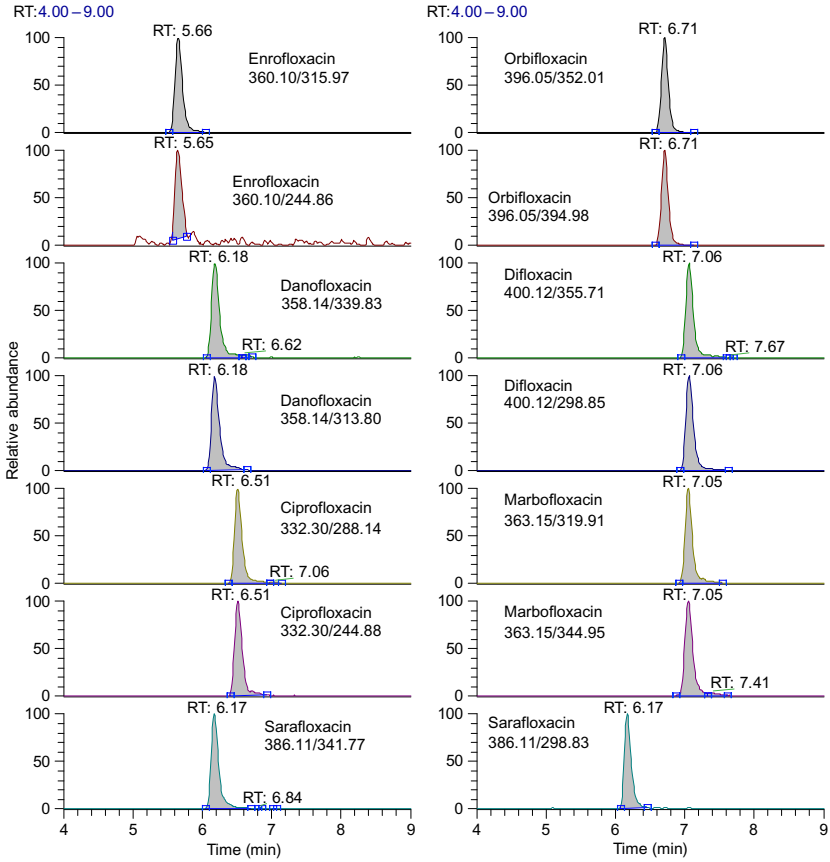

FIG. 11.2 The MRM chromatograms of seven kinds of fluoroquinolone standard working solutions (*Note*: The first chromatogram is qualitative and the other is quantitative).

ranges and the repeatability equations of seven kinds of fluoroquinolone residues in milk and milk powder are shown in Tables 11.10 and 11.11.

If the difference is above the repeatability limit (r), the results of the experiment should be abandoned and two independent experiments should be redone.

(2) Reproducibility

Under reproducibility conditions, the difference of absolute value of two independent results is not above the reproducibility limit (R). The content ranges and the reproducibility equations of seven kinds of fluoroquinolone residues in milk and milk powder are shown in Tables 11.10 and 11.11.

TABLE 11.10 Content Ranges and the Repeatability and Reproducibility Equations of Seven Kinds of Analytes for Milk Sample

Analyte	Content Ranges (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Enrofloxacin	1.0–10.0	$r = 0.1313m - 0.0985$	$R = 0.2018m - 0.1500$
Danofloxacin	1.0–10.0	$r = 0.0942m - 0.0351$	$\lg R = 1.1637 \lg m - 0.9122$
Ciprofloxacin	1.0–10.0	$r = 0.0737m + 0.0168$	$R = 0.1221m + 0.1099$
Sarafloxacin	1.0–10.0	$r = 0.0783m - 0.0281$	$R = 0.1240m + 0.0584$
Orbifloxacin	1.0–10.0	$r = 0.0690m - 0.0087$	$\lg R = 1.0398 \lg m - 0.8377$
Difloxacin	1.0–10.0	$r = 0.0905m - 0.0343$	$R = 0.1710m - 0.0612$
Marbofloxacin	1.0–10.0	$\lg r = 1.0992 \lg m - 1.0940$	$\lg R = 1.0162 \lg m - 0.8046$

Note: m *is the mean determination result of the duplicate test.*

TABLE 11.11 Content Ranges and the Repeatability and Reproducibility Equations of Seven Kinds of Analytes for Milk Sample

Analyte	Content Ranges (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
Enrofloxacin	4.0–40.0	$\lg r = 0.9319 \lg m - 0.9356$	$\lg R = 1.0695 \lg m - 0.9456$
Danofloxacin	4.0–40.0	$\lg r = 1.0358 \lg m - 1.0438$	$R = 0.1782m - 0.0346$
Ciprofloxacin	4.0–40.0	$\lg r = 0.7928 \lg m - 0.8237$	$\lg R = 0.9275 \lg m - 0.7228$
Sarafloxacin	4.0–40.0	$r = 0.0746m + 0.1785$	$\lg R = 0.8883 \lg m - 0.6491$
Orbifloxacin	4.0–40.0	$r = 0.0712m + 0.0610$	$R = 0.1109m + 0.2602$
Difloxacin	4.0–40.0	$\lg r = 0.9401 \lg m - 0.9084$	$\lg R = 0.9051 \lg m - 0.6383$
Marbofloxacin	4.0–40.0	$\lg r = 0.9285 \lg m - 0.8998$	$\lg R = 0.8448 \lg m - 0.5742$

Note: m *is the mean determination result of the duplicate test.*

11.5.8 RECOVERY

Under optimized condition, the recoveries of enrofloxacin, danofloxacin, ciprofloxacin, sarafloxacin, orbifloxacin, difloxacin, and marbofloxacin residues in milk and milk powder using this method are listed in Table 11.12.

TABLE 11.12 The Recovery Ranges of the Analytes in Milk and Milk Powder at Four Fortifying Levels

	Milk		Milk Powder	
Compound	Spiked Level (μg/kg)	Recovery Range (%)	Spiked Level (μg/kg)	Recovery Range (%)
Marbofloxacin	1	80.8–108.0	4	76.0–101.2
	2	86.6–100.6	8	86.9–101.5
	5	77.3–95.0	20	78.8–96.0
	10	81.2–92.0	40	83.8–93.4
Ciprofloxacin	1	76.8–90.1	4	89.0–100.3
	2	73.2–92.6	8	75.2–95.3
	5	73.8–100.2	20	76.0–98.6
	10	79.1–92.3	40	82.5–94.6
Danofloxacin	1	87.4–109.4	4	70.4–101.3
	2	84.0–97.6	8	81.6–90.7
	5	77.0–102.1	20	74.6–93.9
	10	78.5–92.1	40	81.8–94.0
Enrofloxacin	1	85.9–102.0	4	75.2–93.8
	2	76.1–91.1	8	77.1–100.1
	5	80.8–101.1	20	78.6–101.5
	10	79.4–92.2	40	81.8–92.7

Continued

TABLE 11.12 The Recovery Ranges of the Analytes in Milk and Milk Powder at Four Fortifying Levels—cont'd

Compound	Milk		Milk Powder	
	Spiked Level (μg/kg)	Recovery Range (%)	Spiked Level (μg/kg)	Recovery Range (%)
Orbifloxacin	1	83.9–105.0	4	80.9–101.7
	2	81.5–96.0	8	82.4–100.5
	5	77.2–98.2	20	77.2–100.2
	10	79.7–93.6	40	79.2–90.4
Sarafloxacin	1	72.1–97.1	4	78.6–100.9
	2	78.2–95.5	8	81.1–94.8
	5	84.6–103.7	20	77.1–97.8
	10	84.5–93.8	40	80.5–94.0
Difloxacin	1	88.9–108.1	4	82.3–96.0
	2	88.1–99.3	8	84.1–96.1
	5	85.9–101.3	20	81.3–108.0
	10	84.4–94.6	40	80.9–92.2

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11.6

Method for the Determination of 14 Quinolone Residues in Honey—LC-MS-MS Method (GB/T 20757-2006)

11.6.1 SCOPE

This method is applicable to the determination of 14 quinolone residues in honey.

The limit of determination of this method for 14 quinolone residues is 2.0 μg/kg.

11.6.2 PRINCIPLE

The quinolone drugs are extracted from honey with phosphate buffer (pH = 3); after filtering, the honey solution is cleaned up by Oasis HLB or equivalent solid extraction columns. The drugs are eluted with aqueous ammonia-methanol solution and dried. The dregs are dissolved with a dissolving solution, and then put through a 0.2-μm filter membrane. The determination of the solution is done by LC-MS-MS, using an external standard method.

11.6.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be analytically pure; "water" is deionized water.

Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Phosphoric acid: G.R.
Acetic acid: G.R.
Aqueous ammonia.
Disodium hydrogen phosphate: G.R.
Potassium dihydrogen phosphate: G.R.
Phosphate buffer: 0.05 mol/L. Place 5.68 g disodium hydrogen phosphate and 1.36 g potassium dihydrogen phosphate into a 1000-mL volumetric flask, dissolve in 800 mL water, adjust pH to 3.0 by phosphoric acid, and dilute solution to volume with water.
Aqueous ammonia-methanol solution: (1 + 19). Mix 10 mL aqueous ammonia and 190 mL methanol.

Dissolve solution: Acetonitrile-0.01 mol/L acetic acid solution (1+4). Mix 100 mL acetonitrile and 400 mL 0.01 mol/L acetic acid solution.
Enoxacin, norfloxacin, marbofloxacin, fleroxacin, ciprofloxacin, ofloxacin, danofloxacin, enrofloxacin, orbifloxacin, sarafloxacin, sparfloxacin, difloxacin, oxolinic acid, and flumequin standards: purity $\geq$95%.
Standard stock solutions of quinolone: 1 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volumetric flask with aqueous ammonia-methanol solution; then dilute with aqueous ammonia-methanol solution separately to prepare the standard stock solutions of 1.0 mg/mL in concentration. The standard stock solutions are stored 6 months at 4°C.
Intermediate mixed standard stock solutions of quinolone:10 mg/L. Pipette 0.1 mL standard stock solutions into 10-mL volumetric flasks respectively. Dilute the standards solution to volume with dissolve solution.
Mixed standard working solutions of quinolone: Prepare the standard working solutions of 5, 10, 50, and 100 ng/mL concentration for quinolone with honey control sample extract. Prepare fresh daily.
Oasis HLB solid extraction columns or equivalent: 500 mg, 6 mL. Condition each column with 5 mL methanol followed by 10 mL water and 5 mL phosphate buffer before use.
Filter membrane: 0.2 μm.

11.6.4 APPARATUS

LC-MS-MS: Equipped with ESI source.
SPE vacuum apparatus.
Nitrogen evaporator.
Vortex mixer.
Analytical balance: capable of weighing to 0.1 mg, 0.01 g.
Vacuum pump: Vacuum should attain up to 80 kPa.
pH Meter: Capable of measuring $\pm$0.02 unit.
Reservoirs and adapters to fit SPE columns: 50 mL.

11.6.5 SAMPLE PRETREATMENT

(1) Preparation of Test Sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath and warm at $\leq$60°C with occasional shaking until liquefied; mix thoroughly and promptly cool to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label.

The test samples should be stored at ambient temperature.

(2) Extraction

Weigh 5-g test sample (accurate to 0.01 g) into 150-mL Erlenmeyer flask. Add 30 mL phosphate buffer solution (4.8) to each sample. Mix vigorously 1 min on vortex mixer, until honey is completely dissolved.

(3) Clean-Up

Connect a reservoir with a glass wool plug to an Oasis HLB solid extraction column. Decant the honey sample solution into the reservoir, and let the solution pass through the Oasis HLB column at reduced pressure, adjusting the flow rate to $\leq$3 mL/min. Wait till the sample solution has thoroughly drained, and then rinse reservoir and cartridge respectively with 5 mL water and 5 mL methanol-water (3+7). Discard all the effluents. Dry cartridge by drawing air through it for 30 min, using 65 kPa vacuum. Finally, elute quinolones with 5 mL aqueous ammonia-methanol solution (1+19) into the 10-mL conical tube; dry the elution on a nitrogen evaporator at 50°C. Accurately add 1 mL dissolve solution to drugs; and then put it through a 0.2-μm filter membrane. Determination is by LC-MS-MS.

According to above-mentioned extraction and cleanup steps to prepare the standard working solutions of different concentrations for 14 quinolones with honey control sample extract.

11.6.6 DETERMINATION

(1) LC Operation Conditions

Chromatographic column: Inertsil pH-3 5 μm 150 mm × 2.1 mm i.d. or equivalent;

Column temperature: 40°C;

Injection volumes: 40 μL;

Mobile phase: A: acetonitrile, B: 0.01 mol/L acetic acid solution, gradient elution condition: Table 11.13.

TABLE 11.13 Gradient Elution Condition

Time (min)	Flow (mL/min)	A: Acetonitrile (%)	B: 0.01 mol/L Acetic Acid Solution (%)
0.00	0.3	85.0	15.0
4.00	0.3	5.0	95.0
8.00	0.3	5.0	95.0
8.01	0.3	85.0	15.0
18.00	0.3	85.0	15.0

Ion source: ESI source;
Scan mode: Positive scan;
Monitor mode: Multiple reaction monitor;
Ionspray voltage: 5500 V;
Nebulizer gas: 0.083 MPa;
Curtain gas: 0.069 MPa;
Turbo ionspray gas rate: 6 L/min;
Source temperature: 500°C;
Declustering potential: 55 V;
MRM transitions for precursor/product ion, quantifying for precursor/product ion, declustering potential, collision energy, declustering potential: see Table 11.14.

(2) LC-MS-MS Determination

The standard working solutions of different concentrations for 14 quinolones are prepared with honey control sample extract on base. Then inject 20 μL of the different concentration working standard solutions, respectively, in duplication under LC and MS conditions. Draw the 7-point standard curves of each quinolone (peak area vs. quinolone concentration). Calculate the concentrations of the corresponding content from the working curve. The responses of 14 quinolones in the standard working solution and sample solution should be in the linear range of the instrumental detection. Under LC and MS conditions, for the reference retention times of the 14 quinolones, see Table 11.15. For total ion chromatograms of the mixed standard working solution of 14 quinolones, see Fig. 11.3.

11.6.7 PRECISION

The precision data of the method for this standard have been determined in accordance with the stipulations of GB/T6379 and GB/T 6379.2. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under repeatability conditions, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for 14 quinolone drugs in honey are shown in Table 11.16.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

TABLE 11.14 MRM Transitions for Precursor/Product Ion, Quantifying for Precursor/Product Ion, Collision Energy, Declustering Potential of 14 Quinolones

Quinolone	MRM Transitions for Precursor/ Product Ion (*m*/*z*)	Quantifying for Precursor/ Product Ion (*m*/*z*)	Collision Energy (V)	Declustering Potential (V)
Enoxacin	321/234 321/257	321/234	32 28	18
Norfloxacin	320/276 320/233	320/276	26 36	18
Marbofloxacin	363/320 363/277	363/320	23 27	18
Fleroxacin	370/326 370/269	370/326	29 39	19
Ciprofloxacin	332/288 332/245	332/288	27 35	19
Ofloxacin	362/318 362/261	362/318	29 40	19
Danofloxacin	358/314 358/283	358/314	27 35	21
Enrofloxacin	360/316 360/245	360/316	29 39	19
Orbifloxacin	396/352 396/295	396/352	27 35	21
Sarafloxacin	386/342 386/299	386/342	28 40	20
Sparfloxacin	393/349 393/292	393/349	29 36	20
Difloxacin	400/356 400/299	400/356	29 42	19
Oxolinic acid	262/244 262/216	262/244	27 41	13
Flumequin	262/202 262/244	262/202	46 28	13

TABLE 11.15 Retention Time of 14 Quinolones

Quinolone	Retention Time (min)
Enoxacin	3.85
Norfloxacin	4.21
Marbofloxacin	4.65
Fleroxacin	4.98
Ciprofloxacin	5.10
Ofloxacin	5.19
Danofloxacin	5.52
Enrofloxacin	5.65
Orbifloxacin	5.70
Sarafloxacin	5.86
Sparfloxacin	5.91
Difloxacin	5.93
Oxolinic acid	6.57
Flumequin	7.01

(2) Reproducibility

Under reproducibility conditions, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for 14 quinolone drugs in honey are shown in Table 11.16.

11.6.8 RECOVERY

Under optimized condition, the recoveries of 14 quinolone residues in honey using this method are listed in Table 11.17.

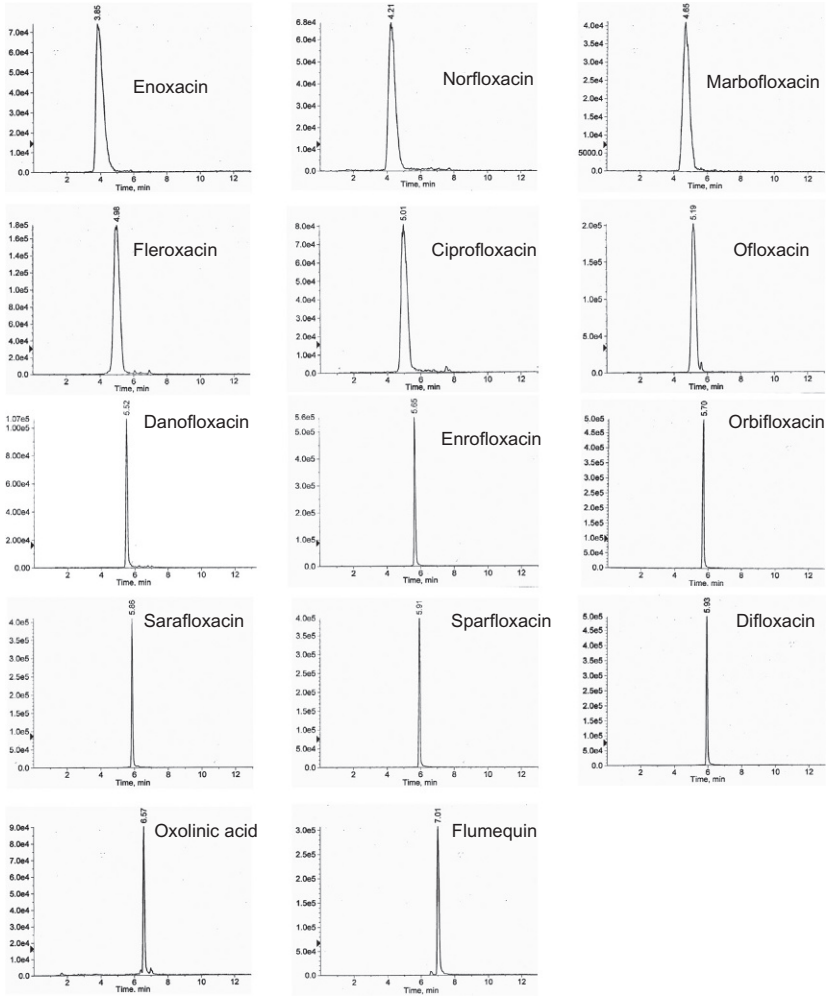

FIG. 11.3 Chromatograms of 14 quinolone standards in MRM mode.

TABLE 11.16 Content Range, Repeatability and Reproducibility (Unit: μg/kg)

Quinolone	Range	Repeatability (r)	Reproducibility (R)
Enoxacin	2.0–20.0	$\lg r = 1.0100$ $\lg m - 0.8398$	$\lg R = 1.1213$ $\lg m - 0.6173$
Norfloxacin	2.0–20.0	$\lg r = 0.9824$ $\lg m - 1.2982$	$\lg R = 0.9984$ $\lg m - 0.6945$
Marbofloxacin	2.0–20.0	$\lg r = 0.9376$ $\lg m - 1.2655$	$\lg R = 1.0068$ $\lg m - 0.5621$

Continued

TABLE 11.16 Content Range, Repeatability and Reproducibility (Unit: μg/kg)—cont'd

Quinolone	Range	Repeatability (r)	Reproducibility (R)
Fleroxacin	2.0–20.0	$\lg r = 1.2342$ $\lg m - 1.4332$	$\lg R = 1.0854$ $\lg m - 0.8657$
Ciprofloxacin	2.0–20.0	$\lg r = 1.0544$ $\lg m - 1.3812$	$\lg R = 0.7999$ $\lg m - 0.5863$
Ofloxacin	2.0–20.0	$\lg r = 0.8264$ $\lg m - 1.2088$	$\lg R = 1.3111$ $\lg m - 0.9888$
Danofloxacin	2.0–20.0	$\lg r = 1.0812$ $\lg m - 1.3742$	$\lg R = 0.8569$ $\lg m - 0.7198$
Enrofloxacin	2.0–20.0	$\lg r = 0.8140$ $\lg m - 1.0683$	$\lg R = 1.1002$ $\lg m - 0.9321$
Orbifloxacin	2.0–20.0	$\lg r = 1.0032$ $\lg m - 1.3366$	$\lg R = 1.2902$ $\lg m - 0.9265$
Sarafloxacin	2.0–20.0	$\lg r = 0.9146$ $\lg m - 1.2046$	$\lg R = 0.7675$ $\lg m - 0.5894$
Sparfloxacin	2.0–20.0	$\lg r = 1.0844$ $\lg m - 1.3444$	$\lg R = 0.8562$ $\lg m - 0.6052$
Difloxacin	2.0–20.0	$\lg r = 0.7067$ $\lg m - 1.0336$	$\lg R = 0.9339$ $\lg m - 0.5986$
Oxolinic acid	2.0–20.0	$\lg r = 1.0998$ $\lg m - 1.4398$	$\lg R = 0.8570$ $\lg m - 0.6139$
Flumequin	2.0–20.0	$\lg r = 0.9411$ $\lg m - 1.1668$	$\lg R = 1.0733$ $\lg m - 0.7792$

Note: m *is average value of parallel test results.*

TABLE 11.17 Test Data of Fortifying Concentration and Average Recovery

Quinolones	Fortifying Concentration (μg/kg)	Average Recovery (%)
Enoxacin	2	110.9
	5	87.4
	20	85.3
	50	71.3

TABLE 11.17 Test Data of Fortifying Concentration and Average Recovery—cont'd

Quinolones	Fortifying Concentration (μg/kg)	Average Recovery (%)
Norfloxacin	2	80.2
	5	76.1
	20	82.9
	50	84.9
Marbofloxacin	2	88.4
	5	89.2
	20	86.7
	50	89
Fleroxacin	2	102.4
	5	102.6
	20	78.6
	50	80.4
Ciprofloxacin	2	85.7
	5	79.9
	20	84.3
	50	80.5
Ofloxacin	2	103.2
	5	102.5
	20	86
	50	80.7
Danofloxacin	2	104.8
	5	94.6
	20	86.2
	50	76.7
Enrofloxacin	2	97.1
	5	90.2
	20	91.3
	50	91.4

Continued

TABLE 11.17 Test Data of Fortifying Concentration and Average Recovery—cont'd

Quinolones	Fortifying Concentration (μg/kg)	Average Recovery (%)
Orbifloxacin	2	90.1
	5	91
	20	78.3
	50	82.4
Sarafloxacin	2	92.9
	5	77.7
	20	84.9
	50	84.7
Sparfloxacin	2	82.7
	5	94
	20	66.3
	50	79.1
Difloxacin	2	94.8
	5	86.1
	20	91.6
	50	93.3
Oxolinic acid	2	100.8
	5	89.8
	20	101.6
	50	95.2
Flumequin	2	100.1
	5	87.6
	20	101.6
	50	97.6

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Chapter 12

Tetracyclines

12.1

Curative Effect and Side Effects

Tetracyclines (TCs) are broad-spectrum antibiotics with activity against Gram-positive and Gram-negative bacteria, including some anaerobes. They have been widely used for the treatment of infectious diseases and as an animal feed additive.

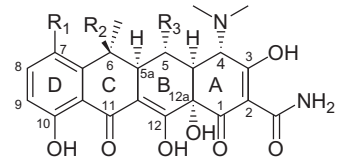
TCs and their degradation products may have harmful effects on consumers, such as possible allergic reactions, liver damage, yellowing of teeth, and gastrointestinal disturbance due to the selective pressure of antibiotics on human gut microflora. To protect humans from the exposure to these drug residues, European Union (EU), Japan and the Chinese Ministry of Agriculture have established maximum residue limits (MRLs) for TCs.

12.2

Chemical Structures and Maximum Residue Limits for Tetracyclines

Fig. 12.1 shows the chemical structure of tetracyclines. Table 12.1 contains chemical formulas and MRLs for tetracyclines.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00012-9



Oxytetracycline: R_1=H, R_2=R_3=OH
Tetracycline: R_1=R_3=H, R_2=OH
Chlortetracycline: R_1=Cl, R_2=OH, R_3=HI
Doxycycline: R_1=R_2=H, R_3=OH

FIG. 12.1 Chemical structure of tetracyclines.

TABLE 12.1 Chemical Formulas and MRLs for Tetracyclines

Compound	Formula	Molecular Weight	Cas. No.	MRL (μg/kg)	
Tetracycline	$C_{22}H_{24}N_2O_8$	444.15	60-54-8	America	300
				China	100
Oxytetracycline	$C_{22}H_{24}N_2O_9$	460.15	2058-46-0	EU	
				Australia	100
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	478.11	64-72-2	Japan	10
				Canada	250
Doxycycline	$C_{22}H_{24}N_2O_8$	444.15	24390-14-5	Japan	50
				EU	100

12.3

Determination of Oxytetracycline, Tetracycline, Chlortetracycline, and Doxycycline Residues in Edible Animal Muscles—LC-UV Method (GB/T 20764-2006)

12.3.1 SCOPE

This method is applicable to the determination of oxytetracycline, tetracycline, chlortetracycline, and doxycycline residues in bovine, mutton, porcine, chicken, and rabbit muscles.

The limit of determination of this method for oxytetracycline, tetracycline, chlortetracycline, and doxycycline is 0.005 mg/kg.

12.3.2 PRINCIPLE

The drugs are extracted from the edible animal muscle with 0.1 mol/L Na_2 EDTA—Mcllvaine buffer (pH = 4.0 ± 0.05). The extracted solution is centrifuged and the supernatant is cleaned up by an Oasis HLB and cation exchange columns. Residues are determined by HPLC with a UV detector at 350 nm.

12.3.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be reagent grade; "water" is deionized water. Methanol, acetonitrile, ethyl acetate: HPLC grade; Disodium hydrogen phosphate: G.R.; Citric acid; Disodium ethylenediamine tetraacetate: $Na_2EDTA{\cdot}2H_2O$; Oxalic acid; Methanol-water solution: 5 + 95. Mix 5 mL methanol and 95 mL water; Mobile phase: acetonitrile-methanol-0.01 mol/L oxalic acid solution (2 + 1 + 7).

Disodium hydrogen phosphate solution: 0.2 mol/L. Place 28.41 g disodium hydrogen phosphate into 1000-mL volumetric flask and dissolve in H_2O. Dilute solution to volume with H_2O;
Citric acid solution: 0.1 mol/L. Place 21.01 g citric acid into 1000-mL volumetric flask and dissolve in H_2O. Dilute solution to volume with H_2O.
Mcllvaine buffer: Combine 1000 mL citric acid solution with 625 mL disodium phosphate solution in 2000-mL Erlenmeyer flask. Adjust pH to 4.0 ± 0.05 by adding dropwise either 0.1 mol/L HCl or 0.1 mol/L NaOH if required.

Mcllvaine buffer–EDTA solution: 0.1 mol/L. Place 60.5 g disodium EDTA dihydrate to 1625 mL Mcllvaine buffer and mix until solid dissolves.
Oxytetracycline, tetracycline, chlortetracycline, doxycycline standards: purity ≥95%.
Standard solutions of oxytetracycline, tetracycline, chlortetracycline, and doxycycline: 0.1 mg/mL. Accurately weigh appropriate amount of oxytetracycline, tetracycline, chlortetracycline, doxycycline standards, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the standard stock solutions of 0.100 mg/mL in concentration. Kept at −18°C.
Mixed standard working solutions: According to the requirement, prepare the mixed standard working solution of 5, 10, 50, 100, 200 ng/mL; prepare the mixed standard working solution fresh daily.
Oasis HLB solid extraction columns: 500 mg, 6 mL. Condition each column with 5 mL methanol followed by 10 mL water before use; Cation exchange column: carboxylic acid, 500 mg, 3 mL. Condition each column with 5 mL ethyl acetate before use.

12.3.4 APPARATUS

Liquid chromatograph: Equipped with UV detector; Analytical balance: capable of weighing to 0.1 mg, 0.01 g; Vortex mixer; Solid phase extraction (SPE) vacuum apparatus; Reservoirs and adapters to fit SPE columns: 50 mL; High speed refrigerator centrifuger: maximum speed 13,000 rpm; Sample tube: 5 mL, accurate to 0.1 mL; Vacuum pump shaker; Flat-flasks: 100 mL; pH meter: Capable of measuring ±0.02 unit.

12.3.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

The representative sample, ca. 1 kg, is taken from whole sample, vigorously stirred, thoroughly mixed, and divided into two equal parts. It is then put in a clean container, which is sealed and labeled. In the course of sampling and sample preparation, precaution should be taken to avoid contamination or any factors that may cause a change of residue content. The test samples should be stored below −18°C.

(2) Extraction

Weigh 6-g test sample (accurate to 0.01 g) into 50-mL PTFE centrifuge tube with plug. Add 30 mL 0.1 mol/L Na_2EDTA—Mcllvaine buffer solution (pH = 4) to each sample. Mix vigorously 1 min on vortex mixer. Shake 10 min on shaker, and centrifugalize the sample solution at 10,000 rpm for 10 min. The supernatant is then decanted into another centrifuge tube. Add 20 mL Na_2EDTA–Mcllvaine buffer solution into the dregs, repeat extraction, and combine the supernatants.

(3) Clean-up

Connect a reservoir to cartridge. Decant the supernatant into the reservoir and dilute it through the Oasis HLB cartridge at up to 3 mL/min, using a vacuum if required. Rinse the reservoir and cartridge with 5 mL methanol-water solution. Discard all eluates from this point. Dry the cartridge by drawing air through it for 40 min, using a 65-kPa vacuum. Place 100-mL flat-flasks in manifold. Elute the TCs from the cartridge with 15 mL ethyl acetate, using gravity only.

The speed of ethyl acetate through the cation exchange cartridge is up to 3 mL/min when using a vacuum. Rinse the reservoir and cartridge with 5 mL methanol. Discard all eluates from this point. Dry cartridge by drawing air through it for 5 min, using a 65-kPa vacuum. Place a 5-mL sample tube in the manifold. Elute the TCs from the cation exchange cartridge with mobile phase and dilute to 4 mL. The solution is ready for HPLC equipped with UV-detector determination.

12.3.6 DETERMINATION

(1) Operation conditions

Chromalographic column: Mightsil RP-18 GP, 3 μm, 150 mm × 4.6 mm, or equivalent; Mobile phase: acetonitril-methanol 0.01 mol/L oxalic acid (20 + 10 + 70); Flow rate of mobile phase: 0.5 mL/min; Column temperature: 25°C; Wavelength: 350 nm; Sample size: 60 μL.

(2) LC determination

Inject the mixed standard working solutions of different concentrations of four TCs, respectively; with concentration as the *X*-axis and peak area as the *Y*-axis, draw the standard working curve. Calculate the concentrations of the corresponding content from the working curve. The responses of the TCs in the standard working solution and sample solution should be in the linear range of the instrumental detection. The standard working solution should be injected in between the injections of the sample solution of equal volume, under the preceding LC conditions. The reference retention times of oxytetracycline, tetracycline, chlortetracycline, and doxycycline are 4.82, 5.42, 10.32, and 15.45 min. For chromatograms of the mixed standard, see Fig. 12.2.

12.3.7 PRECISION

The precision data of the method have been determined in accordance with the stipulations of GB/T6379 and GB/T 6379.2. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

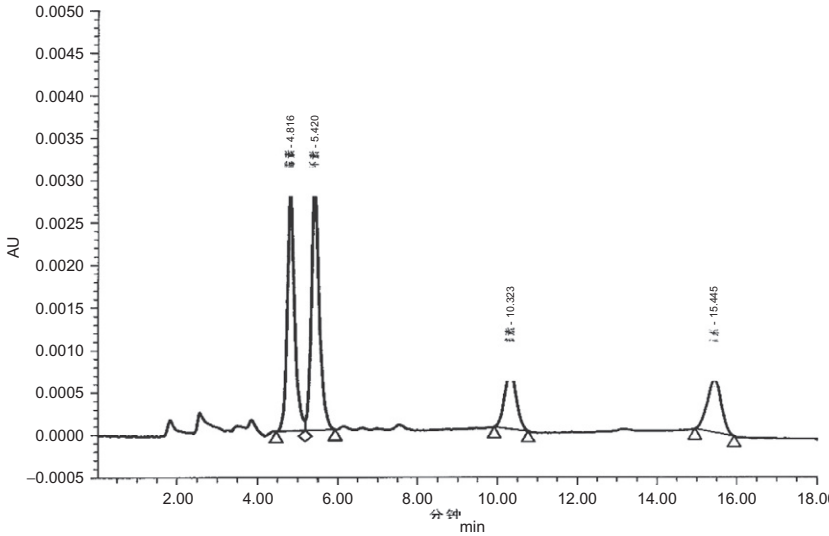


FIG. 12.2 Chromatograms of the mixed standard.

(1) Repeatability

Under repeatability conditions, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for tetracycline drugs in edible animal muscles are shown in Table 12.2.

TABLE 12.2 Range, Repeatability and Reproducibility

Analytes	Range (mg/kg)	Repeatability	Reproducibility
Oxytetracycline	0.005–0.100	$\lg r=0.8738\lg m-0.9302$	$\lg R=0.9322$ $\lg m-0.7613$
Tetracycline	0.005–0.100	$\lg r=0.8725\lg m-0.8862$	$\lg R=0.9504$ $\lg m-0.8435$
Chlortetracycline	0.005–0.100	$\lg r=0.9107\lg m-0.7725$	$\lg R=0.8911$ $\lg m-0.3712$
Doxycycline	0.005–0.100	$\lg r=0.9218\lg m-0.7395$	$\lg R=0.8761$ $\lg m-0.3350$

Note: m *is average value of parallel test results.*

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under reproducibility conditions, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for tetracycline drugs in edible animal muscles are shown in Table 12.2.

12.3.8 RECOVERY

Under optimized conditions, the fortifying concentrations of oxytetracycline, tetracycline, chlortetracycline, and doxycycline and the corresponding average recoveries of this method are listed in Table 12.3.

TABLE 12.3 The Fortifying Concentrations and Corresponding Recoveries

Analytes	Fortifying Concentrations (mg/kg)	Recovery (%)
Oxytetracycline	0.005	84.6
	0.010	95.3
	0.050	94.5
	0.100	96.8
Tetracycline	0.005	75.1
	0.010	75.6
	0.050	83.5
	0.100	89.3
Chlortetracycline	0.005	77.2
	0.010	77.0
	0.050	86.6
	0.100	90.8
Doxycycline	0.005	82.2
	0.010	82.3
	0.050	81.8
	0.100	84.9

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12.4

Determination of Oxytetracycline, Tetracycline, Chlortetracycline, and Doxycycline Residues in Milk and Milk Powder—HPLC-UV Method (GB/T 22990-2008)

12.4.1 SCOPE

This method is applicable to the determination of oxytetracycline, tetracycline, chlortetracycline, and doxycycline residues in milk and milk powder.

The limits of determination of this method in milk are 5 μg/kg for oxytetracycline and tetracycline, and 10 μg/kg for chlortetracycline and doxycycline; in milk powder, the limits are 25 μg/kg for oxytetracycline and tetracycline and 50 μg/kg for chlortetracycline and doxycycline.

12.4.2 PRINCIPLE

The TC residues in the test sample are extracted with 0.1 mol/L Na_2EDTA-McIlvaine buffer solution. The extracts are cleaned up with an Oasis HLB SPE cartridge and carboxylic acid cation exchange cartridge and then are determined by HPLC, using the external standard method.

12.4.3 REAGENTS AND MATERIALS

Unless specifically noted, all reagents used should be of analytical grade; "water" is deionized water.

Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$).
Citric acid monohydrate ($C_6H_8O_7{\cdot}H_2O$).
Ethylenediamine tetraacetic acid disodium salt ($Na_2EDTA{\cdot}2H_2O$).
Oxalic acid ditydrate ($C_2H_2O_4{\cdot}2H_2O$).
0.2 mol/L Disodium hydrogen phosphate solution: weight 71.63 g disodium hydrogen phosphate dodecahydrate, dissolved with water and diluted to 1000 mL.
0.1 mol/L citric acid solution: weight 21.04 g citric acid monohydrate (4.4), dissolved with water and diluted to 1000 mL.
Mcllvaine buffer solution: 625 mL 0.2 mol/L disodium hydrogen phosphate solution and 1000 mL 0.1 mol/L citric acid solution are mixed; adjusted pH = 4.0 ± 0.05 with NaOH or HCL.
0.1 mol/L Na_2EDTA-Mcllvaine buffer solution: weight 60.50 g ethylenediamine tetraacetic acid disodium put into 1625 mL Mcllvaine solution, dissolved and mixed.
0.01 mol/L Oxalic acid solution: weight 1.26 g oxalic acid ditydrate, dissolved with water and diluted to 1000 mL.
Methanol-water (5 + 95, V/V): mix 5 mL methanol and 95 mL water.
0.01 mol/L Oxalic acid-Acetonitrile solution (50 + 50, V/V): mix 50 mL 0.01 mol/L Oxalic acid solution and 50 mL acetonitrile.
Standards of oxytetracycline (CAS 2058-46-0), tetracycline (CAS 64-75-5), chlortetracycline (CAS 64-72-2), and doxycycline (CAS 24390-14-5): purity ≥96%.
0.1 mg/mL Stock standard solution: Accurately weigh 10 mg (accurate to 0.1 mg) of each standard in separate 100-mL volumetric flasks, dilute to volume with methanol, and mix. The concentration of the solutions is 0.1 mg/mL. The solutions should be stored at approximately −20°C.
Mixed standard working solution: According to the concentration required, dilute stock standard solution with mobile phase.
Oasis HLB SPE cartridge: 500 mg, 6 mL, conditioned with 5 mL methanol and 10 ml water before use; keep wet.
Carboxylic acid cation exchange cartridge: 500 mg, 6 ml, conditioned with 5 mL methanol before use; keep wet.

12.4.4 APPARATUS

High performance liquid chromatography-UV detection.
Electronic balance.

Vortex mini-shaker.
Centrifuge: max speed 5000 rpm.
SPE equipment.
Vacuum pump.
Nitrogen evaporator.
pH meter.
Scale tube: 10 mL.

12.4.5 SAMPLE PRETREATMENT

(1) Sample preparation

Obtain combined primary sample of milk or milk powder, mix, and label.

The test sample of milk should be stored at −18°C and protected from light. The test sample of milk powder should be stored at room temperature and should be airproof.

(2) Extraction

Weigh 10 g milk sample (accurate to 0.01 g) into a 50-mL centrifuge tube. Weigh 2 g milk powder (accurate to 0.01 g) into a 50-mL centrifuge tube. Add 20 mL 0.1 mol/L Na_2EDTA-Mcllvaine buffer solution, shake for 2 min with vortex minishaker, and then centrifuge 10 min at 10°C, 5000 rpm. Filter the extract into a new tube. Add another 20 mL buffer solution and extract once more.

(3) Clean-up

Let the extraction solution flow through an Oasis HLB cartridge. Then wash the cartridge with 5 mL methanol-water and dry 5 min in a vacuum. Elute with 5 mL methanol and collect the elute solution in a 10-mL scale tube.

Let the elute solution go through a carboxylic acid canion exchange cartridge and then wash the cartridge with 5 mL methanol. Dry 5 min in a vacuum. Elute with 4 mL 0.01 mol/L oxalic acid-acetonitrile solution; collect the elute solution in a 10-mL graduated tube. Blow about 1.5 mL under 45°C with nitrogen gas and dilute to 2 mL with mobile phase. Filter through a 0.45-μm membrane and then it is ready for HPLC determination.

12.4.6 DETERMINATION

(1) Operating conditions

Column: Kromasil 100-5C_{18}, 150 mm × 4.6 mm (i.d.), particle size 5 μm, or equivalent;
Mobile phase: 0.01 mol/L oxalic acid-acetonitrile-methanol (77 + 18 + 5, *V/V/V*);
Flow rate: 1.0 mL/min;
Column temperature: 40°C;
Detection wavelength: 350 nm;
Injection volume: 60 μL.

(2) HPLC determination

Inject mixed standard working solution separately; concentration is the abscissa and area is the ordinate. Create the standard curve and quantify the test sample solution with the standard curve. The responses of the analyte in the sample solution should be within the linear range of the instrument detection. Under these operating conditions, the reference retention times of oxytetracycline, tetracycline, chlortetracycline, and doxycycline are about 3.09 min, 3.73 min, 8.27 min, and 12.53 min respectively. For the chromatogram of the standard, see Fig. 12.3.

12.4.7 PRECISION

The precision data of this part are confirmed according to GB/T 6379.1 and GB/T 6379.2. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under repeatability conditions, the difference of absolute value of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equations of the four analytes in milk and milk powder are shown in Tables 12.4 and 12.5.

If the difference is above the repeatability limit (r), the results of the experiment should be abandoned and two independent experiments should be redone.

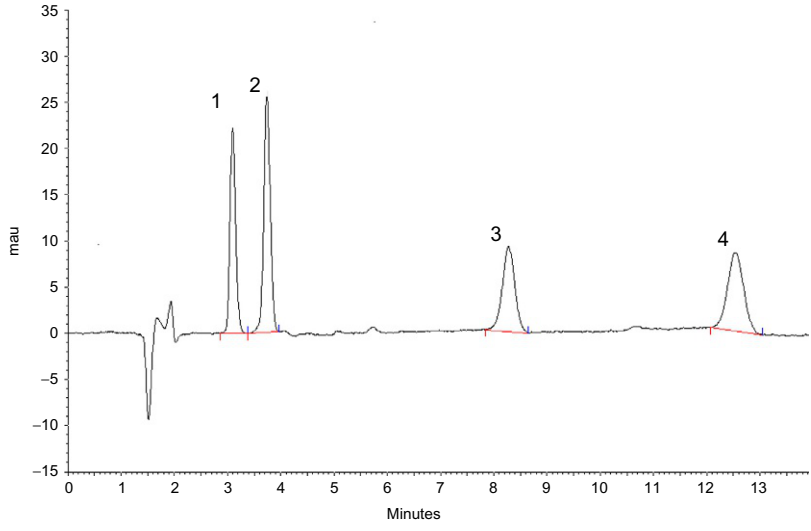

FIG. 12.3 Liquid chromatogram of oxytetracycline, tetracycline, chlortetracycline, and doxycycline.

TABLE 12.4 Content Ranges and the Repeatability and Reproducibility Equations for Milk Sample

Analyte	Content Ranges (μg/kg)	Repeatability Limit r	Reproducibility Limit R
Oxytetracycline	5–50	lg r=0.881 lg m−0.812	lg R=0.899 lg m−0.574
Tetracycline	5–50	lg r=1.06 lg m−1.00	lg R=1.00 lg m−0.612
Chlortetracycline	10–100	lg r=1.08 lg m−1.15	lg R=0.819 lg m−0.430
Doxycycline	10–100	lg r=0.963 lg m−0.903	lg R=0.838 lg m−0.465

Note: m *equals to the average of two results.*

TABLE 12.5 Content Ranges and the Repeatability and Reproducibility Equations for Milk Powder Sample

Analyte	Content Ranges (μg/kg)	Repeatability Limit r	Reproducibility Limit R
Oxytetracycline	25–250	lg r=0.872 lg m−0.791	lg R=0.834 lg m−0.500
Tetracycline	25–250	lg r=0.996 lg m−1.07	lg R=0.877 lg m−0.542
Chlortetracycline	50–500	lg r=0.997 lg m−1.12	lg R=1.12 lg m−1.04
Doxycycline	50–500	lg r=0.817 lg m−0.738	lg R=0.860 lg m−0.464

Note: m *equals to the average of two results.*

(2) Reproducibility

Under reproducibility conditions, the difference of absolute value of two independent results is not above the reproducibility limit (R). The content ranges and the reproducibility equations of the five analytes in milk and milk powder are shown in Tables 12.4 and 12.5.

For liquid chromatograms of oxytetracycline, tetracycline, chlortetracycline, and doxycycline, refer to Fig. 12.3.

TABLE 12.6 Fortifying Concentration and Recoveries of Oxytetracycline, Tetracycline, Chlortetracycline, and Doxycycline

	Milk		Milk Powder	
Analyte	Fortifying Concentration (μg/kg)	Recovery (%)	Fortifying Concentration (μg/kg)	Recovery (%)
Oxytetracycline	5	71.3–87.6	25	71.9–98.7
	10	81.2–94.6	50	76.5–90.0
	25	77.0–98.4	125	80.5–95.1
	50	80.1–93.8	250	81.4–99.2
Tetracycline	5	63.7–74.3	25	71.9–84.8
	10	71.3–84.6	50	70.3–89.5
	25	70.2–80.3	125	80.2–93.6
	50	70.0–86.3	250	81.8–92.9
Chlortetracycline	10	71.7–85.7	50	71.5–84.3
	20	70.6–88.3	100	81.4–90.8
	50	72.5–89.9	250	80.5–92.9
	100	80.3–90.9	500	82.8–90.7
Doxycycline	10	71.1–85.7	50	72.6–93.6
	20	70.8–90.5	100	80.2–89.1
	50	74.0–88.5	250	82.8–94.5
	100	82.1–94.8	500	81.4–90.7

The peaks of 1, 2, 3, 4 sequentially are oxytetracycline, tetracycline, chlortetracycline, and doxycycline.

For fortifying concentrations and recoveries of oxytetracycline, tetracycline, chlortetracycline, and doxycycline in this method, refer to Table 12.4.

12.4.8 RECOVERY

Under optimized condition, the recoveries of oxytetracycline, tetracycline, chlortetracycline, and doxycycline in milk and milk powder using this method are listed in Table 12.6.

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12.5

Determination of Oxytetracycline, Tetracycline, Chlortetracycline, and Doxycycline Residues in Fugu and Eel—HPLC-UV Method (GB/T 22961-2008)

12.5.1 SCOPE

This method is applicable to the determination of oxytetracycline, tetracycline, chlortrtracycline, and doxycycline residues in fugu and eel.

The limit of determination of this method for oxytetracycline, tetracycline, chlortetracycline, and doxycycline is 0.010 mg/kg.

12.5.2 PRINCIPLE

The drugs are extracted from edible fugu and eel with 0.1 mol/L Na_2EDTA–McIlvaine buffer ($pH = 4.0 \pm 0.05$). The edible animal muscle solution is centrifugalized, and the supernatant is cleaned up by Oasis HLB and Anion exchange columns. Determine the residues by HPLC with a UV detector at 350 nm. Calculate the results by comparing peak height of the sample with the corresponding standard peak height.

12.5.3 REAGENTS AND MATERIALS

Water: GB/T6682, First-level.
Methanol: HPLC grade.
Acetonitrile: HPLC grade.
Ethyl acetate: HPLC grade.
n-Hexane: HPLC grade.
Citric acid: G.R.
Disodium hydrogen phosphate: G.R.

Disodium ethylenediamine tetraacetate: $Na_2EDTA{\cdot}2H_2O$.
Oxalic acid: G.R.
Formic acid: G.R.
Citric acid solution: 0.1 mol/L. Place 21.01 g citric acid into 1000-mL volumetric flask and dissolve in H_2O. Dilute solution to volume with H_2O.
Disodium hydrogen phosphate solution: 0.2 mol/L. Place 28.41 g disodium hydrogen phosphate into 1000-mL volumetric flask and dissolve in H_2O. Dilute solution to volume with H_2O.
Mcllvaine buffer: Combine 1000 mL citric acid solution with 625 mL disodium phosphate solution in 2000-mL Erlenmeyer flask. Adjust pH to 4.0 ± 0.05 by adding dropwise either 0.1 mol/L HCl or 0.1 mol/L NaOH if required.
Mcllvaine buffer–EDTA solution: 0.1 mol/L. Place 60.5 g disodium EDTA dihydrate to 1625 mL Mcllvaine buffer and mix until solid dissolves.
0.01 mol/L Oxalic acid solution: Place 2.10 g citric acid into 1000-mL volumetric flask and dissolve in H_2O. Dilute solution to volume with H_2O.
2% Formic acid solution: Mix 10 mL formic acid and 490 mL water.
5% Methanol-water solution: Mix 5 mL methanol and 95 mL water.
Eluate: Acetonitrile-methanol-0.01 mol/L oxalic acid solution (2+1+7, v/v/v).
Oxytetracycline (CAS 2058-46-0), tetracycline (CAS 64-75-5), chlortetracycline (CAS 64-72-2), doxycycline (CAS 24390-14-5) standards: purity $\geq$95%.
Standard stock solutions of oxytetracycline, tetracycline, chlortetracycline, and doxycycline: 0.1 mg/mL. Accurately weigh appropriate amount of oxytetracycline, tetracycline, chlortetracycline, and doxycycline standards. Dissolve in a small volume of methanol and then dilute with methanol separately to prepare the standard stock solutions of 0.100 mg/mL in concentration. Keep at $-18°C$.
6.0 μg/mL mixed standards solutions: Respectively draw 0.6 mL standard stock solutions into 10-mL volumetric flask and dilute 6.0 μg/mL standard mixed solution with methanol. Keep at $-18°C$.
Mixed standard working solutions: According to the requirement, prepare the mixed standard working solution of 5, 10, 50, 100, and 200 ng/mL; the mixed standard working solution is prepared fresh daily.
Oasis HLB solid extraction columns: 500 mg, 6 mL. Condition each column with 5 mL methanol followed by 10 mL water before use.
Anion exchange column: carboxylic acid, 500 mg, 3 mL. Condition each column with 5 mL ethyl acetate before use.
Filter diaphragm: 0.2 μm.

12.5.4 APPARATUS

UPLC: Equipped with TUV Detector.
Analytical balance: capable of weighing to 0.1 mg, 0.01 g.
Vortex mixer.

Solid phase extraction vacuum apparatus.
Reservoirs and adapters to fit SPE columns: 50 mL.
High speed refrigerator centrifuger: maximum speed 13,000 rpm.
Sample tube: 5 mL, accurate to 0.1 mL.
Vacuum pump: Vacuum should attain 80 kPa.
Shaker.
PTFE centrifuge tube: 50 mL.
pH meter: Capable of measuring ±0.02 unit.

12.5.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

The representative sample, ca. 1 kg, is taken from the whole sample. It is vigorously stirred, thoroughly mixed, and divided into two equal parts and then put into a clean container, which is sealed and labeled, respectively. In the course of sampling and sample preparation, precautions should be taken to avoid contamination or any factors that might cause a change of residue content.

The test samples should be stored below −18°C.

(2) Extraction

Weigh 6-g test sample (accurate to 0.01 g) into a 50-mL PTFE centrifuge tube with plug. Add 20 mL 0.1 mol/L Na_2EDTA–Mcllvaine buffer solution (pH = 4) to each sample. Mix vigorously 1 min on vortex mixer. Shake 10 min on shaker and then centrifugalize the sample solution at 10,000 rpm for 10 min. Decant the supernatant into another centrifuge tube. Add 15 mL Na_2EDTA–Mcllvaine buffer solution into the dregs, repeat the extraction, and combine the supernatants. To the eel sample extracts add 20 mL n-hexane and shake 5 min on a shaker. Centrifugalize the sample solution at 4000 rpm for 5 min. Discard the n-hexane and use the aqueous phase part for clean-up.

(3) Clean-up

Connect a reservoir to a cartridge. Decant the supernatant into the reservoir and dilute it through the Oasis HLB cartridge at up to 3 mL/min, using a vacuum if required. Rinse the reservoir and cartridge with 5 mL methanol-water solution. Discard all eluates from this point. Dry the cartridge by drawing air through it for 40 min, using a 65-kPa vacuum. Elute the TCs from the cartridge with 15 mL ethyl acetate through the anion exchange cartridge at up to 3 mL/min, using a vacuum. Rinse the reservoir and cartridge with 5 mL methanol. Discard all eluates from this point. Dry the cartridge by drawing air through it for 5 min, using a 65-kPa vacuum. Place a 5-mL sample tube in the manifold. Elute the TCs from the anion exchange cartridge with eluate; dilute to 4 mL. The solution is ready for determination by UPLC equipped with UV detector.

TABLE 12.7 Gradient Elution Condition

Time (min)	Flow (mL/min)	A (%)	B (%)
0.00	0.4	0	100
0.50	0.4	10	90
1.50	0.4	30	70
2.50	0.4	74	26
2.51	0.4	0	100
4.00	0.4	0	100

12.5.6 DETERMINATION

(1) LC operation conditions
Chromatographic column: BEH C18, 1.7 μm, 50 mm × 2.1 mm (i.d.), or equivalent.
Flow: 0.5 mL/min.
Column temperature: 25°C.
Wavelength: 350 nm.
Sample size: 60 μL.
Mobile phase: A: acetonitrile, B: 2% Formic acid solution; for gradient elution condition, see Table 12.7.

(2) Determination
Inject the mixed standard working solutions of different concentrations of four TCs, respectively; concentration is the *X*-axis and peak area is the *Y*-axis. Draw the working curve. Calculate the concentrations of the corresponding content from the working curve. The responses of the TCs in the standard working solution and sample solution should be in the linear range of the instrumental detection. The standard working solution should be injected in between the injections of the sample solution of equal volume, under the preceding LC conditions. The reference retention times of the TCs are in Table 12.8.

12.5.7 PRECISION

The precision data of the method for this standard have been determined in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability
Under the conditions of repeatability, the difference of the absolute values obtained from two independent determination results shall

TABLE 12.8 The Reference Retention Times of Oxytetracycline, Tetracycline, Chlortetracycline, and Doxycycline

Drug	Retention Time (min)
Oxytetracycline	1.25
Tetracycline	1.35
Chlortetracycline	1.67
Doxycycline	1.77

TABLE 12.9 Range, Repeatability and Reproducibility (Unit: mg/kg)

Drug	Fortifying Concentration	Repeatability	Reproducibility
Oxytetracycline	0.010–0.100	lg r=0.7662 lg m−0.9133	lg R=0.7471 lg m−0.5893
Tetracycline	0.010–0.100	lg r=0.8236 lg m−1.0007	lg R=0.7912 lg m−0.6623
Chlortetracycline	0.010–0.100	lg r=0.6573 lg m−0.7361	lg R=0.6480 lg m−0.4106
Doxycycline	0.010–0.100	lg r=1.1171 lg m−1.5142	lg R=1.0096 lg m−1.0132

Note: m *is average value of parallel test results.*

not exceed the limit of repeatability (r); the content range and repeatability equations for tetracycline drugs in fugu and eel are shown in Table 12.9.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the conditions of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for tetracycline drugs in fugu and eel are shown in Table 12.9.

TABLE 12.10 Fortifying Concentration and Recoveries of Oxytetracycline, Tetracycline, Chlortetracycline, and Doxycycline

Analyte	Fortifying concentration (mg/kg)	Recovery (%)
Oxytetracycline	0.010	81.6
	0.020	82.7
	0.050	81.6
	0.100	85.0
Tetracycline	0.010	89.0
	0.020	84.9
	0.050	85.8
	0.100	81.6
Chlortetracycline	0.010	73.2
	0.020	79.8
	0.050	76.1
	0.100	66.4
Doxycycline	0.010	67.0
	0.020	69.0
	0.050	68.7
	0.100	65.1

12.5.8 RECOVERY

Under optimized condition, the recoveries of oxytetracycline, tetracycline, chlortetracycline, and doxycycline in fugu using this method are listed in Table 12.10.

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12.6

Determination of Oxytetracycline, Tetracycline, Chlortetracycline, and Doxycycline Residues in Honey—LC-UV Method (GB/T 18932.4-2002)

12.6.1 SCOPE

This method is applicable to the determination of oxytetracycline, tetracycline, chlortetracycline, and doxycycline residues in honey.

The limit of determination of this method for oxytetracycline, tetracycline, chlortetracycline, and doxycycline is 0.010 mg/kg.

12.6.2 PRINCIPLE

The drugs are extracted from the honey with 0.1 mol/L Na_2EDTA–Mcllvaine buffer ($pH = 4.0 \pm 0.05$). The honey solution is centrifuged, and the supernatant is cleaned up by Oasis HLB and Anion exchange columns. The residues are analyzed by HPLC with UV detection at 350 nm. Calculate the results by comparing peak height of the sample with the corresponding standard peak height.

12.6.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents used should be reagent grade; "water" is deionized water.

Methanol, acetonitrile: HPLC grade; ethyl acetate: restilled; disodium hydrogen phosphate: G.R.; Citric acid; Disodium ethylenediamine tetraacetate: $Na_2EDTA{\cdot}2H_2O$; Oxalic acid; Methanol-water solution: 5 + 95. Mix 5 mL methanol and 95 mL water.

Disodium hydrogen phosphate solution: 0.2 M. Place 28.41 g disodium hydrogen phosphate into 1000-mL volumetric flask and dissolve in H_2O. Dilute solution to volume with H_2O.

Citric acid solution: 0.1 M. Place 21.01 g citric acid into a 1000-mL volumetric flask and dissolve in H_2O. Dilute solution to volume with H_2O.

Mcllvaine buffer: Combine 1000 mL citric acid solution with 625 mL disodium phosphate solution in a 2000-mL Erlenmeyer flask. Adjust pH to 4.0 ± 0.05 by adding dropwise either 0.1 mol/L HCl or 0.1 mol/L NaOH if required.

Mcllvaine buffer–EDTA solution: 0.1 M. Place 60.5 g disodium EDTA dihydrate to 1625 mL Mcllvaine buffer and mix until solid dissolves.
Oasis HLB solid extraction column: 500 mg, 6 mL. Condition each column with 5 mL methanol followed by 10 mL water before use; Cation exchange column: carboxylic acid, 500 mg, 3 mL. Condition each column with 10 mL ethyl acetate.
Oxytetracycline, tetracycline, chlortetracycline, doxycycline standards: purity $\geq$95%.
Standard solutions of oxytetracycline, tetracycline, chlortetracycline, and doxycycline: 0.1 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the standard stock solutions of 0.100 mg/mL in concentration. Kept at -18°C. According to the requirement, prepare the mixed standard working solution of appropriate concentrations. Mixed standard solutions are stored at 4°C and can be used for 3 days.

12.6.4 APPARATUS

Liquid chromatograph: Equipped with UV detector; Analytical balance: capable of weighing to 0.1 mg, 0.01 g; Vortex mixer; SPE vacuum apparatus; Reservoirs and adapters to fit SPE columns: 50 mL; Microsyringes: 25 μL, 100 μL; Sample tube: 5 mL, accurate to 0.1 mL; Vacuum pump; Centrifuge tubes: 50 mL; Flat-flasks: 100 mL; pH meter: Capable of measuring $\pm$0.02 unit.

12.6.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath and warm at $\leq$60°C with occasional shaking until liquefied. Mix thoroughly and promptly cool it to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label. The test samples should be stored at 20°C.

(2) Extraction

Weigh 6-g test sample (accurate to 0.01 g) into 150-mL Erlenmeyer flask. Add 30 mL 0.1 mol/L Mcllvaine buffer–EDTA solution to each sample. Mix vigorously 1 min on vortex mixer until honey is completely dissolved. Then transfer it to a 50-mL centrifuge tube, and the honey solution is centrifugalized at 1500 $\times$ g for 5 min.

(3) Clean-up

Connect a reservoir to a cartridge. Decant the supernatant into the reservoir and dilute it through the cartridge at up to 3 mL/min, using a vacuum if required. Rinse reservoir and cartridge with 5 mL methanol-water solution. Discard all eluates from this point. Dry cartridge by drawing air through it

for 20 min, using a 65-kPa Vacuum. Place a 100-mL flat-flask in manifold. Elute the TCs from the cartridge with 15 mL ethyl acetate, using gravity only.

Put the ethyl acetate through the cation exchange cartridge at up to 3 mL/min, using a vacuum. Rinse reservoir and cartridge with 5 mL methanol. Discard all eluates from this point. Dry cartridge by drawing air through it for 5 min, using 65-kPa vacuum. Place a 5-mL sample tube in manifold. Elute the TCs from the cation exchange cartridge with mobile phase and dilute to 4 mL. The solution is ready for HPLC determination.

12.6.6 DETERMINATION

(1) Operation conditions

Chromalographic column: μBondapakC_{18}, 10 μm, 300 mm × 3.9 mm, or equivalent; Column temperature: 25°C; Mobile phase: acetonitrile-methanol-oxalic acid (20+10+70); Flow rate of mobile phase: 1.5 mL/min; Wavelength: 350 nm; sample size: 100 μL.

(2) LC determination

According to the appropriate concentrations of the TCs in the sample solution, select the corresponding mixed standard working solution with similar peak heights to that of the sample solution. The responses of the TCs in the standard working solution and sample solution should be in the linear range of the instrumental detection. The standard working solution should be injected in between the injections of the sample solution of equal volume. Under these LC conditions, the reference retention times of oxytetracycline, tetracycline, chlortetracycline, and doxycycline are 4.0, 4.8, 9.6, and 14.0 min, and the chromatogram is shown in Fig. 12.4.

12.6.7 PRECISION

The precision data of the method for this standard have been determined from the four fortification samples tested by 13 laboratories in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at a 95% confidence level.

(1) Repeatability

Under the conditions of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for tetracycline drugs in honey are shown in Table 12.11.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the conditions of reproducibility, the difference of the absolute values obtained from two independent determination results shall not

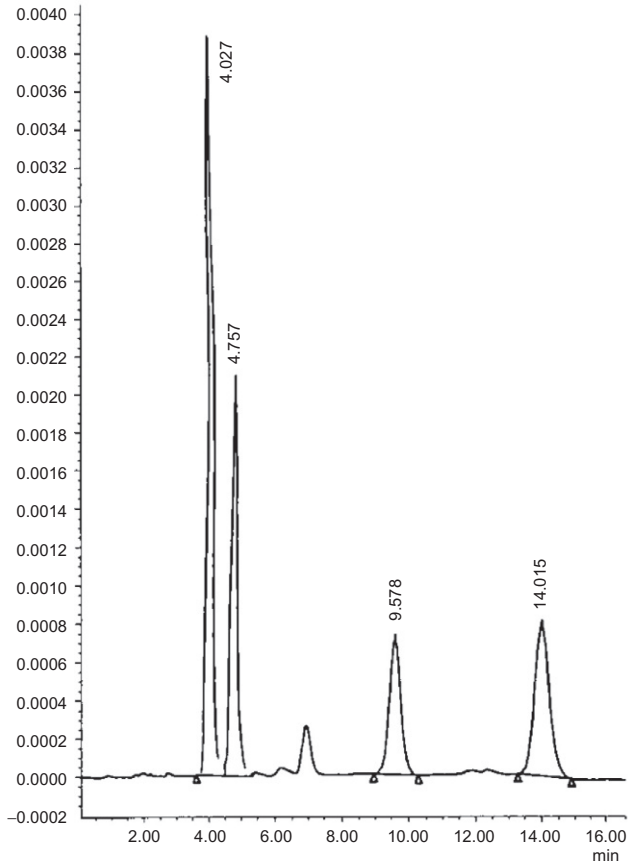

FIG. 12.4 Chromatogram of the TCs.

TABLE 12.11 Range, Repeatability, and Reproducibility

Analytes	Range (mg/kg)	Repeatability	Reproducibility
Oxytetracycline	0.010–0.20	lg r=0.8520 lg m−1.5307	lg R=0.9393 lg m−0.9810
Tetracycline	0.010–0.20	lg r=0.5242 lg m−1.9931	lg R=0.8693 lg m−0.8950
Chlortetracycline	0.010–0.20	r=0.0267m−0.0012	lg R=0.7271 lg m−1.1455
Doxycycline	0.010–0.20	r=0.0236m−0.0035	lg R=0.6227 lg m−1.2716

Note: *The* m *is average value of parallel test results.*

TABLE 12.12 Recoveries of TCs in Honey

Analytes	Fortifying Concentration (mg/kg)	Recovery (%)
Oxytetracycline	0.010	87.8
	0.050	93.0
	0.50	99.5
Tetracycline	0.010	89.0
	0.050	87.2
	0.50	93.3
Chlortetracycline	0.010	84.6
	0.050	80.9
	0.50	87.5
Doxycycline	0.010	97.1
	0.050	85.6
	0.50	85.3

exceed the limit of reproducibility (R); the content range and reproducibility equations for tetracycline drugs in honey are shown in Table 12.11.

12.6.8 RECOVERY

Under optimized conditions, the recoveries of TCs in honey using this method are listed in Table 12.12.

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FURTHER READING

[1] Gilliam M, Argauer RJ. Honeybee Sci 1983;3:55.

[2] MacNeil JD, Martz VK, Korsrud GO, Salisbury CDC, Oka H, Epstein RL, et al. J AOAC 1996;79:405.

12.7

Determination of Oxytetracycline, Tetracycline, Chlortetracycline, and Doxycycline Residues in Honey—LC-MS-MS Method (GB/T 18932.23-2003)

12.7.1 SCOPE

This method is applicable to the determination of oxytetracycline, tetracycline, chlortetracycline, and doxycycline residues in honey.

The limit of determination of liquid chromatography-tandem mass spectrometry (LC-MS-MS) for oxytetracycline and tetracycline is 0.001 mg/kg; for chlortetracycline and doxycycline it is 0.002 mg/kg.

12.7.2 PRINCIPLES

The drugs are extracted from honey with 0.1 mol/L Na_2EDTA–Mcllvaine buffer (pH = 4.0 ± 0.05). The honey solution is centrifuged, and the supernatant is cleaned up by Oasis HLB and Anion exchange columns. The residues are analyzed by LC-MS-MS using an external standard.

12.7.3 REAGENTS AND MATERIALS

Methanol, acetonitrile: HPLC grade; Ethyl acetate: restilled; Disodium hydrogen phosphate: G.R.; Citric acid; Disodium ethylenediamine tetraacetate: $Na_2EDTA{\cdot}2H_2O$; Oxalic acid; Disodium hydrogen phosphate solution: 0.2 M; Citric acid solution: 0.1 mol/L; Mcllvaine buffer: Combine 1000 mL citric acid solution with 625 mL disodium phosphate solution in 2000-mL Erlenmeyer flask. Adjust pH to 4.0 ± 0.05 by adding dropwise either 0.1 M HCl or 0.1 mol/L NaOH if required; Mcllvaine buffer–EDTA solution: 0.1 M: Place 60.5 g disodium EDTA dihydrate to 1625 mL Mcllvaine buffer and mix until solid dissolves; methanol-water solution: 5 + 95; Oasis HLB solid extraction columns: 500 mg, 6 mL; Condition each column with 5 mL methanol followed by 10 mL water before use; Anion exchange column: carboxylic acid, 500 mg, 3 mL. Condition each column with 5 mL methanol followed by 10 mL water before use;

Oxytetracycline, tetracycline, chlortetracycline, doxycycline standards: purity ≥95%.

Standard solutions of oxytetracycline, tetracycline, chlortetracycline, and doxycycline: 0.1 M. Accurately weigh appropriate amount of each drug

standard, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the standard stock solutions of 0.100 mg/mL in concentration. Keep at −18°C.

According to the requirement, prepare the mixed standard working solution of appropriate concentrations. Mixed standard solutions are stored at 4°C and can be used for 3 days.

12.7.4 APPARATUS

LC-MS-MS: Equipped with ESI source; Analytical balance: Capable of weighing to 0.1 mg, 0.01 g; Vortex mixer; SPE vacuum apparatus; Reservoirs and adapters to fit SPE columns: 50 mL; Microsyringes: 25 μL, 100 μL; Sample tube: 5 mL, accurate to 0.1 mL; Vacuum pump: Vacuum to 80 kPa; Centrifuge tubes: 50 mL; Flat-flasks: 100 mL; pH Meter: Capable of measuring ±0.02 unit.

12.7.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath and warm at ≤60°C with occasional shaking until liquefied. Mix thoroughly and promptly cool to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label. The test samples should be stored at ambient temperature.

(2) Extraction

Weigh 6-g test sample (accurate to 0.01 g) into 150-mL Erlenmeyer flask. Add 30 mL 0.1 mol/L McIlvaine buffer–EDTA solution to each sample. Mix vigorously 1 min on vortex mixer until honey is completely dissolved. Then transfer it to a 50-mL centrifuge tube, and centrifuge the honey solution at 3000 rpm for 5 min.

(3) Clean-up

Connect a reservoir to a cartridge. Decant the supernatant into the reservoir and dilute it through the cartridge at up to 3 mL/min, using vacuum if required. Rinse reservoir and cartridge with 5 mL methanol-water solution. Discard all eluates from this point. Dry cartridge by drawing air through it for 20 min, using 65-kPa Vacuum. Place a 100-mL flat-flask in manifold. Elute the TCs from the cartridge with 15 mL ethyl acetate, using gravity only.

Let ethyl acetate pass through the anion exchange cartridge at up to 3 mL/min, using a vacuum. Rinse the reservoir and cartridge with 5 mL methanol. Discard all eluates from this point. Dry cartridge by drawing air through it for 5 min, using a 65-kPa vacuum. Place a 5-mL sample tube

in manifold. Elute the TCs from the anion exchange cartridge with mobile phase, redissolve to 4 mL, and analyze by LC-MS-MS.

12.7.6 DETERMINATION

(1) Operating conditions

LC column: Inertsil C_{18} 3.5 μm, 150 mm × 2.1 mm (i.d), or equivalent; Mobile phase: acetonitrile-methanol 0.4% formic acid solution (18+4+78); Flow rate: 0.2 mL/min; Column temperature: 25°C; Injection volumes: 20 μL. Ion source: ESI source; Scan mode: Positive scan; Monitor mode: Multiple reaction monitor; Ionspray voltage: 5500 V; Nebulizer gas: 0.055 MPa; Curtain gas: 0.079 MPa; Turbo ionspray gas rate: 6 L/min; Source temperature: 430°C; Declustering potential: 55 V; Precursor/product ion combinations, collision energy: see Table 12.13.

(2) LC-MS-MS analysis

The standard working solutions of different concentrations for oxytetracycline, tetracycline, chlortetracycline, and doxycycline are

TABLE 12.13 Precursor/Product ion Combinations, Declustering Potential, Collision Energy of Oxytetracycline, Tetracycline, Chlortetracycline, and Doxycycline

Analytes	Qualifying Parent/Production (*m*/*z*)	Quantifying Parent/Production (*m*/*z*)	Collision Energy (V)
Oxytetracycline	461/426	461/426	29
	461/443		20
	461/381		35
Tetracycline	445/410	445/410	28
	445/154		40
	445/428		20
Chlortetracycline	479/444	479/444	32
	479/154		40
	479/462		27
Doxycycline	445/428	445/428	27
	445/410		27
	445/154		40

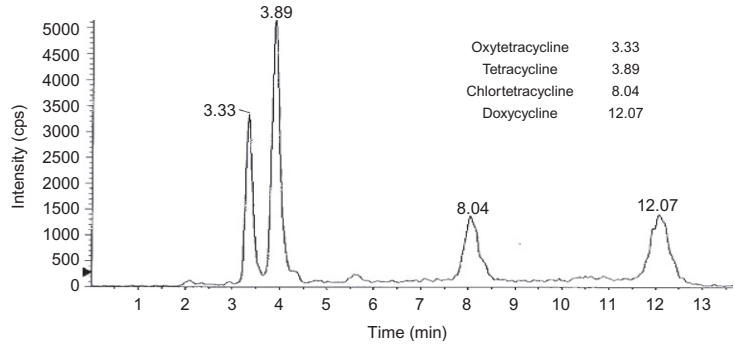

FIG. 12.5 Total ion chromatogram of oxytetracycline, tetracycline, chlortetracycline, and doxycycline standard.

prepared with honey control sample extract; then 20 μL of the different concentration working standard solutions are injected, respectively, in duplication under LC and MS conditions. Draw the standard curves of oxytetracycline, tetracycline, chlortetracycline, and doxycycline (peak area vs. sulfonamide concentration). Calculate the concentrations of the corresponding content from the working curve. The responses of oxytetracycline, tetracycline, chlortetracycline, and doxycycline in the standard working solution and sample solution should be in the linear range of the instrumental detection. For total ion chromatogram and mass spectrum of the standard working solution of oxytetracycline, tetracycline, chlortetracycline, and doxycycline, see Fig. 12.5. Under the LC and MS conditions, the reference retention times of the oxytetracycline, tetracycline, chlortetracycline, and doxycycline are listed in Table 12.14.

TABLE 12.14 Reference Retention Times of Oxytetracycline, Tetracycline, Chlortetracycline, and Doxycycline

Analytes	Reference Retention Times (min)	Analytes	Reference Retention Times (min)
Oxytetracycline	3.33	Chlortetracycline	8.04
Tetracycline	3.89	Doxycycline	12.07

TABLE 12.15 Analytical Range, Repeatability, and Reproducibility

Analytes	Analytical Range (mg/kg)	Repeatability (r)	Reproducibility (R)
Oxytetracycline	0.002–0.100	lg r=0.966 8 lg m−0.868 6	Lg R=1.009 6 lg m−0.659 7
Tetracycline	0.002–0.100	lg r=0.948 4 lg m−0.837 0	Lg R=1.161 1 lg m−0.231 7
Chlortetracycline	0.002–0.100	lg r=0.839 5 lg m−1.087 2	Lg R=0.906 0 lg m−0.813 6
Doxycycline	0.002–0.100	lg r=0.920 9 lg m−0.846 2	Lg R=0.989 9 lg m−0.650 4

Note: m—*the average values obtained from two independent determination results.*

12.7.7 PRECISION

The precision data of the method for this standard have been determined in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at a 95% confidence level.

(1) Repeatability

Under the conditions of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r). For repeatability and content range of this method, see Table 12.15.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); for reproducibility and content range of this method, see Table 12.15.

If the difference of values exceeds the limit of reproducibility, the testing results should be discarded and two individual testing determinations shall be reconducted and completed.

12.7.8 RECOVERY

Under optimized conditions, the recoveries of TCs in honey using this method are listed in Table 12.16.

TABLE 12.16 The Recoveries of TCs in Honey

Analytes	Fortifying Concentration (mg/kg)	Average Recovery (%)
Oxytetracycline	0.002	88
	0.01	95.3
	0.05	93.6
	0.1	95.8
Tetracycline	0.002	81.9
	0.01	82.6
	0.05	84.5
	0.1	89.3
Chlortetracycline	0.002	87.2
	0.01	86
	0.05	86.6
	0.1	90.8
Doxycycline	0.002	85.2
	0.01	85.3
	0.05	86.8
	0.1	87.9

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[2] MacNeil JD, Martz VK, Korsrud GO, Salisbury CDC, Oka H, Epstein RL, et al. J AOAC 1996;79:405.

[3] Official Method of Analysis of AOAC International 17th Edition (2000) Appendix D, Appendix E.

Chapter 13

Sedative

13.1

Curative Effects and Side Effects of Tranquilizers

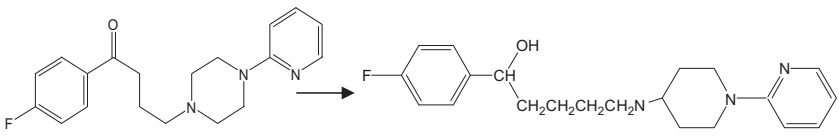
Acetopromaizine, chlorpromazine, haloperidol, propionylpromazine, xylazine, azaperone, azaperol, and carazolol residues are neuroleptic tranquilizers belonging to the class of butyrophenones. These drugs are considered to be particularly effective in the management of hyperactivity, agitation, and mania, and are also administered to animals for sedation prior to being transported to the market. Stress in animals is known to reduce meat quality and pigs, in particular, easily become stressed during transport. The presence of residues of these drugs in edible tissues (e.g., kidney or liver) represents a potential consumer risk given their bioactivity. These drugs may cause low blood pressure, liver failure, anxiety, restlessness, depression, confusion, dizziness, and so on. This fact has been recognized by the European Union (EU) and these compounds have been included among those that should be analyzed in meat-producing animals and their products.

13.2

Pharmacokinetics of Azaperone

Metabolism of azaperone in microbial organisms leads primarily to the production of its reduced metabolite azaperol (Fig. 13.1). Azaperone displays a potency that is 4–30 times the biological potency of azaperol in mice. Residues of azaperone are evaluated as the sum of azaperone and azaperol, as the activity of azaperol may be associated with its ready interconversion with azaperone.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00013-0



Azaperone Azaperol

FIG. 13.1 Metabolism of azaperon.

13.3

Chemical Structures and Maximum Residue Limit of Tranquilizers

Table 13.1 shows chemical structures and maximum residue limit of tranquilizers.

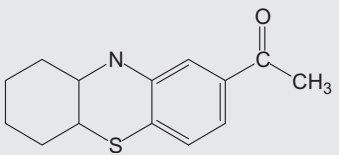
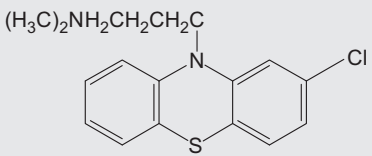
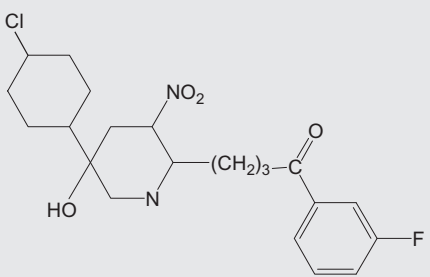
13.4

Determination of Acetopromaizine, Chlorpromazine, Haloperidol, Propionylpromazine, Xylazine, Azaperone, Azaperol, and Carazolol Residues in Porcine Kidney and Muscle Tissues—LC-MS-MS Method (GB/T 20763-2006)

13.4.1 SCOPE

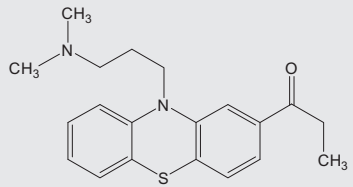
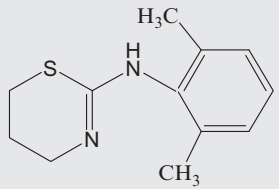
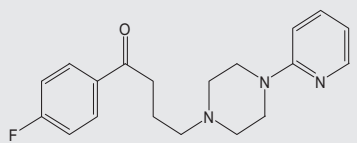
This method is applicable to the determination of acetopromaizine, chlorpromazin, haloperidol, propionylpromazine, xylazine, azaperone, azaperol, and carazolol in porcine kidney and muscle tissues.

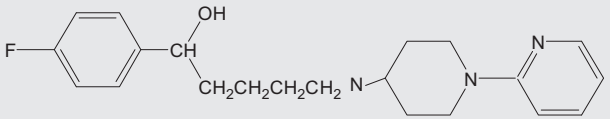
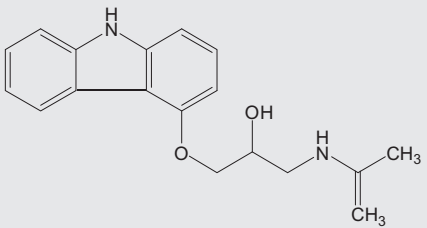
TABLE 13.1 Chemical Structures and Maximum Residue Limits of Tranquilizers

Compound Names	Structure	Molecular Weight	Cas. No.	MRL (μg/kg)
Acetopromaizine		326.46	61-00-7	–
Chlorpromazin		318.86	50-53-3	–
Haloperidol		375.87	52-86-8	–

Continued

TABLE 13.1 Chemical Structures and Maximum Residue Limits of Tranquilizers—cont'd

Compound Names	Structure	Molecular Weight	Cas. No.	MRL (μg/kg)
Propionylpromazine		340.55	3568-24-9	–
Xylazine		220.33	7361-61-7	JP:20
Azaperone		327.40	1649-18-9	EU: 100 (liver, muscle, kidney, fat of pork) CN: 100 (liver, kidney of pork), 60 (muscle and fat of pork) Codex: 100 (liver, kidney of pork), 60 (muscle and fat of pork)

Azaperol		329.30	2804-05-9	–
Carazolol		298.38	57775-29-8	EU: 15 (liver and kidney of cattle), 5 (muscle, fat of cattle and pork), 1 (milk), 25 (liver and kidney of pork)

The limit of determination of the method of acetopromaizine, carazolol, propionylpromazine, chlorpromazin is 0.50 μg/kg; xylazine is 0.25 μg/kg; azaperone is 0.20 μg/kg; azaperol is 0.15 μg/kg; haloperidol is 0.1 μg/kg.

13.4.2 PRINCIPLE

The tranquilizer residues in the test sample are made basic with aqueous NaOH and extracted with *tert*-butyl methyl ether (TBME). A pH 3 phosphate buffer is added to the extract and cleaned up with TBME. The remaining buffer is made basic with aqueous NaOH and the analytes are quantitatively back-exacted from the aqueous buffer into TBME. Concentrate and bring to volume of the volumetric flask. The solution is analyzed by LC-MS-MS, using an external standard method.

13.4.3 REAGENTS AND MATERIALS

Methanol: HPLC grade; Acetonitrile: HPLC grade; Absolute ethanol: HPLC grade; Hydrochloric acid: G.R.; Methyl-butyl ether; Sodium hydroxide; Sodium hydroxide: 5 M; Potassium dihydrogen phosphate monobasic, KH_2PO_4:G.R.; 1 M Potassium dihydrogen phosphate monobasic solution, pH = 3; Ammonium formate; Formic acid: HPLC grade; 0.1 M Ammonium formate buffer, pH = 4; 0.01 M Ammonium formate buffer, pH = 4; Mobile phase A: 0.01 M ammonium formate buffer solution, pH = 4. Filter the solution prepared in step under vacuum through a 0.2-μm filter; Mobile phase B: Acetonitrile. Filter acetronitrile under vacuum through a 0.2-μm filter; Mobile phase C: Methanol. Filter methanol under vacuum through a 0.2-μm filter; Composite Mobile Phase: Combine 70 mL of mobile phase A, 15 mL of mobile phase B, 15 mL of mobile phase C.

Reference standards: Acetopromaizine Maleate: Purity ≥99%; Azaperone: Purity, 99%; Carazolol: Purity, 99%; Chlorpromazine•HCl: Purity, 99%; Haloperidol: Purity ≥99%; Propionylpromazine•HCl: Purity, 98 %; Xylazine•HCl:Purity, 99%; Azaperol: 10 ng/μL (Methanol), purity, 96.5%.

Standard stock solutions of acetopromaizine, chlorpromazin, haloperidol, propionylpromazine, xylazine, azaperone, azaperol, and carazolol: 100 μg/mL. Accurately weigh adequate amount of acetopromaizine, chlorpromazin, haloperidol, propionylpromazine, xylazine, azaperone, azaperol, and carazolol standards. Separately prepare stock standard solutions of 100 μg/mL with absolute ethanol. The solutions should be stored at 2–4°C in the dark Prepare annually.

Standard stock solution of azaperol: 1 μg/mL. Store at 2–4°C in the dark, Prepare annually.

Mixed standard stock solution: 1 μg/mL. Prepare by diluting appropriate volume of each of the stock solution in step 4.19.1 into a volumetric flask with acetonitrile. Mix thoroughly and store at 2–4°C in the dark. Prepare every 6 months.

Mixed standard working solutions: Prepare the mixed working standard solution in different concentrations with blank tissue extracts as various sensitivity and linear range of each reference standard. Store at 2–4°C in the dark. The solutions must be freshly prepared.

13.4.4 APPARATUS

LC-MS-MS: Equipped with ESI source; Balances: Electronic, 0.1 g sensitivity, and 0.1 mg sensitivity; Centrifuge: Benchtop with 15- and 50-mL tube carriers, capable of 10,000 rpm; Centrifuge tubes: Conical-bottom, 15-mL glass with plug; conical-bottom, 10-mL glass with plug; conical bottom, 50 mL polypropylene with screw cap; Filters:Polytetrafluoroethylene membrane filter (0.2 μm × 13 mm) and nylon membrane filter (0.2 μm × 47 mm); Liquid dispensers: Adjustable, 1–10 mL and 5–50 mL; Micropipettors: Adjustable, 10–100 μL and 100–1000 μL; Mobile phase filtration unit; Nitrogen evaporator; Vortex mixer: Variable, high speed; Shaker: Adjustable; Meat chopper; pH Metre: Capable of measuring of ±0.02 unit; Ultrasonic; Autoinjector sample vial: 2.0 mL.

13.4.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Fat and other non-kidney tissue must be removed for kidney of pigs, skin and bone for pork. Mix the sample completely, and then separate about 0.5 kg as the test sample. Seal and mark the test sample accordingly. The test samples are stored at −18°C.

(2) Extraction

Weigh 2 g (accurate to 0.01 g) test sample into 50-mL polypropylene centrifuge tube. Add 200 μL of acetonitrile and vortex. Add 400 μL of 5 mol/L NaOH and vortex mix about 30 s. Place in water bath at 80 ± 5°C for 1 h. Vortex sample solution a couple of times during this step. Remove from water bath and let cool to room temperature. Add 12 mL *tert*-Butyl Methyl Ether (TBME) to sample solution. Mechanically shake at high speed for 15 min, centrifuge for 15 min at 4000 rpm, and transfer the supernatant into a clean 15-mL glass centrifuge tube.

(3) Clean-up

Add 3 mL 1 mol/L KH_2PO_4 solution to centrifuge tube prepared in previous step. Shake for 10 min and centrifuge 10 min at 4000 rpm. Aspirate the TBME to waste. Add 2 mL TBME to the phosphate buffer, shake 5 min, and centrifuge for 5 min at 4000 rpm. Aspirate the TBME to waste. Repeat previous step once. Add 10 mL of TBME and 1 mL of 5 M NaOH, shake 15 min and centrifuge 5 min at 4 000 r/min. Transfer the TBME to a clean 10 mL glass centrifuge tube. Evaporate the TBME to dryness with nitrogen at 40°C. Add 1 mL of mobile phase, vortex-mix, and sonicate

for 10 min. Filter the sample through a syringe filter (PTFE 0.2 μm × 13 mm) into a sample vial for HPLC-MS/MS.

13.4.6 DETERMINATION

(1) Operation condition

Column: Inertsil ODS-3, 5 μm 150 × 2.1 mm, or the equivalent; Column temperature: 35°C; Injection volume: 20 μL; Total column flow: 200 μL/min; Mobile phase and pump gradient: See Table 13.2.

Ionization mode: Electrospray mode; Scan mode: Positive scan; Monitor mode: Multiple reaction monitor; Ionspray voltage: 5000 V; Nebulizer gas: 70.00 psi; Curtain gas: 10.00 psi; GS2: 70.00 psi; Source temperature: 700°C; Interface heater: ON; Other MS parameters: see Table 13.3.

(2) LC-MS-MS analysis

Inject the working standard mixed solutions of acetopromaizine, chlorpromazine, xylazine, propionylpromazine, haloperidol, azaperone, azaperol, and carazolol in sequence. Draw the standard curve with peak area vs. sample concentration. The sample is quantified with the standard curve. The responses of each standard in the sample solution should be in the linear range of the instrumental detection. Under LC and MS conditions, for the reference retention times of tranquilizer standards, see Table 13.4. For total ion chromatograms of the mixed standard working solution of tranquilizers, see Fig. 13.2.

13.4.7 PRECISION

The precision data of the method have been determined according to the stipulations of GB/T 6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

TABLE 13.2 Mobile Phase and Gradient Elution Program

Time	Mobile Phase A (%)	Mobile Phase B (%)	Mobile Phase C (%)
0	70	15	15
4.00	70	15	15
9.00	40	15	45
25.00	35	15	50
28.00	20	15	50
30.00	70	15	15
60.00	70	15	15

TABLE 13.3 Qualifier/Target Ion Pairs of Tranquilizers

Analytes	Qualifier Ion Pairs	Target Ion Pairs	Decluster Potential (V)	Collision Energy (V)	Entrance Potential (V)	Exit potential (V)
Acetopromaizine	327.40/58.20	327.40/58.20	61.00	68.00	5.00	10.00
	327.40/86.20	327.40/86.20	61.00	68.00	5.00	15.60
Chlorpromazine	319.30/58.20	319.30/58.20	60.00	66.00	10.00	10.00
	319.30/86.20	319.30/86.20	60.00	30.00	10.00	7.00
Haloperidol	376.40/165.10	376.40/165.10	70.00	35.00	11.00	11.00
	376.40/122.90	376.40/122.90	70.00	57.00	11.00	11.00
Propionylpromazine	341.20/58.20	341.20/58.20	64.00	59.80	10.60	9.70
	341.20/86.30	341.20/86.30	64.00	30.00	10.60	15.00
Xylazine	221.30/90.10	221.30/90.10	85.00	33.00	11.00	8.00
	221.30/164.40	221.30/164.40	85.00	38.00	11.00	13.00
Azaperone	328.40/121.00	328.40/121.00	60.00	30.00	11.00	10.00
	328.40/147.10	328.40/147.10	60.00	30.00	11.00	10.00
Azaperol	330.30/121.00	330.30/121.00	60.00	35.00	11.00	12.00
	330.30/149.10	330.30/149.10	60.00	40.00	11.00	12.00
	330.30/312.20	330.30/312.20	60.00	25.00	11.00	10.00
Carazolol	299.50/116.20	299.50/116.20	83.00	30.00	11.00	11.00
	299.50/222.40	299.50/222.40	83.00	30.00	11.00	6.00

TABLE 13.4 Reference Retention Time of Tranquilizers

Analytes	Retention Time (min)	Analytes	Retention Time (min)
Xylazine	5.09	Haloperidol	15.87
Azaperol	9.17	Acetopromaizine	16.55
Carazolol	11.33	Propionylpromazine	18.82
Azaperone	12.99	Chlorpromazin	19.72

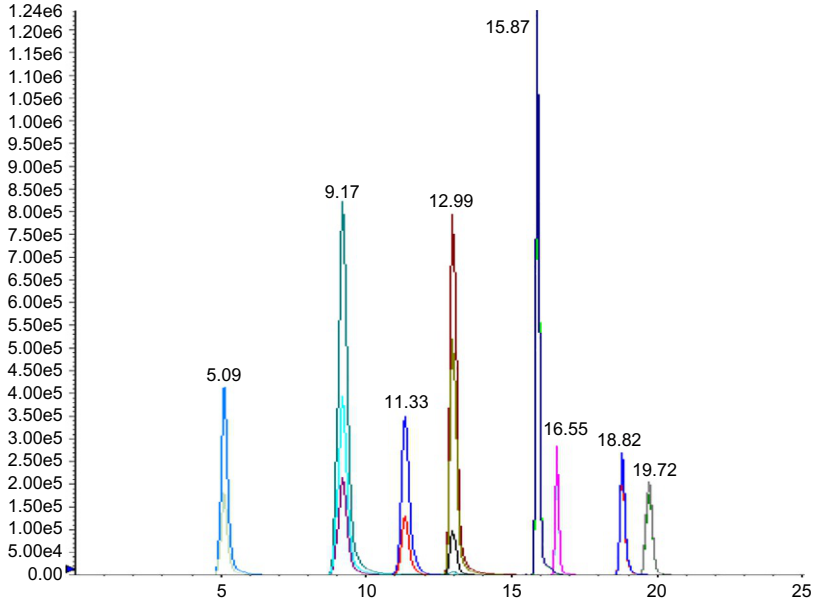


FIG. 13.2 Chromatogram of xylazine, azaperol, carazolol, azaperone, haloperidol, acetopromaizine, propionylpromazine, and chlorpromazin, and standards.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results should not exceed the limit of repeatability (r); the content range and repeatability equations of acetopromaizine, chlorpromazin, xylazine, propionylpromazine, haloperidol, azaperone, azaperol and carazolol in porcine kidney and muscle tissues are shown in Table 13.5.

TABLE 13.5 The Analytical Range, and Repeatability and Reproducibility Equations

Analytes	Content Range (kg)	Repeatability (r)	Reproducibility (R)
Acetopromaizine	0.750–4.800	$r=0.5481m-0.2456$	$\lg R=0.9549\lg m+0.0396$
Chlorpromazin	0.750–4.800	$r=0.4736m-0.1775$	$R=0.4384m-0.1224$
Haloperidol	0.150–0.960	$\lg r=0.9429\lg m-0.2700$	$\lg R=0.0702\lg m-0.2137$
Propionylpromazine	0.750–4.800	$r=0.3609m-0.0858$	$R=0.4821m+0.0048$
Xylazine	0.375–2.400	$r=0.3492m-0.0062$	$R=0.4150m+0.0020$
Azaperone	0.300–1.920	$r=0.4314m-0.0707$	$R=0.4547m+0.0756$
Azaperol	0.225–1.440	$\lg r=1.3616\lg m-0.5490$	$\lg R=0.9467\lg m-0.4548$
Carazolol	0.750–4.800	$r=0.2433m-0.0504$	$\lg R=1.1332\lg m-0.4501$

TABLE 13.6 The Fortifying Concentrations and Corresponding Recoveries

Analytes	Fortifying concentration (μg/kg)	Average recovery (%)
Acetopromaizine	0.75	100.991
	1.5	78.744
	3	85.198
	4.8	86.734
Chlorpromazin	0.75	99.873
	1.5	80.636
	3	93.255
	4.8	91.697
Haloperidol	0.15	81.946
	0.3	83.721
	0.6	98.905
	0.96	91.867
Propionylpromazine	0.75	101.222
	1.5	81.507
	3	87.957
	4.8	85.183
Xylazine	0.375	87.009
	0.75	95.477
	1.5	98.012
	2.4	85.696
Azaperone	0.3	82.205
	0.6	93.616
	1.2	103.21
	1.92	99.63
Azaperol	0.225	88.46
	0.45	91.94
	0.9	95.774
	1.44	102.429

TABLE 13.6 The Fortifying Concentrations and Corresponding Recoveries—cont'd

Analytes	Fortifying concentration (μg/kg)	Average recovery (%)
Carazolol	0.75	95.469
	1.5	83.287
	3	102.091
	4.8	97.688

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determination reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results should not exceed the limit of reproducibility (R); the analytical range and reproducibility equations of acetopromaizine, chlorpromazin, xylazine, propionylpromazine, haloperidol, azaperone, azaperol and carazolol in porcine kidney and muscle tissues are shown in Table 13.5.

13.4.8 RECOVERY

Under optimized conditions, the fortifying concentrations of acetopromaizine, chlorpromazin, xylazine, propionylpromazine, haloperidol, azaperone, azaperol, and carazolol in porcine kidney and muscle tissues and their corresponding average recoveries of this method are listed in Table 13.6.

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FURTHER READING

[1] Yagi S, Nakayama K, Kurosak Y, Higaki K, Kimura T. Biol Pharm Bull 1998;21:1195.

[2] Wu SM, Ko WK, Wu HL, Chen SH. J Chromatogr A 1999;846:239.

[3] Delahaut P, Levaux C, Eloy P, Dubois M. Anal Chim Acta 2003;483:335.

13.5

Determination of Eight Tranquilizer Residues in Bovine Milk and Milk Powder—LC-MS-MS Method (GB/T 22993-2008)

13.5.1 SCOPE

This method is applicable to the determination of acetopromaizine, chlorpromazin, haloperidol, propionylpromazine, xylazine, azaperone, azaperol, and carazolol in bovine milk and milk powder.

The limit of determination of the method for this method of acetopromaizine, carazolol, propionylpromazine, and chlorpromazin is 0.50 μg/kg; for xylazine it is 0.25 μg/kg; for azaperone it is 0.20 μg/kg; for azaperol it is 0.15 μg/kg; for haloperidol it is 0.1 μg/kg in bovine milk.

The limit of determination of the method for this method of acetopromaizine, carazolol, propionylpromazine, and chlorpromazin is 4.0 μg/kg; for xylazine it is 2.0 μg/kg; for azaperone it is 1.6 μg/kg; for azaperol it is 1.2 μg/kg; for haloperidol it is 0.8 μg/kg in milk powder.

13.5.2 PRINCIPLE

The tranquilizer residues in the test sample are made basic with aqueous NaOH and extracted with *tert*-butyl methyl ether (TBME). A pH 3 phosphate buffer is added to the extract and cleaned up with TBME. The remaining buffer is made basic with aqueous NaOH and the analytes are quantitatively back-extracted from the aqueous buffer into TBME. Concentrate and bring to volume for the volumetric flask. The solution is analyzed by LC-MS-MS, using an external standard method.

13.5.3 REAGENTS AND MATERIALS

Unless otherwise specified, all of the reagents used should be analytically pure. "Water" is the first grade of GB/T6682 specified.

Acetonitrile: HPLC grade.
Methanol: HPLC grade.
Absolute ethanol: HPLC grade.

Hydrochloric acid: G.R.
Tert-buty1 methy1 ether.
Sodium hydroxide.
Sodium hydroxide: 5 mol/L. Weigh 50.0 g and dissolve in water; let cool to room temperature and dilute to a final volume of 250 mL with water in a volumetric flask.
Potassium dihydrogen phosphate monobasic, KH_2PO_4; G.R.
Potassium dihydrogen phosphate monobasic solution: 1 mol/L pH = 3; weigh 68.045 g potassium dihydrogen phosphate monobasic and dissolve in water. Adjust the pH of the solution to 3 with concentrated HCl. Dilute to a final volume of 500 mL in a volumetric flask.
Ammonium formate.
Formic acid: HPLC grade.
Ammonium formate buffer: 0.1 mol/L pH = 4. Accurately weigh 6.306 g ammonium formate and dissolve in water. Adjust the pH of the solution to 4 with formic acid. Transfer quantitatively into a 1.0 L volumetric flask and make up to volume with water.
Ammonium formate buffer: 0.01 mol/L pH = 4. Transfer 100 mL of the solution prepared in the previous step into a 1.0-L volumetric flask and dilute to volume with water.
Mobile phase A: 0.01 mol/L ammonium formate buffer solution, pH = 4. Filter the solution prepared in step under vacuum through a 0.2-μm filter.
Mobile phase B: Acetonitrile. Filter acetronitrile under vacuum through a 0.2-μm filter.
Mobile phase C: Methanol. Filter methanol under vacuum through a 0.2-μm filter.
Composite Mobile Phase: Combine 70 mL of mobile phase A, 15 mL of mobile phase B, 15 mL of mobile phase C.
Reference and its metabolite, and the β-blocker standards
Acetopromaizine Maleate: Purity ≥99%.
Azaperone: Purity, 99%.
β-blocker: Carazolol, Purity, 99%.
Chlorpromazine•HCl: Purity, 99%.
Haloperidol: Purity ≥99%.
Propionylpromazine•HCl: Purity, 98%.
Xylazine•HCl: Purity, 99%.
The metabolite of azaperone: Azaperol, 10 ng/μL (methanol).
Standard stock solutions of acetopromaizine, chlorpromazin, haloperidol, propionylpromazine, xylazine, azaperone, azaperol, and carazolol: 0.10 mg/mL. Accurately weigh adequate amount of acetopromaizine, chlorpromazin, haloperidol, propionylpromazine, xylazine, azaperone, azaperol, and carazolol standards. Separately prepare stock standard solutions of 0.10 mg/mL with absolute ethanol. The solutions should be stored at 2–4° C in the dark. Prepare annually.

Standard stock solution of azaperol: 1 μg/mL. Transfer 1 mL of solution of azaperol into a 10-mL volumetric flask and dilute to volume with methanol. Store at 2–4°C in the dark. Prepare annually.
Mixed standard stock solution: Prepare by diluting appropriate volume of each of the stock solutions in step 4.19.1 into a volumetric flask with acetonitrile. Mix thoroughly and store at 2–4°C in the dark.
Mixed standard working solutions: Prepare the mixed working standard solutions in different concentrations with blank tissue extracts as various sensitivity and linear ranges of each reference standard. Store at 2–4°C in the dark. The solutions must be freshly prepared.
Note: The weights have been corrected for purity and/or adjusted for salt content.

13.5.4 APPARATUS

LC-MS-MS: Equipped with ESI source.
Balances: Electronic, 0.1 g sensitivity, and 0.1 mg sensitivity.
Centrifuge: Benchtop with 15 and 50 mL tube carriers, capable of 10,000 rpm.
Centrifuge tubes: Conical-bottom, 15-mL glass with plug; conical-bottom, 10-mL glass with plug; conical-bottom, 50-mL polypropylene with screw cap.
Filters: Polytetrafluoroethylene membrane filter (0.2 μm × 13 mm) and nylon membrane filter (0.2 μm × 47 mm).
Liquid dispensers: Adjustable, 1–10 mL and 5–50 mL.
Micropipettors: Adjustable, 10–100 μL and 100–1000 μL.
Mobile phase filtration unit.
Nitrogen evaporator.
Vortex mixer: Variable, high speed.
Shaker: Adjustable.
One-off straw.
pH Meter: Capable of measuring ±0.02 unit.
Ultrasonic.
One-off injector: 1 mL.
Autoinjector sample vial: 2.0 mL.

13.5.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Mix the bovine milk and milk powder sample completely, and then separate about 0.5 kg as the test sample. Seal and mark the test sample accordingly.

The test samples are stored at 4°C.

(2) Extraction

Bovine milk sample

Weigh 2 g (accurate to 0.01 g) test sample into 50-mL polypropylene centrifuge tube. Add 200 μL of acetonitrile and vortex. Add 400 μL of 5 mol/L NaOH; vortex mix about 30 s. Place in water bath at 80 ± 5°C for 1 h. Vortex sample solution a couple of times during this step. Remove from water bath and let cool to room temperature. Add 12 mL *tert*-butyl methyl ether (TBME) to sample solution. Mechanically shake at high speed for 15 min, centrifuge for 15 min at 5000 r/min, and transfer the supernatant into a clean 15-mL glass centrifuge tube.

Milk powder sample

Weigh 0.25 g of milk powder (accurate to 0.01 g) and 1.75 g of water into a 50-mL centrifuge tube to sonicate. The operation is the same as the above-mentioned extraction step for milk sample.

(3) Clean-up

Add 3 mL 1 mol/L KH_2PO_4 solution to centrifuge tube prepared in previous step; shake for 10 min and centrifuge 10 min at 5000 rpm. Aspirate the TBME to waste. Add 2 mL TBME to the phosphate buffer, shake 5 min, and centrifuge for 5 min at 4000 rpm; aspirate the TBME to waste. Repeat previous step once. Add 10 mL of TBME and 1 mL of 5 mol/L NaOH; shake 15 min and centrifuge 5 min at 5000 rpm. Transfer the TBME to a clean 10-mL glass centrifuge tube. Evaporate the TBME to dryness with nitrogen at 40°C. Add 1000 μL of mobile phase, vortex-mix, and sonicate for 10 min. Filter the sample through a syringe filter (PTFE 0.2 μm × 13 mm) into an HPLC sample vial for HPLC-MS-MS.

13.5.6 DETERMINATION

(1) Operating conditions:

Column: Inertsil ODS-3, 5 μm 150 × 2.1 mm, or the equivalent;
Column temperature: 35°C;
Injection volume : 20 μL;
Total column flow: 200 μL/min;
Mobile phase and pump gradient: See Table 13.2.
Ionization mode: Electrospray mode;
Scan mode: Positive scan;
Monitor mode: Multiple reaction monitor;
Ionspray voltage: 5000 V;
Nebulizer gas: 482.6 kPa;
Curtain gas: 68.9 kPa;
GS2: 482.6 kPa;
Source temperature: 700°C;
Interface heater: ON;
Other MS parameters: Transitions for confirmation and quantitation of Virginiamycin M1 Dwell, DP, CE, EP, CXP, See Table 13.3.

(2) Qualitative determination

Under LC-MS-MS conditions, the working solution and sample solution are injected. If the retention time of the sample chromatogram peak is consistent with that of the standard solution, and the selective ions all appear, choose a mother-ion and two product-ions. The relative abundance of the qualitative ion of the component in the sample chromatogram is compared with the standard solution with the near constant; if the deviation is no more than the given range of Table 13.7, then the corresponding analyte must be present in the sample.

(3) Quantitative determination

Inject the working standard mixed solutions of acetopromaizine, chlorpromazin, xylazine, propionylpromazine, haloperidol, azaperone, azaperol, and carazolol in sequence. Draw the standard curve with peak area vs. sample concentration. The sample is quantified with the standard curve. The responses of each standard in the sample solution should be in the linear range of the instrumental detection. Under LC and MS conditions, for the reference retention times of tranquilizer standards, see Table 13.8. For total ion chromatograms of the mixed standard working solution of tranquilizers, see Fig. 13.3.

TABLE 13.7 Maximum Permitted Tolerances for Relative Ion Intensities During Confirmation (%)

Relative intensity, k	$k > 50$	$20 < k < 50$	$10 < k < 20$	$k \leq 10$
Permitted tolerances	±20	±25	±30	±50

TABLE 13.8 Reference Retention Time of Tranquilizers

Tranquilizers	Retention Time (min)	Tranquilizers	Retention Time (min)
Xylazine	5.12	Haloperidol	15.90
Azaperol	9.21	Acetopromaizine	16.56
Carazolol	11.38	Propionylpromazine	18.84
Azaperone	13.01	Chlorpromazin	19.74

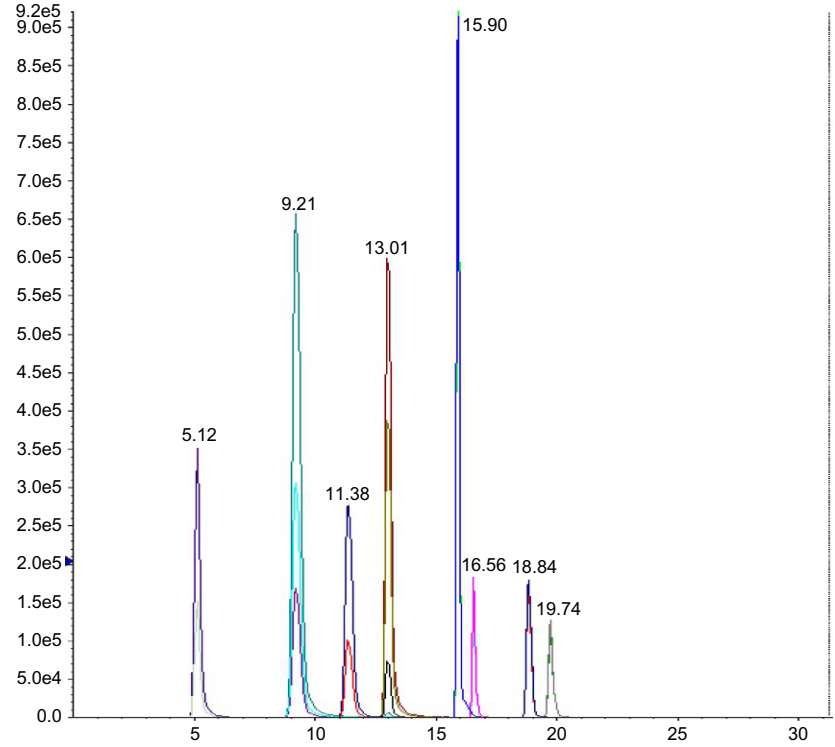

FIG. 13.3 Chromatograms of xylazine, azaperol, carazolol, azaperone, haloperidol, acetopromaizine, propionylpromazine, and chlorpromazin, and standards.

13.5.7 PRECISION

The precision data of the method for this standard have been determined according to the stipulations of GB/T 6379.1 and GB/T 6379.2. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results should not exceed the limit of repeatability (r); the content range and repeatability equations of acetopromaizine, chlorpromazin, xylazine, propionylpromazine, haloperidol, azaperone, azaperol, and carazolol in bovine milk and milk powder are shown in Tables 13.9 and 13.10.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

TABLE 13.9 Eight Tranquilizers of Content Range and Repeatability and Reproducibility Equations in Bovine Milk

Tranquilizers	Content Range (μg/kg)	Repeatability (r)	Reproducibility (R)
Acetopromaizine	0.50–4. 00	lg r=0.749 lg m−1.113	lg R=0.662 lg m+0.436
Chlorpromazin	0.50–4. 00	lg r=0.5971 lg m−1.0663	lg R=0.7682 lg m+0.4355
Haloperidol	0.10–0.80	lg r=0.97153 lg m−1.39365	lg R=0.92256 lg m+0.44711
Propionylpromazine	0.50–4. 00	lg r=1.0188 lg m−1.4323	lg R=1.5916 lg m+2.2075
Xylazine	0.25–2. 00	lg r=2.4448 lg m−3.9187	lg R=6.0859 lg m+3.4365
Azaperone	0.20–1.60	lg r=0.6706 lg m−0.1095	lg R=0.6997 lg m+0.3546
Azaperol	0.15–1.20	lg r=1.03479 lg m−1.0999	lg R=1.106 lg m−2.1312
Carazolol	0. 50–4. 00	lg r=0.3726 lg m−0.6302	lg R=0.4622 lg m+0.4401

TABLE 13.10 Eight Tranquilizers of Content Range and Repeatability and Reproducibility Equations in Milk Powder

Tranquilizers	Content Range (μg/kg)	Repeatability (r)	Reproducibility (R)
Acetopromaizine	4.0–16.0	$\lg r=0.520\lg m+0.055$	$\lg R=0.621\lg m+0.045$
Chlorpromazin	4.0–16.0	$\lg r=0.458\lg m+0.030$	$\lg R=0.559\lg m+0.019$
Haloperidol	0.8–3.2	$\lg r=5.967\lg m-2.785$	$\lg R=3.761\lg m-1.684$
Propionylpromazine	4.0–16.0	$\lg r=0.467\lg m+0.031$	$\lg R=0.569\lg m+0.020$
Xylazine	2.0–8.0	$\lg r=0.148\lg m+0.130$	$\lg R=0.310\lg m+0.105$
Azaperone	1.6–6.4	$\lg r=0.249\lg m-0.254$	$\lg R=0.127\lg m-0.257$
Azaperol	1.2–4.6	$\lg r=0.753\lg m-0.419$	$\lg R=0.603\lg m-0.416$
Carazolol	4.0–16.0	$\lg r=0.345\lg m+0.066$	$\lg R=0.445\lg m+0.057$

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results should not exceed the limit of reproducibility (R); the content range and reproducibility equations of acetopromaizine, chlorpromazin, xylazine, propionylpromazine, haloperidol, azaperone, azaperol, and carazolol in bovine milk and milk powder are shown in Tables 13.9 and 13.10.

13.5.8 RECOVERY

According to the experimental data, the fortifying concentrations of acetopromaizine, chlorpromazin, xylazine, propionylpromazine, haloperidol, azaperone, azaperol, and carazolol in porcine kidney and muscle tissues and the corresponding recoveries of this method are as seen in Tables 13.11 and 13.12.

TABLE 13.11 Fortifying Concentrations and Recoveries

Tranquilizers	Fortifying Concentration (μg/kg)	Bovine milk/Average Recovery (%)
Acetopromaizine	0.5	88.1
	1	89.2
	2	89.93
	4	91.94
Chlorpromazin	0.5	89.61
	1	86.29
	2	89.39
	4	90.29
Haloperidol	0.1	87.6
	0.2	89.28
	0.4	86.9
	0.8	90.72
Propionylpromazine	0.5	88.52
	1	91.39
	2	92.37
	4	90.38

Continued

TABLE 13.11 Fortifying Concentrations and Recoveries—cont'd

Tranquilizers	Fortifying Concentration (µg/kg)	Bovine milk/Average Recovery (%)
Xylazine	0.25	89.9
	0.5	91.38
	1	92.12
	2	90.59
Azaperone	0.2	89.35
	0.4	88.15
	0.8	90.07
	1.6	90.26
Azaperol	0.15	91.44
	0.3	91.45
	0.6	87.11
	1.2	87.42
Carazolol	0.5	89.72
	1	88.58
	2	92.49
	4	90.39

TABLE 13.12

Tranquillizers	Fortifying Concentration (µg/kg)	Bovine Milk/Average Recovery (%)
Acetopromaizine	4	92.85
	8	91.68
	12	90.3
	16	94.13
Chlorpromazin	4	90.71
	8	83.68
	12	84.11
	16	88.61

TABLE 13.12 —cont'd

Tranquillizers	Fortifying Concentration (μg/kg)	Bovine Milk/Average Recovery (%)
Haloperidol	0.8	88.79
	1.6	93.52
	2.4	88.02
	3.2	87.91
Propionylpromazine	4	92.18
	8	90.41
	12	87.24
	16	87.54
Xylazine	2	90.62
	4	86.98
	6	87.52
	8	81.59
Azaperone	1.6	90.8
	3.2	85.89
	4.8	86.05
	6.4	94.65
Azaperol	1.2	85.15
	2.4	89.98
	3.6	93.36
	4.8	97.45
Carazolol	4	92.87
	8	91
	12	88.04
	16	91.72

RESEARCHERS

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Chapter 14

Pyrazolone

14.1

Curative Effects and Side Effects of Phenylbutazone

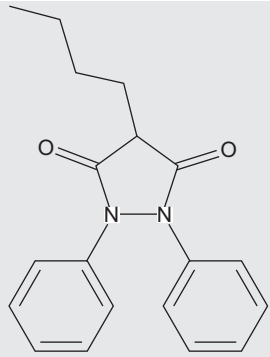
Phenylbutazone is a nonsteroidal anti-inflammatory drug (NSAID) and cylooxygenase inhibitor. It is a potent pain reliever, antipyretic, and anti-inflammatory. In animals, including food-producing animals, it is commonly used for lameness resulting from soft tissue injury, muscle soreness, bone and joint problems, and laminitis. NSAIDs work by inhibiting the body's production of prostaglandins, thromboxane, and other inflammatory mediators. But toxicity and side effects such as gastrointestinal bleeding, aplastic anemia, and agranulocytosis might be produced after a routine or prolonged treatment. Many countries have not yet established an MRL for this substance, which is considered to be a banned compound whose exhaustive control in animal products is, at present, required.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00014-2

14.2

Chemical Structure of Phenylbutazone

TABLE 14.1 Chemical Structure for Phenylbutazone

Compound Name	Chemistry Structure	Molecular Weight	Cas. No	MRL (μg/kg)
Phenylbutazone	 PBZ	307.36	129-18-0	US: – CN: – EU: –

14.3

Determination of Phenylbutazone in Livestock and Poultry Muscles—LC-UV Method (GB/T 20754-2006)

14.3.1 SCOPE

This method is applicable to the determination of phenylbutazone residues in muscle tissues in bovine, porcine, ovine, and gallinaceous species.

The limit of detection of this method: 5.0 μg/kg.

14.3.2 PRINCIPLES

Phenylbutazone residues in bovine, porcine, hivcine, and chicken muscles are extracted by methanol-ethyl acetate of dithiothreitol solution (1+7). The extraction solution is concentrated and diluted by methanol-ammonia-ethyl acetate-ethyl acetate of dithiothreitol solution and is purified by Florisil solid phase extraction (SPE) column. The drug is eluted using the mixture solution of glacial acetic acid-methanol-ethyl acetate-ether and evaporated to near dryness. The residue is dissolved in mobile phase and analyzed by liquid chromatography with UV detection using an external standard.

14.3.3 REAGENTS AND MATERIALS

All of the reagents used are reagent-grade, except illuminated. The water is deionized water. Methanol, acetonitrile, ethyl acetate, methylene dichloride: HPLC grade; Glacial acetic acid; Ammonium acetate; Ammonia; Ether; Dithiothreitol: 99% purity.

Ammonium acetate: 0.05 M. This solution is prepared by weighing 1.95 g of ammonium acetate and dissolving in 500 mL of water.
Solution A: 0.25 mg/mL. This solution is prepared by weighing 1.25 g dithiothreitol and dissolving in 500 mL of ethyl acetate.
Solution B: mixture with methanol and the solution by 1:7 (v/v), prepare fresh before using.
Solution C: methanol-ammonia-methylene dichloride-solution A (1+1+70 +70). This solution is prepared just before using.
Solution D: 2 mL of glacial acetic acid and 4 mL of methanol are dissolved in 100 mL of methylene dichloride. This solution is mixed with 80 mL of ether.
Florisil solid phase extraction column: 2 g, 12 mL. It is diluted with 5 mL of solution C before using it, and the column is protected wet.
Phenylbutazone standards: purity $\geq$95%.
Phenylbutazone stock solution: prepared by weighing 5.0 mg phenylbutazone and putting it into a 100-mL flask and dissolving it in 80 mL of methanol and 5 mL of solution A. Methanol is added to the line and then shaken. This solution is stored at 4°C.
Phenylbutazone middle concentration standard solution: 2.0 μg/mL. 0.4 mL of phenylbutazone stock solution is added to a flask and diluted to 10 mL with mobile phase. This solution is stored at 4°C.
Phenylbutazone working standard solution: Calibration standards at different concentrations (ng/mL) are prepared by dilution of the working standard with blank sample extraction solution according to phenylbutazone sensitivity and the linear range of the instrument. This solution is stable for 1 week when stored at 4°C.

14.3.4 APPARATUS

Liquid chromatagraphy with ultraviolet detector; Analytical balance: capable of weighing to 0.01 g, 0.0001 g; Nitrogen evaporator; Centrifuge tubes: 50 mL, polypropylene with screw cap; SPE apparatus.

14.3.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Take representative samples of 1 kg from the total defrosted samples of beef, pork, mutton, and chicken; shred and mix them thoroughly, break into two equal parts, and load respectively into clean containers. These will serve as test samples after being sealed and shall be clearly marked or labeled. Precautions should be taken to prevent the samples from being contaminated or undergoing any change of the residue content in the sampling and sample preparation operations. The test samples should be stored below −20°C.

(2) Extraction

Weigh 2-g test sample (accurate to 0.01 g) into 50-mL centrifuge tube. Add 8 mL solution B, vortex to mix, and shake for 10 min on high speed horizontal shaker; centrifuge at 4200 rpm and below 5°C for 5 min. Transfer the supernatant to a second centrifuge tube. Repeat the extraction step twice and combine the extracts in the second tube. Evaporate the extract under nitrogen at 55°C to minimum volume and wait for clean-up.

(3) Clean-up

Reconstitute residues with 5 mL solution C and add the extract to the Florisil SPE cartridge. Process the extract through the cartridge at below 1.0 mL/min. After the extract has flowed out, wash the cartridge with 1 mL solution C. Discard all eluates. Finally, elute phenylbutazone with 15 mL solution D into a 30-mL conical flask. Evaporate the extract under nitrogen at 55°C to minimum volume. Reconstitute the residues in 1 mL mobile phase solution. Filtrate the solution with 0.2-μm filter membrane and detect by liquid chromatograph.

14.3.6 DETERMINATION

(1) Operation conditions

Chromatographic column: Mightysil C18, 150 mm × 4.6 mm, 3 μm, or equivalent; Mobile phase: 0.05 M ammonium acetate-methanol-acetonitrile (50+37+13); Flow rate of mobile phase: 0.8 mL/min; Column temperature: 40°C; Injection volume: 40 μL; Wavelength: 270 nm.

(2) LC determination

A standard working curve shall be drawn with the injection of phenylbutazone standard working solutions, and the standard working curve is

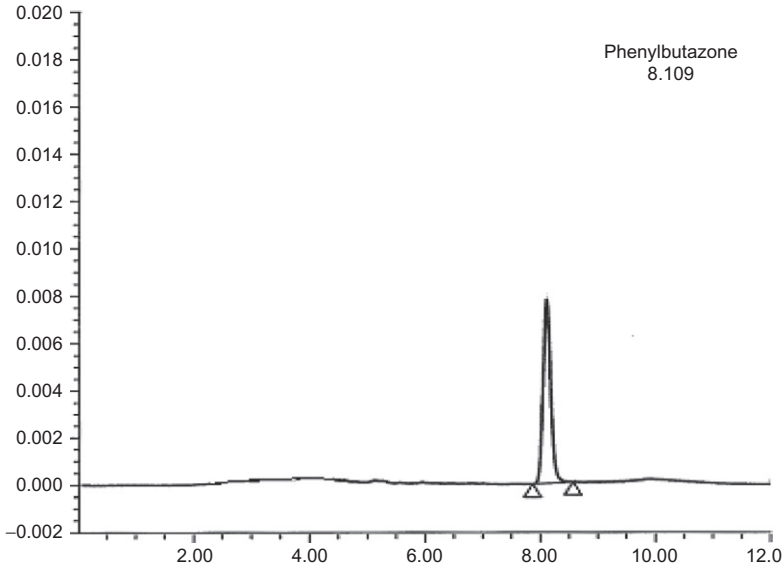


FIG. 14.1 Chromatogram of the standard working solution of phenylbutazone.

then used for quantifying purposes, with the response values of phenylbutazone in the sample solutions falling within the linear range of the instrument detection. Under the previously mentioned chromatic conditions, the retention time of phenylbutazone shall be 8.1 min and the chromatogram of phenylbutazone standard LC is shown in Fig. 14.1.

14.3.7 PRECISION

The precision data of the method have been determined in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the conditions of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); the content range and repeatability equations for phenylbutazone in muscle tissues are shown in Table 14.2.

If the difference of values exceeds the limit of repeatability (r), the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the conditions of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R); the content range and reproducibility equations for phenylbutazone in muscle tissues are shown in Table 14.2.

TABLE 14.2 Range and Repeatability/Reproducibility

Analyte	Range (μg/kg)	Repeatability (r)	Reproducibility (R)
Phenylbutazone	5.0–150.0	lg r=0.7649 log m−0.8285	lg R=0.7120 log m−0.7601

Note: m *is average value of parallel test results.*

TABLE 14.3 Fortifying Concentrations and Corresponding Recoveries

Analyte	Fortifying Concentration (μg/kg)	Recovery (%)
Phenylbutazone	5.0	86.9
	10.0	82.2
	50.0	91.4
	100.0	90.3

14.3.8 RECOVERY

Under optimized conditions, the recoveries of phenylbutazone using this method are listed in Table 14.3.

RESEARCHERS

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FURTHER READING

[1] He J, Shibukama A, Tokunaga S, Nakagawa T. J Pharm Sci 1997;86:120.

14.4

Curative Effects and Side Effects of Dipyrone

Commonly used in the past as a powerful painkiller and fever reducer, dipyrone (noramidopyrine methanesulfonate sodium, metamizole) is a water-soluble pyrazolone derivative drug available in oral and parenteral forms. Dipyrone has significant analgesic and antipyretic properties. Traditionally, dipyrone has been thought to also have antispasmodic properties on the smooth muscle of the gastrointestinal tract, a basis for its common use in cases of mild colic. Similar to that of other nonsteroidal anti-inflammatory drugs, the mode of action of dipyrone involves the inhibition of prostaglandin synthesis. In acute studies, dipyrone showed no potential for the therapy of gastric ulceration.

The use of dipyrone has been associated with bone-marrow toxicity, severe agranulocytic anemia, dose-independent teratogenicity, induction of the microsomal enzyme system, and a tendency to increase bleeding time by suppressing the formation of prothrombin. Extralabel use of dipyrone in food animals is specifically prohibited by the FDA-CVM. Dipyrone has also been banned in some regions (UK, Sweden) because of its adverse drug reactions. In Japan, it is ascribed to the Uniform List of chemical residues (0.01 μg/kg).

14.5

Pharmacokinetics of Dipyrone

Dipyrone pharmacokinetics has been studied in healthy volunteers. In humans, dipyrone is nonenzymatically hydrolyzed in the gastrointestinal tract to 4-methylaminoantipyrine (MAA), which is then rapidly and extensively absorbed. MAA undergoes metabolism in the liver to 4-aminoantipyrine (AA) by demethylation and to 4-formylaminoantipyrine (FAA) by oxidation of the N-methyl side chain. The specific forms of cytochrome P450 subfamilies involved in these reactions have not yet been identified. AA is transformed into 4-acetylaminoantipyrine (AAA) by the polymorphic N-acetyltransferase (NAT2) system. None of these four dipyrone products is extensively bound to plasma proteins, and all are excreted by the kidney, accounting for 65%–70% of the administered dose of dipyrone. Metabolites of dipyrone are shown in Fig. 14.2.

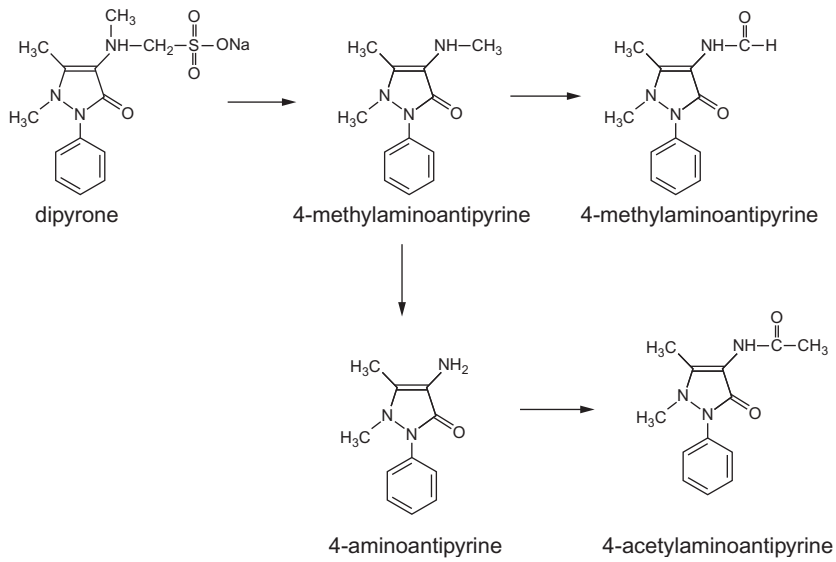


FIG. 14.2 Main metabolites of dipyrone.

14.6

Chemical Structure and Maximum Residue Limit for Dipyrone

TABLE 14.4

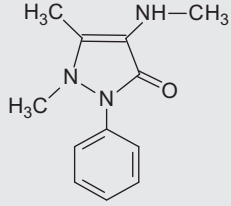
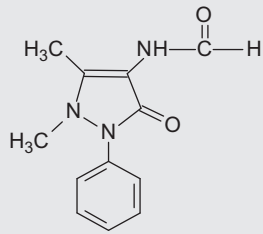
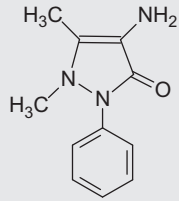
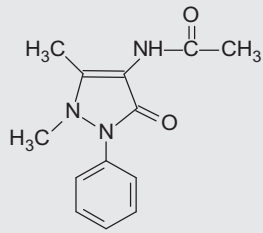
Compound Names	Molecular Structure	Molecular Weight	Cas. No.	MRLs (μg/kg)	
MAA		217.27	519-98-2	America, Sweden	banned
				China	200
				EU	100
				Japan	10

TABLE 14.4 —con'd

Compound Names	Molecular Structure	Molecular Weight	Cas. No.	MRLs (μg/kg)
FAA		231.26	1672-58-8	–
AA		203.24	83-07-8	–
AAA		245.28	83-15-8	–

14.7

Determination of Metabolite Residues of Dipyrone in Bovine and Porcine Muscle Tissues—LC-UV and LC-MS-MS Method (GB/T 20747-2006)

14.7.1 SCOPE

This method is applicable to the qualified determination and confirmation of three metabolite residues of dipyrone (4-formylaminoantipyrine, 4-methylaminoantipyrine and 4-aminioantipyrine) in beef and pork muscle tissues.

The detection limits of the LC-UV method are: 12.5 μg/kg for 4-formylaminoantipyrine, 15.0 μg/kg for 4-aminioantipyrine, and 20.0 μg/kg for 4-methylaminoantipyrine.

The detection limits of the LC-MS-MS method are: 1.8 μg/kg for 4-formylaminoantipyrine, 1.5 μg/kg for 4-methylaminoantipyrine and 4-aminioantipyrine, and 1.0 μg/kg for 4-acetylaminoantipyrine.

14.7.2 PRINCIPLE

The residues of dipyrone in the test samples are extracted with sodium sulfate buffer (pH 7.0). After filtration, the extracts are cleaned by Bond Elut C_{18} SPE column or other equivalent SPE column, and then eluted with methanol. The eluate is dried on an N-Evap, and the residue is dissolved in methanol-water solvent. The samples are determined by liquid chromatography–ultraviolet (LC-UV) or liquid chromatography–tandem mass spectrometry (LC-MS-MS); 4-methylaminoantipyrine and 4-acetylaminoantipyrine are quantitated by an external standard method, while 4-aminioantipyrine and 4-methylaminoantipyrine are quantitated by an internal standard method.

14.7.3 REAGENTS AND MATERIALS

Methanol, acetonitrile, and ammonium acetate are all of HPLC grade; sodium sulfate and sodium sulfite are of analytical grade; filter paper, glass fiber; sodium sulfate + sodium sulfite buffer: accurately weigh 14.20 g sodium sulfate and 2.52 g sodium sulfite into 1000-mL volumetric flask, dilute with water to 950 mL, adjust the pH of the buffer to 7.0 with 0.5 M sulfuric acid, and

then dilute to 1000 mL. Mix to homogeneity; rinse solvent: methanol-water (5/95, v/v); sample dissolved solvent: methanol-water (1/9, v/v).

Bond Elut C_{18} column or other equivalent SPE column: 500 mg, 3 mL. The column is activated with 6 mL methanol and equilibrated with 6 mL water before use. The column should always be kept wet.

Standard chemicals: 4-formylaminoantipyrine, 4-acetylaminoantipyrine, 4-methylaminoantipyrine, and 4-aminoantipyrine; the purity of these standards is more than 99%.

Internal standard chemical: 4-isopropylaminoantipyrine; the purity of the standard is more than 99%.

Standard stock solutions: 100 mg/L. Accurately weigh standard chemicals of each metabolite of dipyrone and dissolve to 100 mg/L with methanol. The solutions can be preserved at temperatures below −18°C for more than 3 months.

Internal standard solution: Accurately weigh 4-isopropylaminoantipyrine standard chemical, dissolve to 100.0 mg/L with methanol. The solutions can be preserved at a temperature below −18°C for more than 3 months.

Mixed standard calibration solution: Accurately take appropriate volumes of standard working solution and internal standard working solution and then mix them up to 1.0 mL with sample dissolved solvent.

Matrix standard calibration solution: Add an appropriate volume of internal solution into standard working solution, blend, and reduce the mixed solution to near dryness on an N-Evap; the residue is then diluted with blank matrix solution.

14.7.4 APPARATUS

Liquid chromatography with UV detector; liquid chromatography–mass spectrometry with electrospray ion source; SPE vacuum manifold; nitrogen evaporator; mechanical shaker; analytical balance with sensitivity of 0.1 mg and 0.01 g; vacuum pump; homogenizer; micropipettor, 10–100 μL and 100–1000 μL; polypropylene centrifuge tube, 50 mL, with screw cup; pH meter with ±0.02 precision; centrifuge with cooler, refrigeration to 4°C; centrifuge tube, 15 mL, glass; 0.45-μm membrane filter.

14.7.5 SAMPLE PRETREATMENT

(1) Sample preparation

Choose the representative sample and prepare it according to analysis requirements in the lab. The prepared sample is divided into two parts, and put into sample containers, pressurized, and labeled.

(2) Extraction

Accurately weigh 5 g of the test sample (accurate to 0.01 g) in a 50-mL polypropylene centrifuge tube; an appropriate volume of 4-isopropylaminoantipyrine is added as an internal standard. Then 15 mL sodium sulfate + sodium sulfite buffer is added into the tube and the mixture is homogenized and centrifuged at 4000 rpm for 5 min. After centrifugation, the supernatant is collected in a new clean 50-mL polypropylene centrifuge tube. Repeat the preceding extraction procedure twice (15 mL/10 mL), combine the supernatants (approximately 40 mL), and dilute the extract to 50 mL with sodium sulfate + sodium sulfite buffer. After complete mixing, the extract is filtered to a new clean 50-mL polypropylene centrifuge tube by glass filter paper for further cleaning.

(3) Clean-up

Attach 25-mL reservoirs onto C_{18} SPE columns (preconditioned by 5 mL methanol and 5 mL water) and place on the vacuum manifold. Allow the sample to pass through at the rate of about 1 mL/min. After all the sample has been processed, wash the column with 5 mL water and 5 mL methanol-water, and discard the elute. Dry the column for 10 min. Elute the analytes from the column into a clean 15-mL glass centrifuge tube with 5 mL of methanol and reduce the methanol to near dryness on an N-Evap set at 55°C. Reconstitute the residue with 1 mL of sample solvent. The solution is filtered through a 0.45-μm membrane and determined by high performance liquid chromatography or liquid chromatography-tandem mass spectrometry.

14.7.6 DETERMINATION

(1) LC-UV operating conditions

Column: Inertial ODS-3 column (250 mm × 4.6 mm, 5 μm) or equivalent; column temperature: 30°C; injection volume: 50 μL; mobile phase and the elution gradient are listed in Table 14.5; detection wavelength: 265 nm.

(2) LC-UV determination

According to the approximate concentration of dipyrone metabolites in the test sample solution, the standard working solution is selected with similar peak height to that of the sample solution. The response of dipyrone metabolites in the standard working solution and sample solution should be in the linear range of the instrumental detection. The standard working solution should be injected randomly between the injections of sample solution of equal volume (Fig. 14.3). Under the chromatography conditions described previously, the reference retention times of

TABLE 14.5 Mobile Phase and Elution Gradient of LC

Time (min)	Flow Rate (mL/min)	Water (%)	Methanol (%)
0	1.0	90	10
15	1.0	30	70
17	1.0	5	95
18	1.0	90	10
23	1.0	90	10

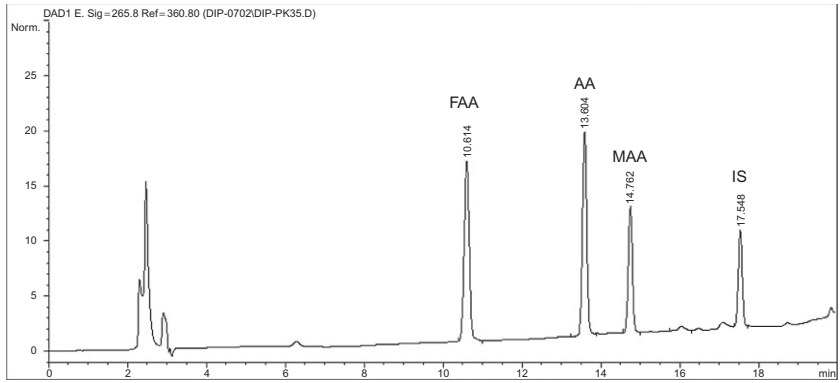


FIG. 14.3 HPLC chromatogram of dipyrone metabolites in standard working solution (10.61 min: 4-formylaminoantipyrine, 13.60 min: 4-aminoantipyrine, 14.76 min: 4-methylaminoantipyrine, 17.55 min: 4-isopropylaminoantipyrine (internal standard)).

4-formylaminoantipyrine, 4-aminoantipyrine, and 4-methylaminoantipyrine are about 10.61 min, 13.60 min, and 14.76 min, respectively. For 4-isopropylaminoantipyrine, which is used as the internal standard, the retention time is 17.55 min.

(3) LC-MS-MS operating conditions

Column: Atlantis dC_{18} column (150 mm × 2.1 mm, 3 μm) or equivalent; column temperature: 30°C; injection volume: 20 μL; Mobile phase and the elution gradient are listed in Table 14.6.

Ion source: ESI; scan mode: positive mode; detection mode: multiple reaction monitoring (MRM); ion spray voltage: 5000.00 V; nebulizer

TABLE 14.6 Mobile Phase and Elution Gradient of LC-MS-MS

Time (min)	Flow Rate (mL/min)	5 mmol/L NH4Ac Buffer, pH 4.5	Acetonitrile (%)
0	0.30	90	10
10	0.30	20	80
12	0.30	5	95
13	0.30	90	10
18	0.30	90	10

TABLE 14.7 MS Parameters for the Detection of Dipyrone Metabolites

Metabolites	Qualitative Ion (*m*/*z*)	Quantitative Ion (*m*/*z*)	Collision Energy (V)	Decluster Potential (V)
4-Formylaminoantipyrine	232/104	232/104	21	36
	232/83		30	36
4-Aminoantipyrine	204/159	204/159	19	35
	204/111		19	35
4-Methylaminoantipyrine	218/56	218/56	40	50
	218/97		23	50
4-Acetylaminoantipyrine	246/228	246/228	20	32
	246/104		35	32
4-Isopropylaminoantipyrine (internal standard)	246/56	246/56	46	35
	246/125		35	35

gas: 8.00 L/min; heat temperature: 500°C; Focus potential: 200 V; collision exit potential: 15 V; qualitative ions, quantitative ion, dwell time, decluster potential, and collision energy are listed in Table 14.7.

(4) LC-MS-MS analysis

(a) Qualitative analysis

Select one parent ion and two or more than two daughter ions for each analyte. If the deviation of relative retention time of the analyte

between the test sample and standard solution is within ±2.5% under the same experimental conditions, and if the difference of relative ion ratio of the analyte between the test sample and standard solution is also within the error allowed (the max deviations allowed for relative ion ratios are listed in Table 2.4), then the corresponding analyte would be considered to be in the sample.

(b) Quantitative determination

Under optimized instrumental working conditions, different mixed matrix calibration solutions are injected into the instrument in a separated run (Fig. 14.4). The content of different metabolites of dipyrone in the sample is quantified by each standard calibration curve. The response of dipyrone metabolites in the standard working solution and sample solution should be in the linear range of the instrumental detection. For 4-formylaminoantipyrine and 4-acetylaminoantipyrine, determination is made with an external standard; for 4-methylaminoantipyrine and 4-aminoantipyrine, determination is made with an internal standard.

14.7.7 PRECISION

The data in this part are acquired under the GB/T 6379 regulations. The values of repeatability and reproducibility are calculated with the confidence coefficient set to 95%.

(1) Repeatability

Under repeatability conditions, the difference of the absolute values of the result from two independent tests should be not more than the repeatability limit (r). The content range and repeatability equation of the three metabolites of dipyrone are shown in Table 14.8.

(2) Reproducibility

Under reproducibility conditions, the difference in the absolute values of the result from two independent tests should be not more than the reproducibility limit (R). The analytical range and repeatability equations of the four metabolites of dipyrone are shown in Table 14.8.

14.7.8 RECOVERY

Under optimized conditions, the recoveries of metabolites of dipyrone using this method are listed in Tables 14.9 and 14.10.

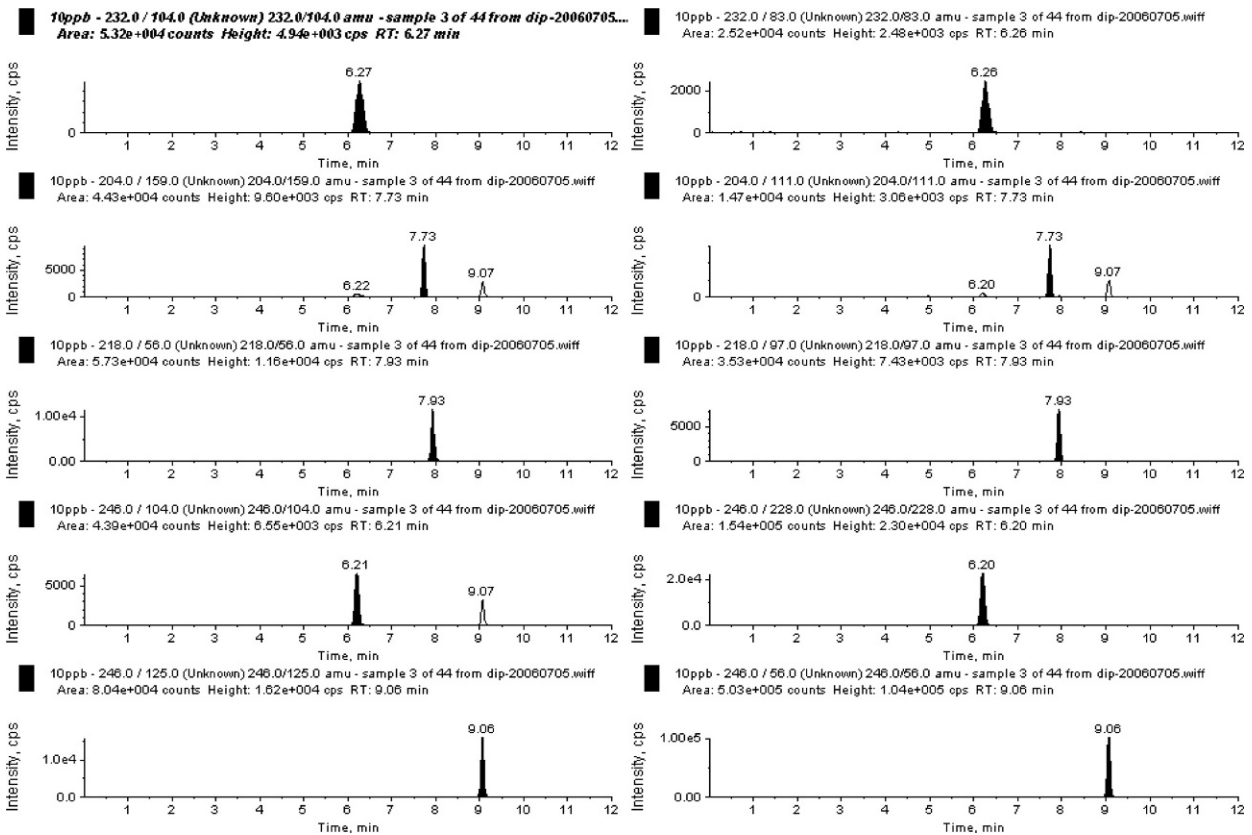


FIG. 14.4 HPLC-MS/MS chromatogram of dipyrone metabolites in standard working solution (6.27 min: 4-formylaminoantipyrine, 6.21 min: 4-acetylaminoantipyrine, 7.73 min: 4-aminoantipyrine, 7.93 min: 4-methylaminoantipyrine, 9.06 min: 4-isopropylamino-antipyrine (internal standard)).

TABLE 14.8 Analytical Range and Repeatability/Reproducibility Equations

Analytes	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
4-Formylaminoantipyrine	5.0–40	$\lg r = 0.3970 \lg m - 0.6061$	$\lg r = 0.7675 \lg m - 0.9967$
4-Methylaminoantipyrine	5.0–40	$\lg r = 0.6830 \lg m - 0.8383$	$\lg r = 0.8660 \lg m - 1.0902$
4-Aminioantipyrine	5.0–40	$\lg r = 0.7378 \lg m - 1.0216$	$\lg r = 1.0072 \lg m - 1.2633$
4-Acetylaminoantipyrine	5.0–40	$\lg r = 0.6076 \lg m - 0.8019$	$\lg r = 0.7973 \lg m - 1.0027$

Note: m *is the arithmetic mean of two test results.*

TABLE 14.9 The Fortifying Concentrations and Corresponding Recoveries (HPLC-UV)

Analytes	Added Level (μg/kg)	Average Recovery (%)
The added level and average recovery of dipyrone metabolites in pork muscle tissue		
4-Formylaminoantipyrine	5	91.2
	10	94
	20	92
	40	92
4-Aminoantipyrine	5	82
	10	87.5
	20	96.5
	40	97.8
4-Methylaminoantipyrine	5	80.4
	10	77.1
	20	75
	40	75.7

Continued

TABLE 14.9 The Fortifying Concentrations and Corresponding Recoveries (HPLC-UV)—con'd

Analytes	Added Level (μg/kg)	Average Recovery (%)
4-Acetylaminoantipyrine	5	90.2
	10	88.2
	20	85
	40	88.8
The added level and average recovery of dipyrone metabolites in bovine muscle tissue		
4-Formylaminoantipyrine	5	81.6
	10	85.5
	20	89
	40	89.1
4-Aminoantipyrine	5	96.2
	10	97.6
	20	101
	40	104
4-Methylaminoantipyrine	5	86.4
	10	80.7
	20	78.5
	40	76.4
4-Acetylaminoantipyrine	5	86.4
	10	88.1
	20	85.9
	40	90.5

TABLE 14.10 The Fortifying Concentrations and Corresponding Recoveries (LC-MS-MS)

Analytes	Added Level (μg/kg)	Average Recovery (%)
The added level and average recovery of dipyrone metabolites in pork muscle tissue		
4-Formylaminoantipyrine	5	91.2
	10	94
	20	92
	40	92
4-Aminoantipyrine	5	82
	10	87.5
	20	96.5
	40	97.8
4-Methylaminoantipyrine	5	80.4
	10	77.1
	20	75
	40	75.7
4-Acetylaminoantipyrine	5	90.2
	10	88.2
	20	85
	40	88.8
The added level and average recovery of dipyrone metabolites in bovine muscle tissue		
4-Formylaminoantipyrine	5	81.6
	10	85.5
	20	89
	40	89.1
4-Aminoantipyrine	5	96.2
	10	97.6
	20	101
	40	104
4-Methylaminoantipyrine	5	86.4
	10	80.7
	20	78.5
	40	76.4

Continued

TABLE 14.10 The Fortifying Concentrations and Corresponding Recoveries (LC-MS-MS)—con'd

Analytes	Added Level (μg/kg)	Average Recovery (%)
4-Acetylaminoantipyrine	5	86.4
	10	88.1
	20	85.9
	40	90.5

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FURTHER READING

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14.8

Determination of Metabolite Residues of Dipyrone in Milk and Milk Powder—LC-MS-MS Method (GB/T 22971-2008)

14.8.1 SCOPE

This method is applicable to the qualified determination and confirmation of four metabolite residues of dipyrone, 4-formylaminoantipyrine, 4-acetylaminoantipyrine, 4-methylaminoantipyrine, and 4-aminioantipyrine in milk and milk powder.

The limit of detection of the standard: for milk, it is 5.0 μg/kg for 4-formylaminoantipyrine, 4-methylaminoantipyrine, 4-aminioantipyrine, and 4-acetylaminoantipyrine; for milk powder, the limit is 40.0 μg/kg for 4-formylaminoantipyrine, 4-methylaminoantipyrine, 4-aminioantipyrine, and 4-acetylaminoantipyrine.

14.8.2 PRINCIPLE

The residues of dipyrone in the test sample are extracted with acetonitrile after the TRIS buffer is added. The extracts are then deesterified by hexane. The samples are determined by LC-MS-MS; 4-methylaminoantipyrine, 4-aminioantipyrine, and 4-acetylaminoantipyrine are quantified by an external standard method, while 4-methylaminoantipyrine is quantified by an internal standard method.

14.8.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents should be of analytical grade; water is the first-grade water as prescribed by GB/T 6682-1992.

Methanol: HPLC Grade.
Isopropanol
Acetonitrile: HPLC Grade.
TRIS (Tris(Hydroxymethyl)aminomethane).
Ammonium acetate: HPLC Grade.
1.0 mol/L TRIS buffer: accurately weigh 12.1 g TRIS; dilute with water to 100 mL.
Sample dissolved solvent: methanol + water (5:95, *V*/*V*).
5.0 mmol/L ammonium acetate buffer: accurately weigh 0.385 g ammonium acetate and dilute with 950 mL water. The pH is adjusted to pH 3.0 by acetic acid. The buffer is then diluted to 1000 mL.
Standard chemicals: 4-formylaminoantipyrine, 4-acetylaminoantipyrine, 4-methylaminoantipyrine, and 4-aminoantipyrine; the purity of these standards is more than 99%.
Internal standard chemical: 4-isopropylaminoantipyrine; the purity of the standard is more than 99%.
100 mg/L standard stock solutions: Accurately weigh appropriate standard chemicals of each metabolite of dipyrone and dissolve to 100 mg/L with methanol. The solutions can be preserved at temperatures below −18°C for more than 3 months.
1.0 mg/L standard working solution: Accurately pipette an appropriate volume of standard stock solution of each metabolite of dipyrone and dilute to

1.0 mg/L with methanol. The solution can be preserved at temperatures below −18°C for 1 month.
100 mg/L internal standard solution: Accurately weigh appropriate 4-isopropylaminoantipyrine standard chemicals and dissolve to 100.0 mg/L with methanol. The solutions can be preserved at temperatures below −18°C for more than 3 months.
1.0 mg/L internal standard working solution: Accurately pipette an appropriate volume of internal standard stock solution and dilute to 1.0 mg/L with methanol. The solution can be preserved at temperatures below −18°C for one month.
Matrix standard calibration solution: Accurately pipette an appropriate volume of internal solution and standard working solution and dilute with blank matrix solution. The concentration of each metabolite of dipyrone in calibration solution is 1.0, 5.0, 10.0, 20.0, 40.0, and 100 ng/mL, respectively. The concentration of the internal standard (4-isopropylaminoantipyrine) in the working solution is 10.0 μg/L.
Membrane filter: 0.22 μm.

14.8.4 APPARATUS

LC-MS, equipped with electrospray ion source.
Rotary evaporator.
Mechanical shaker.
Analytical balance, sensitivity: 0.1 mg, 0.01 g.
Micropipettor, 10–100 μL and 100–1000 μL.
Polypropylene centrifuge tube: 15 mL and 50 mL, with screw cap.
Centrifuge with cooler, refrigeration to 4°C.
Volumetric flask: 25 mL.
Conical flask.

14.8.5 SAMPLE PRETREATMENT

(1) Preparation of sample

Choose a representative sample greater than 500 g (milk or milk powder) and mix thoroughly. The prepared sample is divided into two parts and put into a sample container, pressurized, and labeled.

Milk should be kept at 4°C in a dark place, while milk powder should be kept in a desiccator at room temperature.

(2) Extraction

Milk

Accurately weigh 5 g of the test sample (accurate to 0.01 g) in a 50-mL polypropylene centrifuge tube and add 100 μL of 1.0 mg/L internal standard working solution. After the adding of 0.5 mL TRIS buffer, 10 mL

acetonitrile is added to the tube, it is vortex blended for 1 min, extracted by shaking for 15 min, and then centrifuged at 5000 rpm for 5 min. After centrifugation, the supernatant is collected in a new clean volumetric flask. Repeat the preceding extract procedure once with a volume of 8 mL; combine the supernatants (approximately 23 mL) and dilute the extract to 25 mL with acetonitrile; mix thoroughly.

Milk powder

Accurately weigh 12.5 g of milk powder (accurate to 0.01 g) in a 100-mL beaker and dissolve the sample by adding an appropriate volume of 35 − 50°C water. After cooling, the sample is diluted to 100 g by water and mixed throughly. Accurately weigh 5 g of the test sample (accurate to 0.01 g) in a 50-mL polypropylene centrifuge tube and prepare it according to the above-mentioned extraction steps of milk sample.

(3) Clean-up

Accurately pipette 5.0 mL of extract solution in a 15-mL polypropylene centrifuge tube, and add 3.0 mL acetonitrile saturated hexane; vortex mix for 3.0 min. The sample is centrifuged at 5000 rpm under 4°C for 5 min, and the hexane portion is discarded. The acetonitrile portion is transferred into a conical flask, and 5.0 mL isopropanol is added; the solution is reduced to near dryness on a rotary evaporator set at 40°C. . Reconstitute the residue with 1 mL of sample dissolved solvent. The solution is filtered through a 0.22-μm membrane and determined by high performance LC-MS-MS.

(4) Preparation of blank matrix solution

Accurately weigh 5 g of negative sample (accurate to 0.01 g) and prepare as described in above. The blank matrix solution is used to prepare the matrix standard calibration solution.

14.8.6 DETERMINATION

(1) Operating conditions

Column: C_{18} column (50 mm × 2.1 mm, 1.7 μm) or equivalent;
Column temperature: 40°C;
Injection volume: 10 μL;
Mobile phase and the elution gradient are listed in Table 14.11.
Ion source: ESI;
Scan mode: Positive mode;
Detection mode: MRM;
Ion spray voltage: 3000 V;
Collision gas: Argon;
Nebulizer gas and flow rate: nitrogen, 750 L/h;
Nebulizer gas temperature: 350°C;
Ion source temperature: 105°C;

TABLE 14.11 Mobile Phase and Elution Gradient of LC-MS-MS

Time (min)	Flow Rate (mL/min)	5 mmol/L NH4Ac Buffer (pH 3.0) (%)	Methanol (%)
0	0.25	95	5
3.0	0.25	85	15
4.0	0.25	10	90
4.1	0.25	95	5
5.5	0.25	95	5

Qualitative ions, quantitative ion, dwell time, cone voltage, and collision energy are listed in Table 14.12.

(2) Qualitative determination

Select one parent ion and two daughter ions for each analyte. If the deviation of relative retention time of the analyte between test sample and standard solution is within ±2.5% under the same experimental conditions, and the difference of the relative ion ratio of the analyte between test sample and standard solution is also within the error allowed (the max deviations allowed for relative ion ratios are listed in Table 14.13), then the corresponding analyte would be considered to be in the sample.

(3) Quantitative determination

External standard method: Under the optimized instrument working conditions, different mixed matrix calibration solutions are injected into the instrument in a separate run. Using peak area or ratio of peak area as *y*-axis, and concentration as *x*-axis, the content of different metabolites of dipyrone in sample is quantified by each standard calibration curve. The response of dipyrone metabolites in the standard working solution and sample solution should be in the linear range of the instrumental detection. The multiple reaction monitoring (MRM) chromatograms of dipyrone metabolites are shown as Fig. 14.5.

14.8.7 PRECISION

The data in this part are acquired under the regulations of GB/T6379.1 and GB/T6379.2. The values of repeatability and reproducibility are calculated at a confidence level of 95%.

(1) Repeatability

Under repeatability conditions, the absolute values of the difference of the result from two independent tests should not be more than the repeatability limit (r). The content range and repeatability equations of the four metabolites of dipyrone are shown in Table 14.14.

TABLE 14.12 MS Parameters for the Detection of Dipyrone Metabolites

Metabolites	Reference Retention Time (min)	Quality ion (*m*/*z*)	Quantity Ion (*m*/*z*)	Collision Energy (V)	Decluster Potential (V)
4-Formylaminoantipyrine	2.76	232/104	232/104	21	28
		232/83		21	28
4-Aminoantipyrine	2.95	204/159	204/159	12	30
		204/111		13	30
4-Methylaminoantipyrine	2.39	218/56	218/56	15	24
		218/97		13	24
4-Acetylaminoantipyrine	2.79	246/228	246/228	13	32
		246/104		22	32
4-Isopropylaminoantipyrine (internal standard)	3.28	246/56	246/56	16	30
		246/125		13	30

TABLE 14.13 Max Deviation Allowed for Relative Ion Ratio in Qualitative Determination

Relative ion ratio k	$k > 50\%$	$20\% < k < 50\%$	$10\% < k < 20\%$	$k \leqq 10\%$
Max deviation allowed	±20%	±25%	±30%	±50%

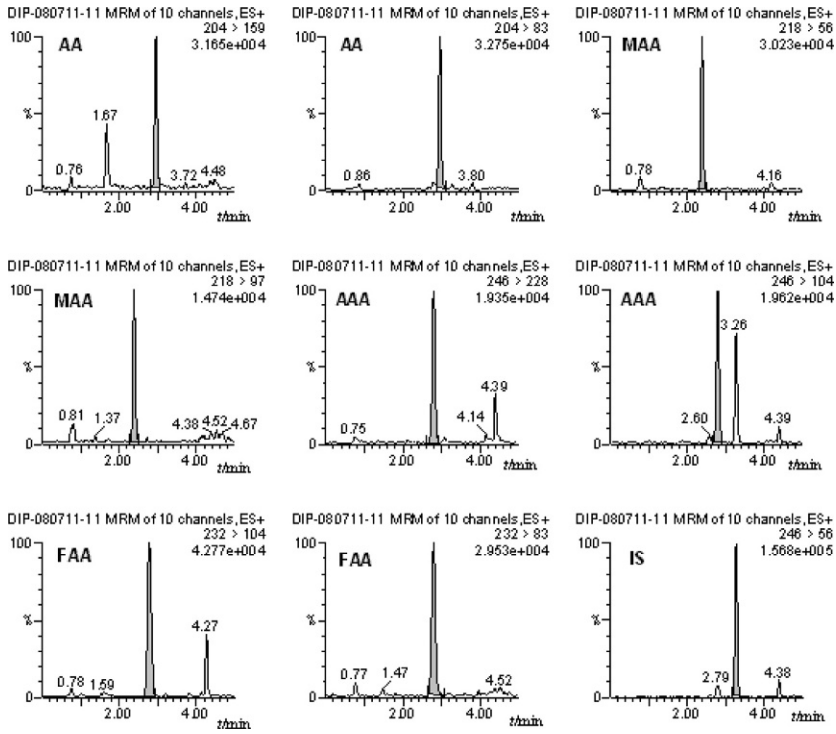

FIG. 14.5 MRM chromatogram of dipyrone metabolites with a concentration of 5.0 µg/L (2.76 min: 4-formylaminoantipyrine, 2.95 min: 4-aminoantipyrine, 2.39 min: 4-methylaminoantipyrine, 2.79 min: 4-acetylaminoantipyrine, 3.28 min: 4-isopropylaminoantipyrine (internal standard)).

TABLE 14.14 Content Range and Repeatability/Reproducibility Equations (Unit: μg/kg)

Compound		Content Range (μg /kg)	Repeatability Limit, r	Reproducibility Limit, R
Milk	4-Formylaminoantipyrine	5.0–50	$\lg r = 0.9814 \lg m - 0.5435$	$\lg R = 0.9031 \lg m - 0.5016$
	4-Aminioantipyrine	5.0–50	$\lg r = 1.0221 \lg m - 0.6365$	$\lg R = 0.9649 \lg m - 0.5252$
	4-Methylaminoantipyrine	5.0–50	$\lg r = 0.8383 \lg m - 0.4033$	$\lg R = 0.8449 \lg m - 0.4098$
	4-Acetylaminoantipyrine	5.0–50	$\lg r = 1.0333 \lg m - 0.6554$	$\lg R = 0.9731 \lg m - 0.5472$
Milk powder	4-Formylaminoantipyrine	40–400	$\lg r = 0.8907 \lg m - 0.2969$	$\lg R = 0.9931 \lg m - 0.5151$
	4-Aminioantipyrine	40–400	$\lg r = 1.1079 \lg m - 0.8764$	$\lg R = 1.0879 \lg m - 0.7730$
	4-Methylaminoantipyrine	40–400	$\lg r = 0.8218 \lg m - 0.1982$	$\lg R = 0.9789 \lg m - 0.5250$
	4-Acetylaminoantipyrine	40–400	$\lg r = 1.1130 \lg m - 0.8071$	$\lg R = 1.0793 \lg m - 0.7428$

Note: m *is the arithmetic mean of two test results.*

(2) Reproducibility

Under reproducibility conditions, the absolute value of the difference of the result from two independent tests should not be more than the reproducibility limit (R). The content range and repeatability equations of the four metabolites of dipyrone are shown in Table 14.14.

TABLE 14.15 The Added Level and Average Recovery of Dipyrone Metabolites in Milk

Metabolite of Dipyrone	Added Level (μg/kg)	Average Recovery (%)
4-Formylaminoantipyrine	5	97.5
	10	92.8
	20	96
	50	91.9
4-Aminoantipyrine	5	96.7
	10	88.6
	20	83.7
	50	85.5
4-Methylaminoantipyrine	5	80.4
	10	84.7
	20	84.2
	50	85.5
4-Acetylaminoantipyrine	5	90.2
	10	95.1
	20	97.9
	50	90.1

TABLE 14.16 The Added Level and Average Recovery of Dipyrone Metabolites in Milk Powder

Metabolite of Dipyrone	Added Level (μg/kg)	Average Recovery (%)
4-Formylaminoantipyrine	40	81.2
	80	97.5
	160	97.9
	400	96.6

TABLE 14.16 The Added Level and Average Recovery of Dipyrone Metabolites in Milk Powder—con'd

Metabolite of Dipyrone	Added Level (μg/kg)	Average Recovery (%)
4-Aminoantipyrine	40	81.5
	80	90.8
	160	98.8
	400	92.7
4-Methylaminoantipyrine	40	78.4
	80	88.1
	160	98.4
	400	96.1
4-Acetylaminoantipyrine	40	79.7
	80	93.6
	160	99.4
	400	94.3

14.8.8 RECOVERY

The recovery of four dipyrone metabolites in milk by this method is listed in Table 14.15.

The recovery of four dipyrone metabolites in milk powder by this method is listed in Table 14.16.

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Chapter 15

Quinoxaline

15.1

Curative Effects and Side Effects of Carbadox and Olaquindox

Carbadox (methyl-3-(2-quinoxalinylmethylene) carbazate-N^1,N^4-dioxide-CBX) is an antimicrobial drug that has been used as a feed additive for the prevention of swine dysentery and bacterial enteritis in young swine, and as a growth promoter. Carbadox has been shown to cause cancer in laboratory animals, but when fed to swine it is rapidly metabolized or transformed. Olaquindox (2-(N-2-hydroxyethylcarbamoyl)-3-methylquinoxaline-N^1,N^4-dioxide-OQX) is a similar quinoxaline-N-dioxide drug used in veterinary medicine. The side effects of olaquindox are extremely long tissue residence times, a strong cumulative toxicity, and a pronounced sensitivity of poultry to olaquindox.

Within the European Union (EU) the product licenses of both drugs were withdrawn in 1998, due to health concerns over possible carcinogenic, mutagenic, and photoallergenic effects of the drugs and their desoxy metabolites. To ensure confidence in the meat industry and to enforce the ban of the compounds, tissues of food-producing animals must be guaranteed free of such residues within the EU. The FDA did not approve Olaquindox to be used in feed. In China, Olaquindox can only be applied in feed for piglets under 35 kg body weight with a 35-day withdrawal period.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00015-4

15.2

Pharmacokinetics of Carbadox and Olaquindox

Carbadox is rapidly metabolized via mono and desoxy compounds to quinoxaline-2-carboxylic acid (QCA). QCA is the longest existing detectable metabolite found in tissue and was, therefore, designated as the marker substance for CBX use in animals. Metabolism of OQX, again via mono and desoxy compounds, produces 3-methyl-quinoxaline-2-carboxylic acid (MQCA), a compound structurally similar to QCA. MQCA is the last major remaining detectable metabolite of OQX, and therefore was designated the marker substance for the drug. Figs. 15.1 and 15.2 show the main metabolites of carbadox and olaquindox in animals.

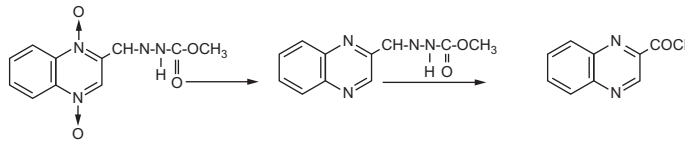


FIG. 15.1 Main metabolites of carbadox in animals.

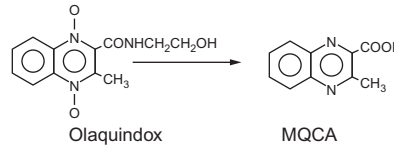


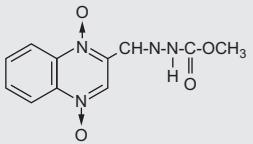
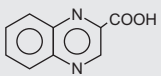
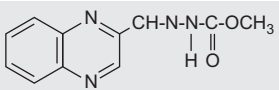
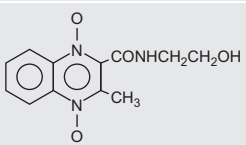
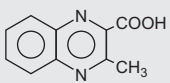
FIG. 15.2 Main metabolites of olaquindox in animals.

15.3

Chemical Structures and Maximum Residue Limits for Carbadox and Olaquindox

See Table 15.1 for chemical structures and maximum residue limits (MRLs) for carbadox and olaquindox.

TABLE 15.1 Chemical Structures and MRLs

Compound Names	Molecular Structure	Molecular Weight	Cas. No	MRLs (μg/kg)	
Carbadox		262.22	6804-07-5	China	Banned
				EU	Banned
				America	30 μg/kg in liver
				CAC	30 μg/kg in liver and 5 μg/kg in meat
				Japan	Banned
Quinoxaline-2-carboxylic acid		174.16	879-65-2	–	
Deoxycarbadox		230.22	55456-55-8	–	
Olaquindox		263.25	23696-28-8	China	50 μg/kg in liver and 4 μg/kg in meat
				EU	Banned
				Australia	300 μg/kg both in liver and meat
				CAC	30 μg/kg in liver and 5 μg/kg in meat
				Japan	300 μg/kg both in liver and meat
3-Methylquinoxaline-2-carboxylic acid		188.18	–	–	

15.4

Determination of the Residues of Carbadox, Olaquindox, and Related Metabolites in Bovine and Porcine Liver and Muscle Tissues—LC-MS-MS Method (GB/T 20746-2006)

15.4.1 SCOPE

This method is applicable to the determination of carbadox, desoxycarbadox (DCBX), quinoxalie-2-carboxylic acid (QCA), and 3-methylquinoxaline-2-carboxylic acid (MQCA) in bovine and porcine muscle and liver tissues.

The limit of determination of this method for carbadox, DCBX, QCA, and MQCA is 0.5 μg/kg.

15.4.2 PRINCIPLE

Carbadox is extracted from animal liver and muscle tissue in acetonitrile/ethyl acetate (1/1, v/v), and then defatted by n-hexane. The extraction is evaporated to dryness and the residue is dissolved in 0.1% formic acid/methanol (95/5, v/v). Portions are analyzed by LC-MS-MS and quantified with internal standard.

.Homogenized liver or muscle tissue is digested with 0.6% formic acid to deactivate naturally occurring enzymes. After overnight enzymatic hydrolysis with protease, the tissue extract is acidified, centrifuged, and filtered. DCBX, QCA, and MQCA are recovered from the tissue extract by using an Oasis MAX solid phase extraction (SPE) cartridge. DCBX is eluted first from the SPE cartridge with methylence chloride and then QCA and MQCA is eluted with ethyl acetate containing 2% formic acid. The eluted solutions are evaporated to dryness and dissolved in 0.1% formic acid/methanol (95/5, v/v). Portions are analyzed by LC-MS-MS and quantitated with an internal standard.

15.4.3 REAGENTS AND MATERIALS

Methanol, acetonitrile, and formic acid are all of HPLC grade. Acetic acid, sodium acetate, N, N-dimethylformamide, ethyl acetate, and neutral aluminum oxide are all of analytical grade. Acetonitrile/ethyl acetate (1/1, v/v);

2% formic acid in ethyl acetate; 0.6% formic acid; 0.1% formic acid; 0.1% formic acid/methanol (95/5, v/v); 0.1 M hydrochloric acid; 0.3 M hydrochloric acid; protease: stored below −18°C; 0.01 g/mL protease solution: stored at 4°C; 10% acetic acid; 0.05 mol/L sodium acetate solution (pH = 7.0): adjust the pH to 7.0 with 10% acetic acid; sodium acetate solution (0.05 mol/L, pH = 7.0)/methanol (95/5); methanol/water (20/80, v/v); 1.0 mol/L Tris solution: stored at 4°C; Waters Oasis MAX SPE column (60 mg, 3 mL), or other equivalent SPE column. The column is activated with 3 mL methanol and equilibrated with 3 mL water before use. The column should be always kept wet.

Carbadox, DCBX, QCA, MQCA, and QCA-d_4: the purity of these standards is more than 99%.

Stock standard solution: 100 mg/L; weigh each of the standards accurately; carbadox is dissolved in N and N-dimethylformamide and the others are dissolved with methanol. The solutions can be preserved in the refrigerator below −18°C for a year.

Working standards solution: according to the sensitivity and requirements, accurately measure an adequate volume of mixed standard working solution and dilute with blank matrix solution (extracted from blank sample) just before use. The solution can be preserved at 4°C for a week.

15.4.4 APPARATUS

LC-MS-MS system: equipped with an electrospray ion source; SPE vacuum apparatus; vacuum rotary evaporator; nitrogen evaporator; vortex mixer; vacuum pump; centrifuge with cooler, refrigeration to 4°C; pipettor: 1 mL, 2 mL; vortex flask: 150 mL; pH detector, with ±0.02 precision.

15.4.5 SAMPLE PRETREATMENT

(1) Sample preparation

Animal liver or muscle tissue is blended in a food blender and homogenized thoroughly; then take 0.5 kg portion for test use, which is then sealed and labeled.

(2) Extraction of carbadox

Accurately weigh 5 g (to the nearest 0.01 g) of the test sample into a 50-mL polytetrafluorothylene tube; then add 5.0 g neutral aluminum oxide. Add 25.0 mL acetonitrile/ethyl acetate (1/1, v/v) into the tube and shake on a vortex mixer for 5 min. Centrifuge the mixture at 5000 rpm for 5 min, transferring the supernatant into another clean 50-mL tube.

Add 10.0 mL n-hexane into the tube and shake on a vortex mixer for 2 min. Centrifuge the mixture at 5000 rpm for 5 min, discard the supernatant, and transfer the underlayer into the 150-mL vortex flask. Repeat the

procedure once and then combine the extracts into the same flask. Add an appropriate volume of d4-QCA as the internal standard and then evaporate to dryness in a 40°C water bath. Dissolve the residue in 1.0 mL 0.1% formic acid/methanol (95/5, v/v); filter through a 0.45-μm PTFE (polytetrafluorothylene) filter for LC-MS/MS analysis.

(3) Extraction and clean-up of DCBX, QCA, and MQCA

Weigh 5 g (to the nearest 0.01 g) test sample and add 10 mL 0.6% formic acid into a 50-mL tube; shake on a vortex mixer and then place the sample in a water or air bath at 47 ± 3°C for 1 h. Add 3.0 mL 1.0 mol/L Tris solution and 0.3 mL protease solution, then vortex mix, place the sample in water or air bath at 47 ±3°C for 16-18 h. Add 20.0 mL 0.3 mol/L hydrochloric acid, shake at vortex mixer for 5 min, centrifuge at 5000 rpm for 15 min below 10°C, filter the supernatant through a filter paper, then load the filtered solution on the SPE cartridge (condition the cartridge with 3.0 mL methanol and 3.0 mL water). Wash the cartridge with 30.0 mL sodium acetate solution (0.05 mol/L, pH = 7.0)/methanol (95/5), dry cartridge under full vacuum for at least 15 min, elute DCBX with 4 × 3.0 mL methylence chloride into a 15 mL tube, and add certain quantity of d_4-QCA into the same tube, then evaporate the tube to near dryness with nitrogen in 45°C water bath. Wash the cartridge consecutively with 3 × 3.0 mL methanol, 3.0 mL water, 3 × 3.0 mL 0.1 mol/L hydrochloric acid, and 2 × 3.0 mL methanol/water (20/80, v/v). Dry cartridge under full vacuum for at least 15 min and then wash the cartridge with 2.0 mL ethyl acetate. Elute QCA and MQCA with 3.0 mL ethyl acetate containing 2% formic acid, into the same tube, then evaporate the tube to dryness with a nitrogen 45°C water bath. Dissolve the residue in 1.0 mL 0.1% formic acid/methanol (95:5, v/v); filter the solution through a 0.45-μm PTFE (polytetrafluorothylene) filter for LC-MS analysis.

15.4.6 DETERMINATION

(1) Operating conditions

LC operating conditions: column: Inertsil ODS-3, 3 μm, 2.1 × 100 mm, or the like; mobile phase: methanol, acetonitrile, and 0.1% formic acid (time gradient program is listed in Table 15.2); flow rate: 0.2 mL/min; column temperature: 30°C; injection volume: 30 μL.

MS/MS operating conditions: Ionization mode: electrospray ionization mode; Scan mode: positive mode; Detection mode: multiple reaction monitoring (MRM); Ion spray voltage: 5000.00 V; Nebulizer gas: 8.00 L/min; Curtain gas: 7.00 L/min; Assistant gas: 5.00 L/min; Ionization temperature: 500°C; other MS parameters are listed in Tables 15.3 and 15.4.

(2) Qualitative analysis

Select one parent ion and two or more than two daughter ions for each analyte. If the deviation of relative retention time of the analyte between

TABLE 15.2 Time Gradient Program

Time (min)	Methanol (%)	Acetonitrile (%)	0.1% Formic Acid (%)
0	13	7	80
10.0	45	15	40
10.1	64	16	20
13.1	13	7	80
20.0	13	7	80

TABLE 15.3 MS/MS Parameters of Each Compound

Analytes	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (ms)	DP	FP	CE	CXP
Carbadox	263.2	90.1	50	31	120	49	8
		231.1	50	31	120	19	18
QCA	175.0	131.2	50	26	120	23	12
		129.2	50	26	120	21	12
DCBX	231.2	143.2	50	26	140	49	8
		199.2	50	31	150	31	10
MQCA	189.2	143.1	50	26	120	23	12
		145.1	50	26	120	21	12
QCA-d4	179.2	133.2	50	26	130	29	10
		135.1	50	26	130	23	10

the test sample and standard solution (the referent retention times of the analytes are listed in Table 15.5) is within ±2.5% under the same experimental conditions, and the difference of relative ion ratio of the analyte between test sample and standard solution is also within the error allowed (the max deviations allowed for relative ion ratios are listed in Table 15.6), then the corresponding analyte would be considered to be in the sample.

TABLE 15.4 Qualitative Ions and Quantitative Ions of Each Compound

Analytes	Qualitative ions (m/z)	Quantitative ions (m/z)	Quantity Internal Standard Ion (m/z)
Carbadox	263.2 > 90.1 263.2 > 231.1	263.2 > 231.1	179.2 > 133.2
QCA	175.0 > 129.2 175.0 > 131.2	175.0 > 131.2	179.2 > 133.2
DCBX	231.2 > 143.2 231.2 > 99.2	231.2 > 143.2	179.2 > 133.2
MQCA	189.2 > 143.1 189.2 > 145.1	189.2 > 145.1	179.2 > 133.2

TABLE 15.5 The Retention Times of the Analytes

Analytes	CBX	QCA	DCBX	MQCA
Retention time (min)	9.11	9.95	14.43	10.97

TABLE 15.6 Allowed Error of Relative Ion Abundance

Relative Abundance k	$20 < k < 50$	$k \leq 10$
Allowed error	±25%	±50%

(3) Quantitative analysis

Mixed standards are injected into the instrument in a separate run. Using peak area or ratio of peak area as y-axis, and concentration as x-axis, the content of the sample is quantified by a standard calibration curve. The response of carbadox, DCBX, QCA, and MQCA in the standard working solution and sample solution should be in the linear range of the instrumental detection. The retention times of the four analytes are listed in Table 15.5. Under the preceding chromatographic conditions, a chromatogram of the standard is shown as Fig. 15.3.

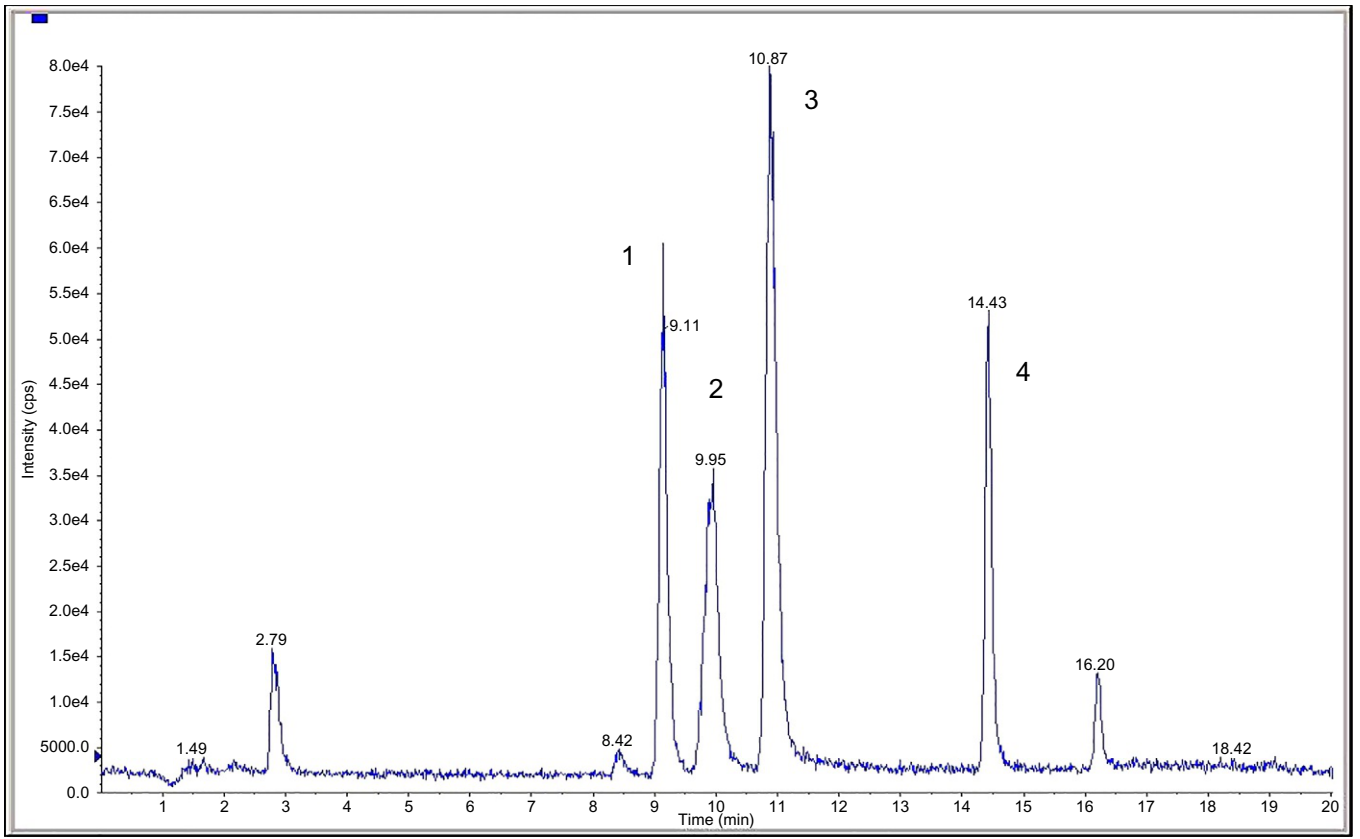


FIG. 15.3 Ion chromatograms of carbadox, DCBX, QCA and MQCA standards (1. Carbadox: 9.11 min; 2. QCA: 9.95 min; 3. MQCA: 10.97 min; 4. DCBX: 14.43 min).

TABLE 15.7 Content Range and Repeatability/Reproducibility Equations

Analytes	Content Range (μg /kg)	Repeatability Limit (r)	Reproducibility Limit (R)
CBX	0.5–2.0	$\lg r = 1.0815 \lg m - 1.0388$	$\lg R = 0.9533 \lg m - 0.6908$
QCA	0.5–2.0	$\lg r = 1.0623 \lg m - 0.9762$	$\lg R = 0.9628 \lg m - 0.7026$
DCBX	0.5–2.0	$\lg r = 0.9356 \lg m - 0.8530$	$\lg R = 0.8030 \lg m - 0.3055$
MQCA	0.5–2.0	$\lg r = 0.9850 \lg m - 0.8650$	$\lg R = 0.8155 \lg m - 0.4586$

Note: m *is the arithmetic mean of two test results.*

15.4.7 PRECISION

The data in this part are acquired under the regulation of GB/T 6379. The values of repeatability and reproducibility are calculated at a confidence level of 95%.

(1) Repeatability

Under repeatability conditions, the absolute value of the difference result from two independent tests should not be more than the repeatability limit (r). The content range and repeatability equations of the four analytes are shown in Table 15.7.

(2) Reproducibility

Under reproducibility conditions, the absolute value of the difference result from two independent tests should not be more than the reproducibility limit (R). The content range and repeatability equations of the four analytes are shown in Table 15.7.

15.4.8 RECOVERY

Under optimized conditions, the recoveries of Carbadox, QCA, DCBX, MQCA using this method are listed in Table 15.8.

TABLE 15.8 The Fortifying Concentrations and Corresponding Recoveries

Analytes	Added Level (μg/kg)	Average Recovery (%)			
		Pork Muscle	Pork Liver	Bovine Muscle	Bovine Liver
Carbadox	0.5	79.8	80.2	81.4	84.8
	1.0	89.1	90.1	89.7	89.0
	2.0	89.6	91.1	92.0	92.3
	5.0	87.2	89.2	85.8	86.8
DCBX	0.5	83.0	82.2	81.8	78.8
	1.0	87.3	87.0	92.1	91.6
	2.0	96.7	89.1	94.9	96.6
	5.0	84.6	83.1	83.0	85.0
QCA	0.5	81.8	80.0	80.8	80.2
	1.0	87.5	88.0	86.7	84.9
	2.0	92.9	94.9	92.7	94.9
	5.0	85.4	84.2	86.0	82.8
MQCA	0.5	76.2	77.6	78.0	76.2
	1.0	86.4	84.8	83.2	85.2
	2.0	94.6	96.2	93.9	92.8
	5.0	82.2	82.8	81.4	84.8

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FURTHER READING

[1] Hutchinson MJ, Young PY, Hewitt SA, Faulkner D, Kennedy DG. Analyst 2002;127:342.

[2] Hutchinson MJ, Young PB, Kennedy DG. J Chomatogr B 2005;816:15.

15.5

Determination of the Residues of Metabolites of Carbadox and Olaquindox in Milk and Milk Powder—LC-MS-MS Method (GB/T 22984-2008)

15.5.1 SCOPE

This method is applicable to the inspection of the metabolites of cabadox-quinoxalie-2-carboxylic acid (QCA) and the metabolites of olaquindox-3-methyl-quinoxaline-2-carboxylic acid (MQCA) in milk and milk powder.

The limit of determination of this method for both QCA and MQCA is 0.5 μg/kg (milk), 4.0 μg/kg (milk powder).

15.5.2 PRINCIPLE

Homogenized milk and milk powder are digested with 0.6% formic acid to deactivate naturally occurring enzymes. After overnight enzymatic hydrolysis with protease, the milk and milk powder are acidified, centrifuged, and filtered. QCA and MQCA are recovered from the milk and milk powder extract by using an Oasis MAX solid phase extraction (SPE) cartridge. Portions are analyzed by LC-MS-MS and quantitated with an internal standard.

15.5.3 REAGENTS AND MATERIAL

Unless otherwise specified, all reagents used should be of analytical grade. "Water" is deionized.

Methanol: HPLC grade
Formic acid: HPLC grade
Sodium acetate
Ethyl acetate
2% Formic acid in ethyl acetate
0.6% Formic acid
0.1% Formic acid
0.1% Formic acid/methanol (19+1)
0.1 mol/L Hydrochloric acid
0.3 mol/L Hydrochloric acid
Protease: stored below −18°C
0.01 g/mL Protease solution: stored at 4°C
10% Acetic acid
0.05 mol/L Sodium acetate solution (pH = 7.0): adjust the pH to 7.0 with 10% acetic acid
Sodium acetate solution (0.05 mol/L, pH = 7.0)/methanol (19+1)
Methanol/water (1+4)
Tris
1.0 mol/L Tris solution: stored at 4°C
Standards of QCA, MQCA and QCA—d4
Stock standard solution: 100 mg/L, accurately weigh each of the standards and dissolve with methanol; the solutions can be preserved in the refrigerator below −18°C for a year.
Working standards solution: according to the sensitivity and requirements, accurately measure an adequate volume of mixed standard working solution and dilute with blank matrix solution (extracted from blank sample) just before use. The solution can be preserved at 4°C for a week.
Working standards solution of QCA—d4
Waters Oasis MAX SPE column (60 mg, 3 mL), or the like
Filter

15.5.4 APPARATUS

LC-MS-MS system: equipped with an electrospray ion source.
SPE vacuum apparatus
Nitrogen evaporator
Vortex mixer
Analytical balance
Vacuum pump
Centrifuge

Pipettor: 10 μL–100 μL, 100 μL–1000 μL.
Ttube
pH detector, with ±0.02 precision.

15.5.5 SAMPLE PRETREATMENT

(1) Sample preparation

Blend milk and milk powder in a food blender and homogenize thoroughly; then take a 0.5-kg portion for the test use; seal and label.

The test sample should be stored below 4°C.

(2) Extraction of QCA and MQCA

Weigh 5 g (to the nearest 0.01 g) milk sample into a 50-mL tube; add an appropriate volume (2.0 ng/g) of d4-QCA into the same tube and add 10 mL 0.6% formic acid; shake on vortex mixer and then place the sample in water or air bath at 47 ± 3°C for 1 h. Add 3.0 mL 1.0 mol/L Tris solution and 0.3 mL protease solution; then vortex mix, place the sample in water or air bath at 47 ± 3°C for 16–18 h. Add 20.0 mL 0.3 mol/L hydrochloric acid and shake on a vortex mixer for 5 min; centrifuge at 5000 rpm for 15 min below 10°C. Filter the supernatant through a filter paper and then load the filtered solution on the SPE cartridge (condition the cartridge with 3.0 mL methanol and 3.0 mL water).

Weigh 12.5 g milk powder into a beaker, dissolve with water (35–50°C), add water to 100 g, and homogenize thoroughly. Then weigh a 5-g (to the nearest 0.01 g) test sample into a 50-mL tube, according to the above-mentioned extraction steps of milk sample.

(3) Clean-up

Wash the cartridge with 15.0 mL sodium acetate solution (0.05 mol/L, pH = 7.0)/methanol (19 + 1). Dry cartridge under full vacuum for at least 15 min. Wash the cartridge consecutively with 5.0 mL methanol, 3.0 mL water, 5.0 mL 0.1 mol/L hydrochloric acid, and 3.0 mL methanol/water (1 + 4) (4.16). Dry cartridge under full vacuum for at least 15 min and then wash the cartridge with 2.0 mL ethyl acetate. Elute QCA and MQCA with 3.0 mL ethyl acetate containing 2% formic acid into the tube; then evaporate the tube to dryness with nitrogen 45°C water bath. Dissolve the residue in 1.0 mL 0.1% formic acid/methanol (19 + 1) and filter the solution through a 0.2-μm PTFE (polytetrafluorothylene) filter for LC-MS analysis.

(4) Blank test

The operation of the blank test is the same as the procedure described previously, but with omission of the sample addition.

15.5.6 DETERMINATION

(1) LC operating conditions:

Column: Inertsil ODS-3, 3 μm, 100 mm × 2.1 mm (i.d.), or equivalent;
Mobile phase: methanol, acetonitrile and 0.1% formic acid (time gradient program is listed in Table 15.9);

TABLE 15.9 Time Gradient Program

Time (min)	Methanol (%)	Acetonitrile (%)	0.1% Formic Acid (%)
0	13	7	80
10.0	45	15	40
10.1	64	16	20
13.1	13	7	80
20.0	13	7	80

Flow rate: 0.2 mL/min;
Column temperature: 30°C;
Injection volume: 30 μL;
Ionization mode: electrospray ionization mode
Scan mode: positive mode
Detection mode: multiple reaction monitoring (MRM)
Ion spray voltage: 5000.00 V
Nebulizer gas: 8.00 L/min;
Curtain gas: 7.00 L/min;
Assistant gas: 5.00 L/min
Ionization temperature: 500°C
Other MS parameters are listed in Table 15.10.

(2) Qualitative determination

If the analyte in the sample has a similar retention time as that in the standard solution under the same experimental conditions and the ion pairs determined both appear in the mass spectrometric chromatography

TABLE 15.10 MS/MS Parameters of Each Compound

Compounds	Qualitative Ions (*m*/*z*)	Quantitative Ions (*m*/*z*)	Quantity Internal Standard Ion (*m*/*z*)	DP	FP	CE	CXP
QCA	175.0/129.2	175.0/131.2	179.2/133.2	26	120	23	12
	175.0/131.2			26	120	21	12
MQCA	189.2/143.1	189.2/145.1	179.2/133.2	26	120	23	12
	189.2/145.1			26	120	21	12

TABLE 15.11 The Referent Retention Times of the Analytes

Analytes	QCA	MQCA
Retention time (min)	10.10	11.11

with the background subtracted, and the difference of ion ratio between analyte and sample is within the error allowed (the ranges of error allowed are listed in Table 15.6), then the sample would be judged to exist in the residue . The referent retention times of the four analytes are listed in Table 15.11.

(3) Quantitative determination

Mixed standards are injected into the instrument in a separate run. Using peak area or the ratio of peak area as the *y*-axis and concentration as the *x*-axis, the content in the sample is quantified by the standard calibration curve. The response of QCA and MQCA in the standard working solution and sample solution should be in the linear range of the instrumental detection. The ion chromatograms of QCA and MQCA standards are shown in Figs. 15.4 and 15.5.

15.5.7 PRECISION

The data in this part are acquired under the regulations of GB/T 6379. The values of repeatability and reproducibility are calculated with the confidence level set to 95%.

(1) Repeatability

Under repeatability conditions, the absolute value of the difference results from two independent tests should not be more than the repeatability limit (r). The content range and repeatability equations of the three analytes are shown in Table 15.12.

(2) Reproducibility

Under reproducibility conditions, the absolute value of the difference results from two independent tests should not be more than the reproducibility limit (R). The content range and repeatability equations of the three analytes are shown in Table 15.12.

15.5.8 RECOVERY

The added level and average recovery of QCA and MQCA in two materials are listed in Table 15.13.

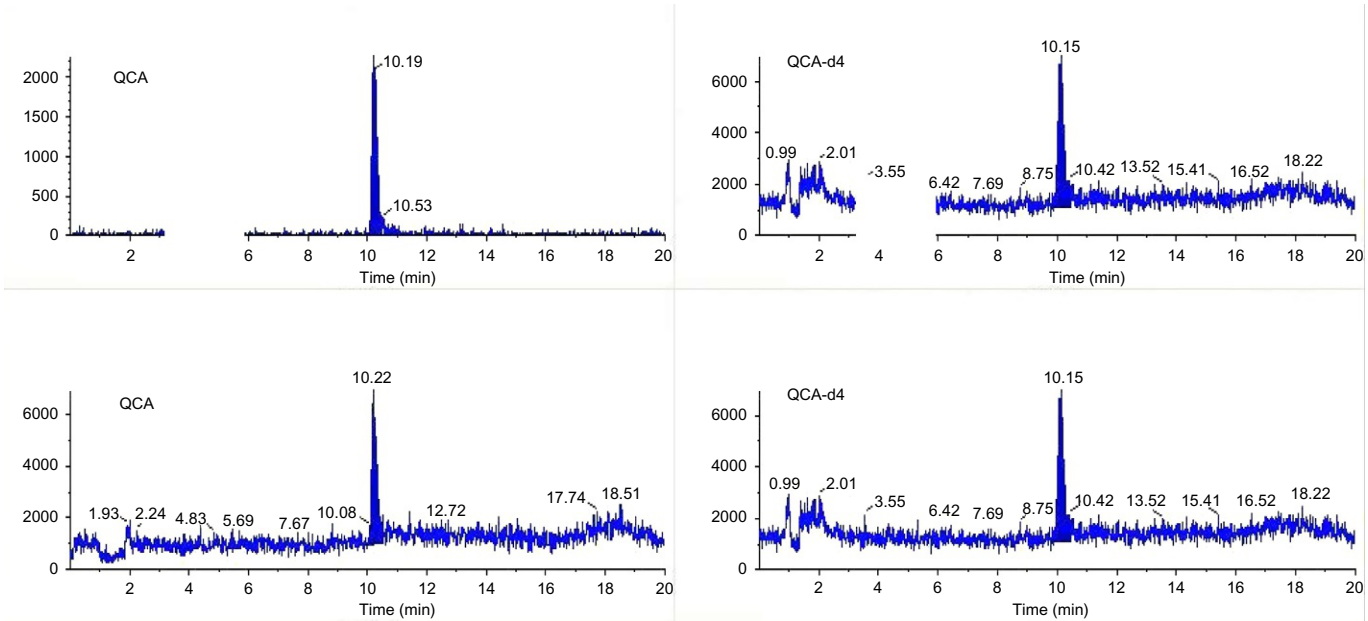

FIG. 15.4 Ion chromatograms of QCA and QCA-d4.

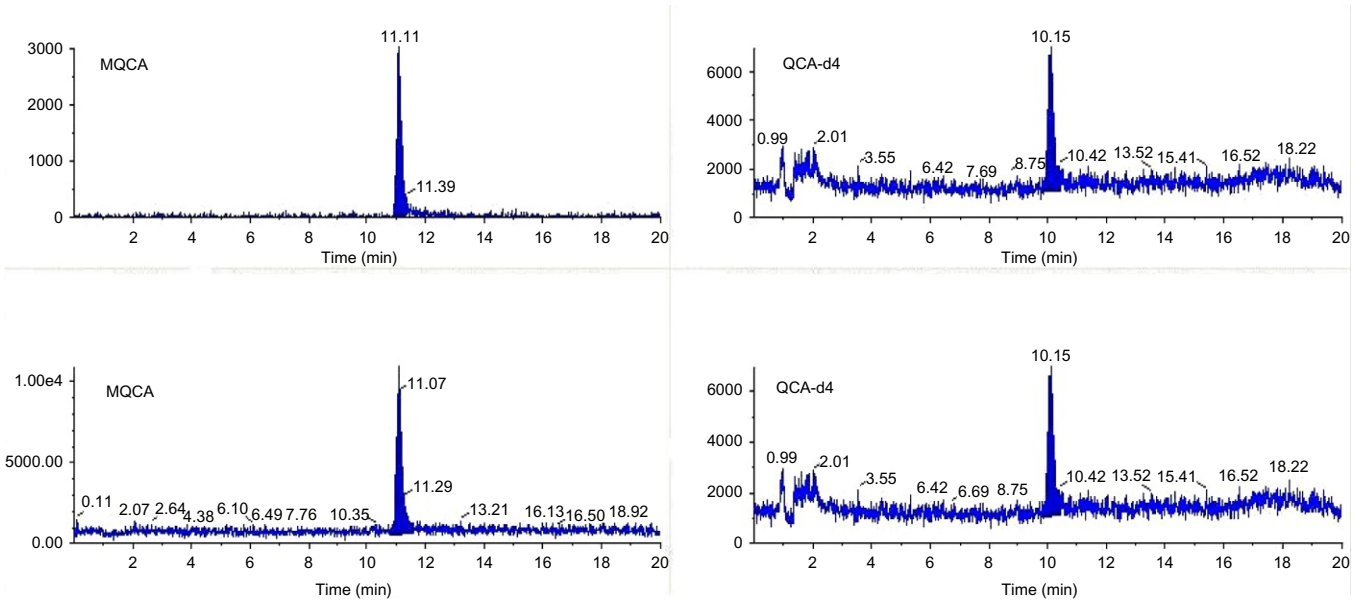

FIG. 15.5 Ion chromatograms of MQCA and QCA-d4.

TABLE 15.12 Content Range and Repeatability/Reproducibility Equations

Compound	Sample	Content Range (μg/kg)	Repeatability Limit, *r*	Reproducibility Limit, *R*
QCA	Milk	0.5–5.0	$\lg r = 0.8797 \lg m - 1.0179$	$\lg R = 0.8445 \lg m - 0.6489$
	Milk powder	4.0–40.0	$\lg r = 0.9561 \lg m - 1.0554$	$\lg R = 0.7578 \lg m - 0.6163$
MQCA	Milk	0.5–5.0	$\lg r = 0.8697 \lg m - 1.0772$	$\lg R = 0.7240 \lg m - 0.6492$
	Milk powder	4.0–40.0	$\lg r = 0.9821 \lg m - 0.9851$	$\lg R = 0.8600 \lg m - 0.5997$

Note: m *is the arithmetic mean of two test results.*

TABLE 15.13 Added Level and Average Recoveries of QCA and MQCA for Milk and Milk Powder

Compounds	Matrix	Added Level (μg/kg)	Average Recovery (%)
QCA	Milk	0.5	83.1
		1.0	75.7
		2.0	80.5
		5.0	89.9
	Milk powder	4.0	79.1
	Milk	8.0	85.6
		16.0	87.7
		40.0	88.7
MQCA	Milk	0.5	77.4
		1.0	85.3
		2.0	72.1
		5.0	79.1
	Milk powder	4.0	75.0
		8.0	85.3
		16.0	88.4
		40.0	77.0

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Chapter 16

Nitroimidazole

16.1

Curative Effect and Side Effects of Nitroimidazoles

Nitroimidazoles are imidazole heterocycles with a nitro group used to prevent and treat histomoniasis and coccidiosis in poultry. They have also been used for the treatment of genital trichomoniasis in cattle and hemorrhagic enteritis in pigs. The most common example is metronidazole. Other heterocycles such as nitrothiazoles (thiazole) are also used for this purpose. Nitroheterocycles may be reductively activated in hypoxic cells, and then undergo redox recycling or decompose to toxic products. They are suspected to be carcinogenic and mutagenic, so nitroimidazoles have been banned from use in food-producing animals within the European Union (EU), the United States, and other countries, including China.

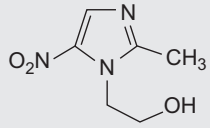
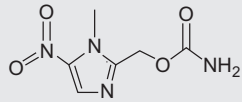
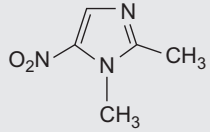
16.2

Chemical Structures and Maximum Residue Limits for Nitroimidazoles

Table 16.1 contains the chemical structures and maximum residue limits (MRLs) for nitroimidazoles.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00016-6

TABLE 16.1 Nitroimidazole Chemical Structures and MRLs

Compound Names	Structure	Molecular Weight	Cas. No.	MRL (μg/kg)
Metronidazole		171.15	443-48-1	CN: ND JP: ND EU: ND US: ND
Ronidazole		200.15	7681-76-7	CN: ND JP: ND EU: ND US: ND
Dimetridazole		141.13	551-92-8	CN: ND JP: ND EU: ND US: ND

16.3

Determination of Metronidazole, Ronidazole, and Dimetridazole Residues in Honey—LC-VU Method (GB/T 18932.26-2005)

16.3.1 SCOPE

This method is applicable to the determination of metronidazole, ronidazole, and dimetridazole residues in honey. The limit of determination of the method for metronidazole, ronidazole, and dimetridazole is 0.010 mg/kg.

16.3.2 PRINCIPLE

Nitromidazole residues in the test sample are extracted with ethyl acetate. The extract is concentrated and the residue is dissolved with water. The solution is

cleaned up with an Oasis HLB cartridge and Bakerbond carboxylic acid cartridge. The solution is analyzed by LC-UV, using an external standard.

16.3.3 REAGENTS AND MATERIALS

Unless otherwise specified, water is the first grade of GB/T6682 specified.

Methanol: solution (40+6+54); Oasis HLB cartridge or equivalent: 500 mg, 6 mL. Condition with 5 mL methanol and 10 mL water and keep the cartridge wet; BAKERBOND Carboxylic Acid cartridge or equivalent: 500 mg, 3 mL. Condition with 5 mL ethyl acetate and keep the cartridge wet; Metronidazole, ronidazole, and dimetridazole standards: Purity $\geq$98%.

Stock standard solutions of metronidazole, ronidazole, and dimetridazole: 1.0 mg/mL. Accurately weigh adequate amount of metronidazole, ronidazole, and dimetridazole standards. Separately prepare stock standard solutions of 1.0 mg/mL with methanol. The solutions should be stored in the dark below 4°C and used within 2 months.

Working standard mixed solutions of metronidazole, ronidazole, and dimetridazole: Separately pipette adequate amount of stock standard solutions of metronidazole, ronidazole, and dimetridazole to prepare working standard mixed solutions with mobile phase. The solutions must be freshly prepared with each batch.

16.3.4 APPARATUS

HPLC: Equipped with UV detector; Analytical balances: Capable of weighing to 0.1 mg, 0.01 g; Vortex mixer; Solid phase extraction (SPE) vacuum apparatus; Shaker; Centrifuge tube: 50 mL, with stopper; Vacuum pump: Vacuum to 80 kPa; Centrifuge; Rotary evaporator; Tube: 5 mL; pH Meter: Capable of measuring $\pm$0.02 unit; Pear-shaped flask: 200 mL.

16.3.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath and warm at $\leq$60°C with occasional shaking until liquefied. Mix thoroughly and promptly cool to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label. Store test samples at room temperature.

(2) Extraction

Weigh 6 g of test sample (accurate to 0.01 g) into a 50-mL centrifuge tube. Add 6 mL water. Mix thoroughly in a vortex mixer. Add 20 mL ethyl acetate. Shake on shaker for 20 min. Centrifuge for 10 min. Pipette supernatant to pear-shaped flask. Proceed twice with

20 mL ethyl acetate and combine the ethyl acetate phases. Evaporate the solution to dryness with rotary evaporator at 45°C. Add 5 mL water to dissolve the residue.

(3) Clean-up

Decant the preceding extract into the Oasis HLB cartridge. Pass through Oasis HLB cartridge at a flow rate of ≤3 mL/min. Rinse the pear-shaped flask with 5 mL water and decant it into the cartridge. Wash the cartridge with 5 mL methanol + water (5+95). Discard the effluents. Dry the cartridge by drawing air through it for 20 min under a 65-kPa vacuum. Connect the Oasis HLB cartridge to the carboxylic acid cartridge. Elute the Oasis HLB cartridge with 5 mL ethyl acetate at a flow rate of ≤3 mL/min and pass through the carboxylic acid cartridge. Discard the Oasis HLB cartridge. Wash the carboxylic acid cartridge with 5 mL ethyl acetate and 5 mL acetonitrile. Discard the effluents. Dry the cartridge by drawing air through it for 5 min under 65-kPa vacuum. Elute the carboxylic acid cartridge with 2 mL eluting solution and collect the eluate in a 5-mL tube. Make up to 2 mL with eluting solution. The solution is ready for LC-UV determination.

16.3.6 DETERMINATION

(1) Operating conditions

Column: Diamonsil C_{18}, 5 μm,250 mm × 4.6 mm, or equivalent; Mobile phase: Acetonitrile + acetate buffer solution (10+90); Flow rate: 1.0 mL/min; Column temperature: 25°C; Injection volume: 50 μL; Detection wavelength: 315 nm.

(2) HPLC determination

Inject the working standard mixed solutions of metronidazole, ronidazole, and dimetridazole in sequence. Draw the standard curve with peak area vs. sample concentration. The sample is quantified with the standard curve. The responses of metronidazole, ronidazole, and dimetridazole in the sample solution should be in the linear range of the instrumental detection. For an LC chromatogram of metronidazole, ronidazole, and dimetridazole standards, see Fig. 16.1. Under the preceding operating conditions, the retention times of metronidazole, ronidazole, and dimetridazole are 12.7 min, 15.0 min, 21.2 min.

16.3.7 PRECISION

The precision data of the method for this standard have been determined according to the stipulations of GB/T 6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

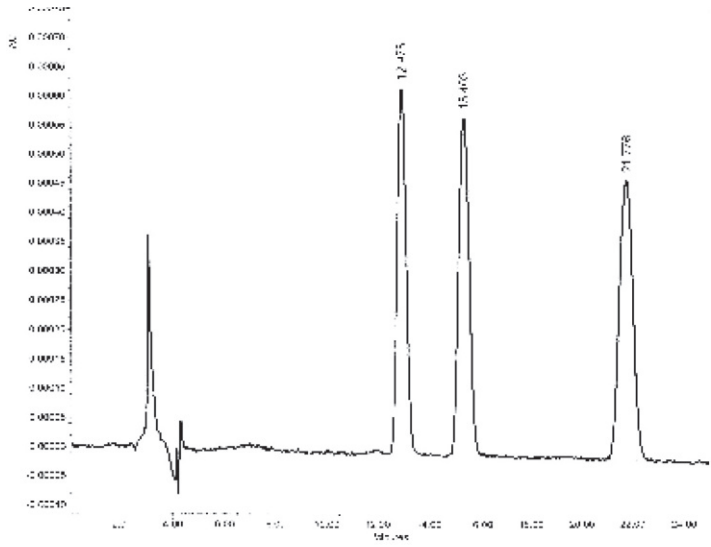


FIG. 16.1 Chromatogram of metronidazole, ronidazole, and dimetridazole standards.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results should not exceed the limit of repeatability (r); the content range and repeatability equations of metronidazole, ronidazole, and dimetridazole in honey are shown in Table 16.2.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations shall be reconducted and completed.

TABLE 16.2 The Analytical Range and Repeatability/Reproducibility Equations

Analytes	Content Range (mg/kg)	Repeatability (r)	Reproducibility (R)
Metronidazole	0.010–0.100	$r=0.0303m+2.1673$	$R=0.0807m+1.0408$
Ronidazole	0.010–0.100	$r=0.0608m+1.8727$	$R=0.0620m+1.7190$
Dimetridazole	0.010–0.100	$r=0.0627m+1.4411$	$\lg R=0.7099\lg m-0.4377$

Note: m *is the average value obtained from two independent determination results.*

TABLE 16.3 The Recoveries of Metronidazole, Ronidazole, and Dimetridazole in Honey

Analytes	Fortifying Concentration (mg/kg)	Recovery (%)
Metronidazole	0.010	86.27
	0.020	87.13
	0.050	87.35
	0.100	86.33
Ronidazole	0.010	84.64
	0.020	89.38
	0.050	82.87
	0.100	87.99
Dimetridazole	0.010	85.96
	0.020	84.86
	0.050	90.70
	0.100	88.61

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results should not exceed the limit of reproducibility (R); the analytical range and reproducibility equations of metronidazole, ronidazole, and dimetridazole in honey are shown in Table 16.2.

16.3.8 RECOVERY

Under optimized conditions, the fortifying concentrations of metronidazole, ronidazole, and dimetridazole in honey and their corresponding average recoveries of this method are listed in Table 16.3.

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16.4

Determination of Metronidazole, Ronidazole, and Dimetridazole Residues in Honey—LC-MS-MS Method (GB/T 20744-2006)

16.4.1 SCOPE

This method is applicable to the determination of metronidazole, ronidazole, and dimetridazole residues in honey.

The limits of determination of the method for this method: 0.1 μg/kg for metronidazole, 0.2 μg/kg for ronidazole and dimetridazole.

16.4.2 PRINCIPLE

The nitromidazole residues in the test samples are extracted with ethyl acetate. The extracts are concentrated and cleaned up on a BAKERBOND carboxylic acid cartridge. The solutions are analyzed by liquid chromatography–tandem mass spectrometry (LC-MS-MS) using an external standard for quantification.

16.4.3 REAGENTS AND MATERIALS

Methanol: HPLC grade; Acetonitrile: HPLC grade; Ethyl acetate: HPLC grade; Formic acid: G.R.; Sodium sulfate: Anhydrous, analytically pure. Ignited at 650°C for 4 h and kepy in a desiccator; Eluting solution: Methanol +

acetonitrile +1% formic acid solution (40+18+42); BAKERBOND Carboxylic Acid cartridge or equivalent: 500 mg, Condition with 5 mL ethyl acetate, keep the cartridge wet; Filter membrane: 0.20 μm.

Metronidazole, ronidazole, and dimetridazole standards: Purity ≥98%.

Stock standard solutions of metronidazole, ronidazole, and dimetridazole: 1.0 mg/mL. Stock standard solutions are prepared by accurately weighing adequate amounts of metronidazole, ronidazole, and dimetridazole standards into 10-mL volumetric flasks and dissolving and diluting to volume with methanol. The solutions should be stored in the dark below 4°C and must be used within 2 months.

Working standard mixed solutions A and B of metronidazole, ronidazole, and dimetridazole: Working standard mixed solutions A are prepared by diluting an adequate amount of stock standard solutions of metronidazole, ronidazole, and dimetridazole to 1.0 μg/mL for metronidazole, 2.0 μg/mL for ronidazole and dimetridazole with methanol. Working standard mixed solution B is prepared by diluting an adequate amount of working standard mixed solution A to 0.010 μg/mL for metronidazole and 0.020 μg/mL for ronidazole and dimetridazole with methanol. The solutions must be freshly prepared.

Working standard mixed solutions of metronidazole, ronidazole, and dimetridazole in matrix: Working standard mixed solutions of metronidazole, ronidazole, and dimetridazole in matrix are prepared by diluting an adequate amount of working standard mixed solutions A and B to 0.25, 0.50, 1.00, and 5.00 ng/mL with blank extract that has been taken through the method with the rest of the samples. The solutions must be freshly prepared.

16.4.4 APPARATUS

LC-MS-MS: Equipped with ESI source; Analytical balances: Capable of weighing to 0.1 mg, 0.01 g; Vortex mixer; SPE vacuum apparatus; Shaker; Centrifuge tube: 50 mL, with stopper; Vacuum pump: Vacuum to 80 kPa; Centrifuge; Rotary evaporator; Tube: 5 mL; Pear-shaped flask: 150 mL; Column funnel.

16.4.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

If the sample is not crystallized, mix it thoroughly by stirring. If crystallized, place the closed container in a water bath, warm at ≤60°C with occasional shaking until liquefied, mix thoroughly, and promptly cool to room temperature. Pour 0.5 kg of the prepared test sample into a clean and dry sample bottle. Seal and label. Store the test samples at room temperature.

(2) Extraction

Weigh 10 g of test sample (accurate to 0.01 g) into a 50-mL centrifuge tube. Add 10 mL water. Mix thoroughly in a vortex mixer. Add 20 mL ethyl acetate. Shake on shaker for 20 min. Centrifuge at 3000 rpm for 5 min. The supernatants are passed through the column funnel containing 25 g sodium sulfate to the pear-shaped flask. Proceed twice with 20 mL ethyl acetate and combine the ethyl acetate phases. Evaporate the solution to about 2 mL with a rotary evaporator at 45°C for clean-up.

(3) Clean-up

The previously described extracts are pipetted into the carboxylic acid cartridge. Wash the pear-shaped flask and the carboxylic acid cartridge with 4 mL ethyl acetate and 4 mL acetonitrile, respectively. Discard all effluents. Dry the cartridge by drawing air through it for 2 min under a 65-kPa vacuum. Elute the carboxylic acid cartridge with 2 mL elution solution at $\leq$3 mL/min and collect the eluate in a 5-mL tube. Make up to 2 mL with elution solution. The solution is ready for LC-MS-MS analysis.

16.4.6 DETERMINATION

(1) Operating conditions

Column: Atlantis dC_{18}, 3 μm, 150 mm × 2.1 mm, or equivalent; Mobile phase: Acetonitrile + 0.1% formic acid solution (30 + 70); Flow rate: 200 μL/min; Column temperature: 30°C; Injection volume: 20 μL.

Ion source: ESI source; Scan mode: Positive scan; Monitor mode: MRM; Ionspray voltage: 5500 V; Nebulizer gas: 0.069 MPa; Curtain gas: 0.069 MPa; Turbo ionspray gas rate: 6 L/min; Source temperature: 400°C; for MRM transitions for precursor/product ion, quantifying for precursor/product ion, declustering potential, and collision energy, see Table 16.4.

(2) HPLC-MS/MS analysis

Inject the working standard mixed solutions of metronidazole, ronidazole, and dimetridazole in sequence. Draw the standard curve with peak area vs. sample concentration. The sample is quantified with the standard curve. The responses of metronidazole, ronidazole, and dimetridazole in the sample solution should be in the linear range of the instrumental detection. For total ion chromatograms of metronidazole, ronidazole, and dimetridazole standards, see Fig. 16.2. Under the preceding operating conditions, for the retention times of metronidazole, ronidazole, and dimetridazole, see Table 16.5.

16.4.7 PRECISION

The precision data of the method for this standard have been determined according to the stipulations of GB/T 6379.1 and GB/T 6379.2. The values

TABLE 16.4 MS Parameters for the Detection of Metronidazole, Ronidazole, and Dimetridazole

Analytes	MRM Transitions for Precursor/Product Ion (*m*/*z*)	Quantifying for Precursor/Product Ion (*m*/*z*)	Declustering Potential (V)	Collision Energy (V)
Metronidazole	172.1/128.1	172.1/128.1	30	19
	172.1/82.1		30	34
Ronidazole	201.1/140.2	201.1/140.2	26	14
	201.1/110.1		26	20
Dimetridazole	142.2/96.1	142.2/96.1	40	22
	142.2/81.2		40	40

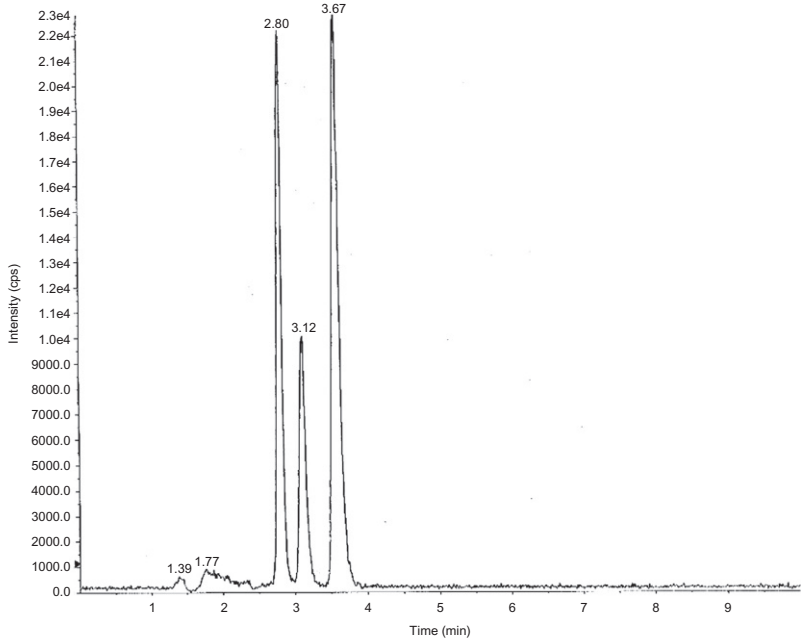

FIG. 16.2 Total ion chromatogram of metronidazole, ronidazole, and dimetridazole standards.

TABLE 16.5 Retention Times of Metronidazole, Ronidazole, and Dimetridazole

Analytes	Retention Time (min)
Metronidazole	2.80
Ronidazole	3.12
Dimetridazole	3.57

of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results should not exceed the limit of repeatability (r); the content range and repeatability equations of metronidazole, ronidazole, and dimetridazole in honey are shown in Table 16.5.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results should not exceed the limit of reproducibility (R); the analytical range and reproducibility equations of metronidazole, ronidazole, and dimetridazole in honey are shown in Table 16.6.

TABLE 16.6 The Analytical Range and Repeatability/Reproducibility Equations

Analytes	Analytical Range (μg/kg)	Repeatability (μg/kg)	Reproducibility (μg/kg)
Metronidazole	0.05–1.0	$\lg r = 0.8439 \lg m - 0.9316$	$\lg R = 0.9502 \lg m - 0.7466$
Ronidazole	0.1–2.0	$\lg r = 1.2008 \lg m - 0.5954$	$\lg R = 1.1100 \lg m - 0.5884$
Dimetridazole	0.1–2.0	$r = 0.1519m + 0.0003$	$\lg R = 0.9557 \lg m - 0.7881$

Note: m *is the average value obtained from two independent determination results.*

TABLE 16.7 The Recoveries of Metronidazole, Ronidazole, and Dimetridazole in Honey

Analytes	Fortifying Concentration (μg/kg)	Recovery (%)
Metronidazole	0.05	76.0
	0.10	79.0
	0.20	77.0
	1.00	82.4
Ronidazole	0.10	69.5
	0.20	78.5
	0.40	73.9
	2.00	71.7
Dimetridazole	0.10	75.6
	0.20	74.9
	0.40	75.4
	2.00	81.7

16.4.8 RECOVERY

Under optimized conditions, the recoveries of metronidazole, ronidazole, and dimetridazole in honey using this method are listed in Table 16.7.

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16.5

Determination of Metronidazole, Ronidazole, and Dimetridazole Residues in Milk and Milk Powder—LC-MS-MS Method (GB/T 22982-2008)

16.5.1 SCOPE

This method is applicable to the determination of metronidazole, ronidazole, dimetridazole and their metabolite residues in milk and milk powder.

The limit of quantification for metronidazole, ronidazole, and dimetridazole in milk is 0.5 μg/kg, metronidazole-OH in milk is 1 μg/kg, and 2-hydroxymethyl-1-methyl-5-nitroim-idazole in milk is 2.5 μg/kg. The limit of quantification for metronidazole, ronidazole, and dimetridazole in milk powder is 2.5 μg/kg, metronidazole-OH in milk powder is 5 μg/kg, and 2-hydroxymethyl-1-methyl-5-nitroim-idazole in milk powder is 12.5 μg/kg.

16.5.2 PRINCIPLE

The residues in the test sample are extracted with acetonitrile-ethyl acetate and cleaned up with strong cation exchange (SCX) solid-phase extraction (SPE). Determination is made by LC-MS-MS using the internal standard method.

16.5.3 REAGENTS AND MATERIALS

Unless specifically noted, all reagents used should be of HPLC grade; "water" is deionized water.

Acetonitrile.
Ethyl acetate.
Methanol.
Acetone.
Acetic acid.
Ammonia water.
Anhydrous sodium sulfate: Ignite at 650°C for 4 h and keep in a tightly closed container after cooling.
Ammonia water-acetonitrile (5+95): Accurately measure 5 mL ammonia water into a 100-mL volumetric flask, dilute with acetonitrile to 100 mL, and mix to homogeneity.

Standards: Metronidazole, CAS: 13182-89-3; Ronidazole, CAS: 7681-76-7; Dimetridazole, CAS: n551-92-8; Metronidazole-OH, CAS: 7681-76-7; 2-hydroxymethyl-1-methyl-5-nitroim-idazole, CAS: 936-05-0; RNZ-D3; MZNOH-D2; HMMNI-D3, purity $\geq$98.0%.
Stock standard solution: Respectively, accurately weigh appropriate amount of standards and dissolve in 100 mL methanol to make a solution concentration of approximately 100 mg/mL.
Mixed standard solution: Accurately measure 1.00 mL stock standard solution respectively into a 100-mL umber volumetric flask, dilute with methanol to 100 mL, and mix to homogeneity. The concentration of the solution is 1 μg/mL.
Standard working solution: According to the requirements, accurately measure an adequate volume of mixed standard solution and dilute with blank matrix extract solution just before use.
Stock internal standard solution: Respectively, accurately weigh appropriate amount of standards and dissolve in 100 mL methanol to make a solution concentration of approximate 100 mg/mL.
Internal standard working solution: Accurately measure internal stock standard solution respectively into a 10-mL umber volumetric flask, dilute with methanol, and mix to homogeneity. The concentration of the solution is 10.0 μg/mL.
0.45-μm filter.
SCX column: 500 mg, 3 mL; condition the column with 3 mL methanol and 3 mL ethyl acetate in sequence before using.

16.5.4 APPARATUS

High performance liquid chromatography tandem mass spectrograph.
Centrifuge.
Analytical balance: Reciprocal sensibility is 0.1 mg and 0.01 g.
Vortex shaker.
Rotary vacuum evaporator.
Nitrogen evaporator.
Vacuum manifold processing station.

16.5.5 SAMPLE PRETREATMENT

(1) Preparation of test sample
Milk

About 250 g representative samples should be taken from all samples, and placed in clean sample containers, sealed, and labeled. The test samples should be stored at −4°C and kept away from light.

Milk Powder

About 250 g representative samples should be taken from all samples and placed in clean sample containers, sealed, and labeled. The test samples should be stored at room temperature and kept away from light.

(2) Extraction

For the milk sample, weigh approximate 5 g (accurate to 0.01 g) of the test sample into 50 mL centrifuge tube, add 25 μL internal standard working solution of 100 ng/mL, and mix for 1 min. For the milk powder sample, weigh approximately 1 g (accurate to 0.01 g) of the test sample, add 5 mL water and 25 μL internal standard working solution of 100 ng/mL, and mix for 1 min by vortex shaker.

Then add 5 mL acetonitrile into the tubes and shake for 2 min to deposit protein. Add 20 mL ethyl acetate and shake the tubes for 2 min; then after centrifuging for 10 min at 3000 rpm, filter the extraction through an anhydrous sodium sulfate layer into a heart-shaped flask. Then use 20 ethyl acetate extract again and mix the extraction into the same flask.

(3) Clean-up

Evaporate the exaction to approximate 2 mL using a rotary vacuum evaporator at 45°C. Transfer the solution into the reservoir above the conditioned SCX cartridge. Dissolve the residues with 5 ml ethyl acetate twice and pour the solution into the same reservoir. Then pass the solution through the cartridge at a speed of less than 2 mL/min. After drop over, wash the cartridge with 3 mL acetone, 3 mL methanole, and 2 mL ammonium hydroxide-acetonitrile (5+95) in turn. Finally, elute the cartridge with 5 mL ammonium hydroxide-acetonitrile (5+95). Evaporate elution to dryness with nitrogen evaporator at 45°C. Residues are dissolved with 1.0 mL deionized water. Then the solution is passed through a 0.45-μm filter and is ready for analysis.

(4) Preparation of blank matrix solution

For milk, accurately weigh a 5 g negative sample and for milk powder, accurately weigh a 1 g negtive sample (accurate to 0.01 g) to prepare the blank matrix solution according to the above-mentioned extraction and cleanup steps.

16.5.6 DETERMINATION

(1) HPLC operating conditions:

Column: C_{18} 150 mm × 2.1 mm (i.d.), 5-μm particle size, or equivalent;
Column temperature: 30°C;
Injection volume: 15 μL.
Mobile phase: The elution gradient and flow rate are listed in Table 16.8;
Ionization mode: ESI+;
Scan mode: MRM;
Sheath gas: 15 units;
Auxiliary gas: 20 units;

Ion spray voltage: 4000V;
Capillary temperature: 320°C;
Source CID: 10V;
Width of Q1 and Q3: 0.4 and 0.7;
Collision gas: Argon;
Collision gas pressure: 1.5 mTorr.
Other mass operating conditions are listed in Table 16.9.

TABLE 16.8 Elution Gradient of LC

Time (min)	Flow Rate (μL/min)	0.1% Acetic Acid Solution (%)	Methanol (%)
0.00	200	80	20
6.00	200	65	35
8.0	200	65	35
8.10	200	80	20
10.0	200	80	20

TABLE 16.9 Scan Segment, Ion Pairs and Collision Energy of the Analytes

Compound	Retention Time (min)	Ion Pairs (*m/z*)	Collision Energy (eV)
MNZOH	3.74	188.04/122.96*	13
		188.04/125.94	18
HMMNI	4.82	158.06/140.01*	12
		158.06/112.05	19
MNZ	4.79	172.09/128.00*	15
		172.09/82.03	25
RNZ	5.76	201.05/139.98*	12
		201.051/110.0	17
DMZ	6.43	142.07/96.03*	16
		142.07/81.03	29
MNZOH-D2	3.74	190.05/124.97	12
HMMNI-D3	4.82	161.06/143.00	13
RNZ-D3	4.79	204.04/143.00	11

Note: **Quantitative ion pair.*

TABLE 16.10 Maximum Permitted Tolerances for Relative Ion Intensities During Confirmation

Relative intensity, K (%base peak)	>50%	20%< K<50%	10%< K<20%	$K \leqq 10\%$
Permitted tolerance	±20%	±25%	±30%	±50%

(2) Qualitative analysis

The qualification ions must include at least one precursor and two daughter ions. Under the same experimental conditions, the variation range of the retention time for the peak of the analyte in the unknown sample and in the matrix standard working solution cannot be out of the range of ±0.25 min. For the same analysis batch and the same compound, the variation of the ion ratio between the two daughter ions for the unknown sample and the matrix standard working solution at a similar concentration cannot be out of the range of Table 16.10; if this is true, then the corresponding analyte must be present in the sample.

(3) Quantitative analysis

Under the best conditions of the apparatus, inject the series of mixed matrix standard working solutions separately. The mixed matrix standard working curves are made by plotting the responses (area of each analyte divided by area of the corresponding internal standard) versus the concentration of standards. Use the curve to quantify each analyte in the unknown sample. The responses of the analyte in the standard working solution and the sample solution should be within the linear range of the instrument detection. Under the preceding operating conditions, the chromatogram of the standard can be seen in Fig. 16.3.

16.5.7 PRECISION

The precision data of this part is confirmed according to GB/T 6379. The values of repeatability and reproducibility are calculated at the 95% confidence level.

(1) Repeatability

Under the repeatability condition, the difference of absolute value of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equations of the five analytes in milk and milk powder are shown in Tables 16.11 and 16.12.

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned, and two independent experiments should be redone.

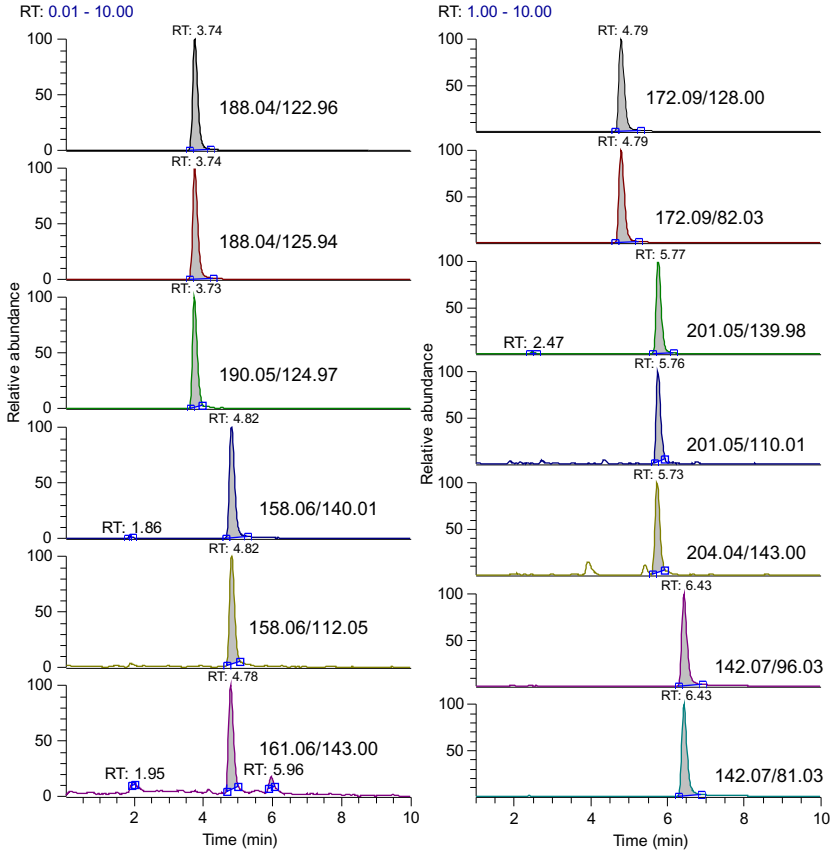

FIG. 16.3 MRM chromatograms of standard working solution

TABLE 16.11 Content Ranges and Repeatability/Reproducibility Equations for Milk Sample

Analyte	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
MNZOH	1–10	$r=0.2259m-0.1764$	$R=0.1812m+0.0496$
HMMNI	2.5–25	$\lg r=0.929\lg m-0.836$	$\lg R=1.009\lg m-0.692$
MNZ	0.5–5	$\lg r=0.952\lg m-0.902$	$\lg R=0.898\lg m-0.680$
RNZ	0.5–5	$\lg r=0.873\lg m-0.670$	$\lg R=0.854\lg m-0.665$
DMZ	0.5–5	$r=0.200m-0.0548$	$R=0.193m+0.0356$

Note: m *equals the average of two results.*

TABLE 16.12 Content Ranges and Repeatability/Reproducibility Equations for Milk Powder Sample

Analyte	Content Range (μg/kg)	Repeatability Limit (r)	Reproducibility Limit (R)
MNZOH	5–50	$\lg r = 1.2597 \lg m - 1.1738$	$\lg R = 1.0540 \lg m - 0.6542$
HMMNI	12.5–125	$r = 0.133m + 1.190$	$R = 0.137m + 3.694$
MNZ	2.5–25	$\lg r = 1.033 \lg m - 0.811$	$\lg R = 0.985 \lg m - 0.622$
RNZ	2.5–25	$\lg r = 0.928 \lg m - 0.687$	$\lg R = 0.924 \lg m - 0.553$
DMZ	2.5–25	$\lg r = 1.133 \lg m - 0.916$	$\lg R = 0.980 \lg m - 0.562$

Note: m *equals the average of two results.*

(2) Reproducibility

Under the reproducibility condition, the difference of absolute value of two independent results is not above the reproducibility limit (R). The content ranges and the reproducibility equations of the five analytes in milk and milk powder are shown in Tables 6.11 and 6.12.

16.5.8 RECOVERY

Under optimized condition, the recoveries of metronidazole, ronidazole, dimetridazole, and their metabolite residues in milk and milk powder using this method are listed in Table 16.13.

TABLE 16.13 The Recovery Ranges of the Analytes in Milk and Milk Powder at Four Fortifying Levels

Analyte	Milk		Milk powder	
	Spiked Level (μg/kg)	Recovery Range (%)	Spiked Level (μg/kg)	Recovery Range (%)
MNZOH	1	89.0–108	5	83.2–105
	2	98.0–110	10	87.4–111
	4	91.0–106	20	89.9–109
	10	83.8–105	50	80.1–101

Continued

TABLE 16.13 The Recovery Ranges of the Analytes in Milk and Milk Powder at Four Fortifying Levels—cont'd

Analyte	Milk		Milk powder	
	Spiked Level (μg/kg)	Recovery Range (%)	Spiked Level (μg/kg)	Recovery Range (%)
MNZ	0.5	82.0–102	2.5	82.8–100
	1	90.0–110	5	91.8–116
	2	85.5–115	10	88.7–105
	5	94.8–106	25	81.5–109
HMMNI	2.5	94.8–108	12.5	94.3–109
	5	98.2–110	50	82.4–105
	10	92.1–112	100	87.0–116
	25	89.6–106	125	94.3–111
RNZ	0.5	82.0–102	2.5	82.0–105
	1	86.0–110	5	89.6–109
	2	91.0–108	10	81.8–97.4
	5	88.2–103	25	82.6–97.3
DMZ	0.5	82.0–98.0	2.5	82.4–104
	1	80.0–97.0	5	84.4–103
	2	86.0–101	10	80.3–103
	5	94.8–106	25	81.6–107

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16.6

Determination of Nitroimidazole Residues in Royal Jelly and Lyophilized Royal Jelly Farina—LC-MS-MS Method (GB/T 22949-2008)

16.6.1 SCOPE

This method is applicable to the qualified and quantified determination of nine nitroimidazole drugs in royal jelly and lyophilized royal jelly farina.

The stated LOQ (S/N > 10) of nitroimidazole residues in the negative honey and royal jelly sample is, respectively, at the level of 0.5 μg/kg.

The stated LOQ (S/N > 10) of nitroimidazole residues in the negative lyophilized royal jelly farina sample is, respectively, at the level of 1.0 μg/kg.

16.6.2 PRINCIPLE

The residues of nitroimidazole in the test sample are extracted with ethyl acetate after dissolving the sample matrix in an acetate natrium buffer. During a rotary evaporation method, followed by reconstituted, constant volume with formic acid aqueous solution, and is cleaned up with carbon tetrachloride. Taking the upper aqueous solution after being purified on the centrifuge, the sample solution is then determined by LC-MS-MS, quantified by a deuterated internal standard method (HMMNI-D^3 and IPZ-OH-D^3), and qualified by the daughter ion ratio.

16.6.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents should be of analytical grade; "water" is the first-grade water prescribed by GB/T 6682-1992.

Acetonitrile: HPLC Grade.
Methanol: HPLC Grade.
Ethyl Acetate: HPLC Grade.
Formic acid: HPLC Grade.
Carbon tetrachloride.
Acetate natrium.
Standard chemicals: Metronidazole (MNZ; CAS No. 443-48-1); Dimetridazole (DMZ; CAS No. 551-92-8); Tindazole (TNZ; CAS No. 19387-91-8),

Ronidazole (RNZ; CAS No. 7681-76-7), Ternidazole (TerNDZ; CAS No.1077-93-6); Ipronidazole (IPZ; CAS No.14885-29-1); 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (Hydroxy-Metronidazole, MNZ-OH; CAS No. 4812-40-2); 2-(1-Methyl-5-nitro-1H-imidazol-2-yl)-propan-2-OL (Hydroxy-Ipronidazole, IPZ-OH; CAS No.35175-14-5); 2-Hydroxymethyl-1-methyl-5-nitro-imidazole (HMMNI; CAS No.936-05-0). All purity is over 98%. Internal standard is HMMNI-D^3 (CAS No. 8061-52-7) and IPZ-OH-D^3 (CAS No. 8061-52-7); Deuterated internal standard purity is over 95%.

Standard stock solution: 1000 μg/mL. Accurately weigh 10.0 mg ±0.05 mg (calibrated by purity) standard chemical of nitroimidazoles respectively, dissolve in 10 mL methanol, and mix to ultrasonic homogeneity. The concentration of the solution is 1000 mg/L. The solution can be preserved at temperatures below −20°C for more than 12 months.

Mixed standard working solution: 100 ng/mL. The standard stock solution is placed at the ambient indoor temperature (about 20°C) for 10 min and dissolved in acetonitrile for distribution concentration just before use.

Acetate natrium buffer solution: 0.1 mol/L. Accurately weigh 8.204 g ± 0.01 g anhydrous acetate natrium and dissolve in water in a 1000-mL volumetric flask. Add 5.83 mL iced acetic acid, then add water to 1000 mL, and mix to ultrasonic homogeneity 15 min. The concentration of the solution is 0.1 mol/L. The solution can be preserved in the HPLC mobile bottle at the indoor temperature for backup.

Formic acid: 0.1%. Accurately add 1 mL formic acid, dissolve in water in a 1000-mL volumetric flask and then add water to 1000 mL and mix to ultrasonic homogeneity 15 min. The concentration of the solution is 0.1%.The solution can be preserved in the HPLC mobile bottle at the indoor temperature for backup.

Mixed standard matrix working solution: according to the sensitivity and the range of the instrument linear requirement, accurately measure an adequate volume of mixed standard solution and dilute with blank matrix extract solution just before use.

Microfiltration membrane: 0.2 μm.

16.6.4 APPARATUS

LC-MS-MS: equipped with atmosphere pressure chemical ion source (or equivalent). HPLC equipped with online degasifier, low residual autosample, control lable temperature column chamber, high precision mixed pump.

Electronic analytical balance: sensitivity: 0.1 mg, 10 mg.

Refrigerated centrifuge: 4000 rpm, 13,000 rpm.

Evaporator with nitrogen flow: water bath, heat control temperature.

Desktop dispersion apparatus.

Rotation concentration apparatus (rotary evaporator): water bath, heat control temperature.
Ultra pure water device.
Ultrasonic extractor.
Multifunctional food grinder.
Mechanical shaker.
Evaporator bottle: 125 mL.
Centrifuge tube: 15 mL and 50 mL; polytetrafluoroethylene material.
Volumetric flask: 10 mL and 1000 mL.

16.6.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

The liquid sample, pulpiness sample, and powder sample are respectively mixed until homogeneous. The cryopreservation solid samples are tested after grinding and size reduction if necessary; make laboratory samples to quarter method shrinkage. Seal and label.

According to sample characteristics and the storage requirements, store test samples in appropriate proper environment and avoid any changes in sample.

The test samples are stored in the refrigerator at −20°C.

(2) Extraction

Weigh 2.5–5.0 g of test sample (weighing 5.0 g of royal jelly and 2.5 g of lyophilized royal jelly farina, respectively, all accurate to 0.2 g) into a 50-mL centrifuge tube. Add HMMNI-D^3 and IPZ-OH-D^3 standard working solution: 100 μL volume, respectively. Add 20 mL acetate natrium buffer solution. Mix thoroughly in a vortex mixer.

Add 20 mL ethyl acetate. Shake on shaker for 20 min, and via liquid-liquid extraction process, centrifuge at 3000 rpm for 5 min. Put ethyl acetate extraction solution into a pear-shaped flask. Proceed twice with 20 mL ethyl acetate and combine the ethyl acetate phases. Evaporate the solution, drying with a rotary evaporator at 40°C, for clean-up.

(3) Clean-up

The previous extracts are reconstituted and made up to a volume of 1 mL for constant volume with formic acid aqueous solution, and cleaned up with carbon tetrachloride. Mix thoroughly 1 min in a vortex mixer. Extract the upper solution via the filter membrane (0.2 μm). The solution is ready for LC-MS-MS determination.

16.6.6 DETERMINATION

(1) Operating condition

Column: Waters Sunfire ODS C18, 5 μm, 250 mm × 4.6 mm, or equivalent;

Column temperature: 30°C;
Mobile phase: Acetonitrile+0.1% formic acid solution (30+70); for HPLC mobile phases gradient program, see Table 16.14.
Flow rate: 800 μL/min;
Injection volume : 10 μL;
Ion source: Atmospheric Pressure Chemical Ionization source (APCI), or equivalent;
Scan mode: Positive scan (+) ;
Monitor mode: Multiple reaction monitor (MRM);
Ionspray voltage: 5250 V;
Nebulizer gas (relative opening flow): 14.00 mL/min;
Curtain gas (relative opening flow): 7.00 mL/min;
Source temperature: 500°C;
Collision exit potential (CXP): 11 V
Dwell time: 50 ms
Entrance potential (EP): 10.0 V
Collision gas (CAD; relative opening flow): 8 mL/min
Accessorial airflow pressure: 60 psi
Decluster potential (DP): 35 V
Focusing potential (FP): 200 V
Nebulizer current (NC): 2 μA
Other instrument parameters: Detector parameters [Positive] DF:−200.0 V;CEM:2300.0 V
For MRM transitions for precursor/product ion, quantifying for precursor/product ion, collision energy, chromatography peak acquisition reten tion time, see Table 16.15.

TABLE 16.14 HPLC Mobile Phases Gradient Program

Time (*t*-min)	A: 0.1% Formic Acid Solution (%)	B: Acetonitrile (%)	Flow (μL/min)
0	95	5	800
2	95	5	800
6	65	35	800
12	10	90	800
14	10	90	800
15	95	5	800
20	95	5	800

TABLE 16.15 Nine Nitroimidazole Drugs Compound, Compound English Name, MRM Transitions for Precursor/Product Ion, Quantifying for Precursor/Product Ion, Collision Energy, Chromatography Peak Acquisition

Compound English Name	Qualitative for Precursor/Product Ion (*m*/*z*)	Quantitative for Precursor/Product Ion (*m*/*z*)	Collision Energy (CE)/V	Chromatography Peak Acquisition Retention Time (min)
Metronidazole-OH;MNZ-OH	188.0/125.9; 188.0/144.0	188.0/125.9	→25 →20	9.01
Metronidazole;MNZ	172.0/128.0; 172.0/82.10	172.0/128.0	→22 →35	9.48
2-Hydroxymethyl-1-methyl-5-nitro-imidazole;HMMNI	158.0/140.0; 158.0/55.1	158.0/140.0	→20 →33	9.67
D3-2-Hydroxymethyl-1-methyl-5-nitro-imidazole; HMMNI-D3	161.0/143.1; 161.0/58.0	161.0/143.1	→24 →32	9.63
Ternidazole;TerNDZ	186.0/128.1; 186.0/82.2	186.0/128.1	→24 →40	9.90
Ronidazole;RNZ	201.0/140.0; 201.0/110.0	201.0/140.0	→18 →25	10.21
Dimetridazole;DMZ	142.0/96.1; 142.0/81.1	142.0/96.1	→25 →37	10.32

Continued

TABLE 16.15 Nine Nitroimidazole Drugs Compound, Compound English Name, MRM Transitions for Precursor/Product Ion, Quantifying for Precursor/Product Ion, Collision Energy, Chromatography Peak Acquisition—cont'd

Compound English Name	Qualitative for Precursor/Product Ion (*m*/*z*)	Quantitative for Precursor/Product Ion (*m*/*z*)	Collision Energy (CE)/V	Chromatography Peak Acquisition Retention Time (min)
Tindazole;TNZ	248.0/121.0; 248.0/93.1	248.0/121.0	→25 →28	11.06
Ipronidazole-OH;IPZ-OH	186.0/168.0; 186.0/122.1	186.0/168.0	→20 →28	11.40
D3-Ipronidazole-OH;IPZ-OH-D3	189.0/171.0; 189.0/125.0	189.0/171.0	→25 →35	11.40
Ipronidazole;IPZ	170.0/124.1; 170.0/109.1	170.0/124.1	→25 →35	12.79

(2) Qualitative determination

The qualification ions for every compound must be found, and must at least include one precursor ion and two daughter ions. For the same analysis batch and the same compound, the variation range of the retention time for the peak of the analyte in the unknown sample and in the standard working solution cannot be out of range of ±0.25 min. The variation range of the ion ratio between the two daughter ions for the unknown sample and the standard matrix working solution at similar concentrations cannot be out of the range of Table 16.10.

(3) Quantitative determination

Inject the mixed standard matrix working solutions of metronidazole (MNZ), dimetridazole (DMZ), tindazole (TNZ), ronidazole (RNZ), ternidazole (TerNDZ), ipronidazole (IPZ), hydroxy-metronidazole (MNZOH), hydroxy-ipronidazole (IPZOH), 2-Hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI) in sequence. Draw the standard curve with peak area vs. sample concentration. The sample is quantified by an internal standard method with standard curve. The responses of nitroimidazole drugs in the sample solution should be in the linear range of the instrumental detection. Over the linear range of the working curve, it must be proper to dilution before analysis.

For multiple reaction monitor (MRM) ion chromatograms of nitroimidazole drug matrix working solutions, see Fig. 16.4. For MRM quantitative ion pair chromatograms of nine nitroimidazole drugs and two deuterated intra-standard methods (HMMNI-D3 and IPZ-OH-D3) matrix working solutions, see Fig. 16.5.

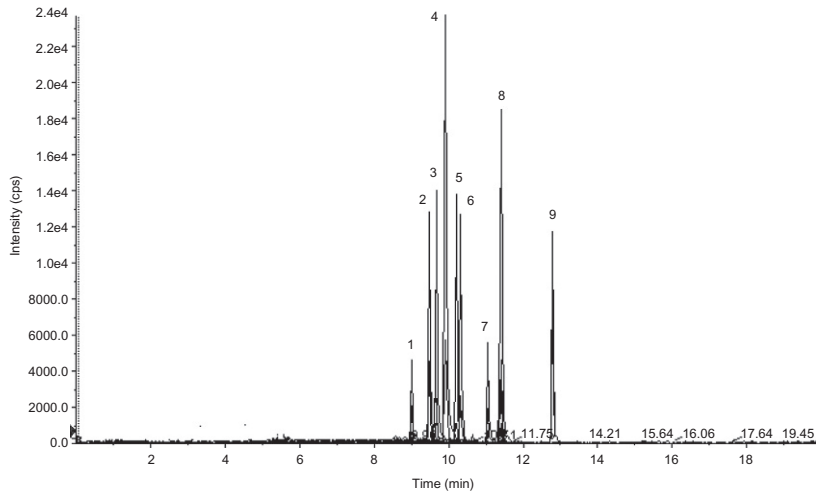

FIG. 16.4 MRM ion chromatograms of nine nitroimidazole drugs (1–9) matrix working solution (1: MNZOH, 2: MNZ, 3: HMMNI HMMNI-D3, 4: TerNDZ, 5: RNZ, 6: DMNZ, 7: TNZ, 8: IPZ-OHIPZ-D3, 9: IPZ).

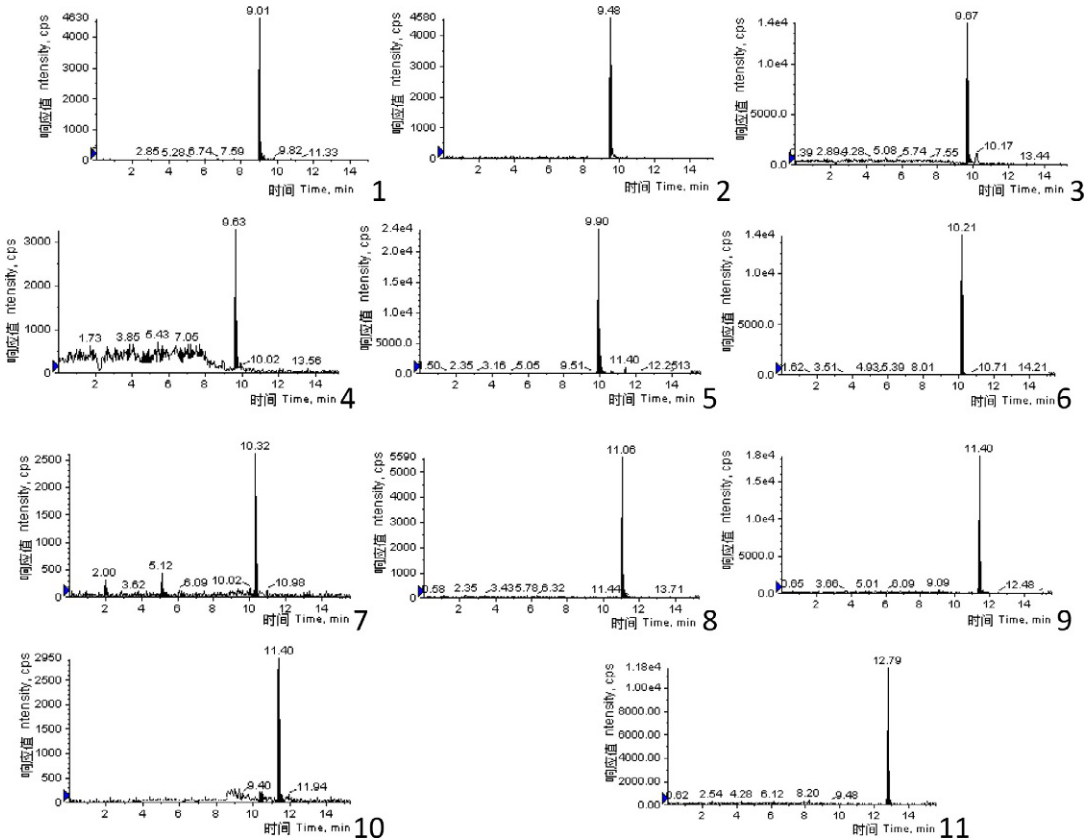

FIG. 16.5 Target quantitative ion pair chromatograms of nine nitroimidazole drugs and two deuterated intra-standard methods (HMMNI-D3 and IPZ-OH-D3)

16.6.7 PRECISION

The precision data of the method for this standard have been determined according to the stipulations of GB/T 6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results should not exceed the limit of repeatability (r); the content range and repeatability equations of nine nitroimidazole drugs in royal jelly and lyophilized royal jelly farina food are shown in Tables 16.16 and 16.17.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations reconducted and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results should not exceed the limit of reproducibility (R); the content range and reproducibility equations of nine nitroimidazole drugs in royal jelly and lyophilized royal jelly farina food are shown in Tables 16.16 and 16.17.

16.6.8 RECOVERY

Under optimized condition, the recoveries of nine nitroimidazole drugs in royal jelly and lyophilized royal jelly farina using this method are listed in Tables 16.18 and 16.19.

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TABLE 16.16 Content Range and Repeatability/Reproducibility Equations of Nine Nitroimidazole Drugs in Royal Jelly (μg/kg)

Name	Content Range (μg/kg)	Repeatability (μg/kg)	Reproducibility (μg/kg)
Metronidazole-OH; MNZ-OH	0.5–25.0	$\lg r = 0.9570 \lg m - 0.8176$	$\lg R = 0.7115 \lg m - 0.5829$
Metronidazole; MNZ	0.5–25.0	$\lg r = 1.0922 \lg m - 0.8648$	$\lg R = 0.7225 \lg m - 0.6263$
2-Hydroxymethyl-1-methyl-5-nitro-imidazole; HMMNI	0.5–25.0	$\lg r = 0.9930 \lg m - 0.7742$	$\lg R = 0.6444 \lg m - 0.5471$
Ternidazole; TerNDZ	0.5–25.0	$\lg r = 0.9067 \lg m - 0.6989$	$\lg R = 0.6619 \lg m - 0.5039$
Ronidazole; RNZ	0.5–25.0	$\lg r = 0.9814 \lg m - 0.7682$	$\lg R = 0.6001 \lg m - 0.5009$
Dimetridazole; DMZ	0.5–25.0	$\lg r = 1.1072 \lg m - 0.8606$	$\lg R = 0.7425 \lg m - 0.5632$
Ipronidazole-OH; IPZ-OH	0.5–25.0	$\lg r = 0.8991 \lg m - 0.7968$	$\lg R = 0.4805 \lg m - 0.4978$
Ipronidazole; IPZ	0.5–25.0	$\lg r = 1.1057 \lg m - 0.8666$	$\lg R = 0.6861 \lg m - 0.5668$

Note: m *is the average values obtained from two independent determination results.*

TABLE 16.17 Content Range and Reproducibility/Repeatability Equations of nine Nitroimidazole Drugs in Lyophilized Royal Jelly Farina Food(μg/kg)

Name	Content Range (μg/kg)	Repeatability (μg/kg)	Reproducibility (μg/kg)
Metronidazole-OH;MNZ-OH	0.5–25.0	$\lg r = 1.2426 \lg m - 0.9564$	$\lg R = 0.7642 \lg m - 0.5940$
Metronidazole;MNZ	0.5–25.0	$\lg r = 0.9895 \lg m - 0.9262$	$\lg R = 0.7042 \lg m - 0.5970$
2-Hydroxymethyl-1-methyl-5-nitro-imidazole; HMMNI	0.5–25.0	$\lg r = 1.0877 \lg m - 0.9873$	$\lg R = 0.7378 \lg m - 0.6479$
Ternidazole;TerNDZ	0.5–25.0	$\lg r = 1.0276 \lg m - 0.8149$	$\lg R = 0.6698 \lg m - 0.5866$
Ronidazole;RNZ	0.5–25.0	$\lg r = 0.9215 \lg m - 0.9994$	$\lg R = 0.7365 \lg m - 0.6546$
Dimetridazole;DMZ	0.5–25.0	$\lg r = 0.8742 \lg m - 0.9267$	$\lg R = 0.4042 \lg m - 0.5805$
Ipronidazole-OH;IPZ-OH	0.5–25.0	$\lg r = 0.8332 \lg m - 0.8668$	$\lg R = 0.6240 \lg m - 0.6499$
Ipronidazole;IPZ	0.5–25.0	$\lg r = 0.4642 \lg m - 0.8126$	$\lg R = 0.1605 \lg m - 0.6116$

Note: m *is the average values obtained from two independent determination results.*

TABLE 16.18 Average Recovery Values, Standard Deviation (SD), Coefficient of Variation of the Average Recovery Test Concentration Data for the Added Nine Nitroimidazole Drugs Corresponding to the Added Concentration Values in Royal Jelly ($n = 10$)

Compound Name	Added Level (μg/kg)	Average Test Values (μg/kg)	Standard Deviation (SD; μg/kg)	Average Recovery Values (%)	Coefficient of Variation (CV; %)
Metronidazole-OH; MNZ-OH	0.5	0.368	0.059	73.6	15.95
	1.0	0.829	0.045	82.9	5.44
	2.0	1.838	0.092	91.9	4.98
Metronidazole; MNZ	0.5	0.445	0.038	88.9	8.50
	1.0	0.924	0.072	92.4	7.79
	2.0	1.889	0.161	94.5	8.53
2-Hydroxymethyl-1-methyl-5-nitro-imidazole; HMMNI	0.5	0.441	0.045	88.1	10.32
	1.0	0.876	0.074	87.6	8.43
	2.0	1.895	0.133	94.8	7.00
Ternidazole; TerNDZ	0.5	0.480	0.025	96.0	5.26
	1.0	0.932	0.071	93.2	7.59
	2.0	1.934	0.106	96.7	5.48
Ronidazole; RNZ	0.5	0.394	0.045	78.8	11.43
	1.0	0.774	0.045	77.4	5.81
	2.0	1.725	0.073	86.3	4.24

Dimetridazole; DMZ	0.5	0.461	0.034	92.1	7.45
	1.0	0.836	0.062	83.6	7.38
	2.0	1.848	0.155	92.4	8.37
Tindazole; TNZ	0.5	0.362	0.025	72.4	6.82
	1.0	0.800	0.067	80.0	8.44
	2.0	1.730	0.163	86.5	9.45
Ipronidazole-OH; IPZ-OH	0.5	0.403	0.051	80.6	12.68
	1.0	0.839	0.065	83.9	7.79
	2.0	1.723	0.113	86.2	6.58
Ipronidazole; IPZ	0.5	0.375	0.043	75.0	11.53
	1.0	0.756	0.078	75.6	10.35
	2.0	1.761	0.117	88.1	6.64

TABLE 16.19 Average Recovery Values, Standard Deviation (SD), Coefficient of Variation of the Average Recovery Test Concentration Data for the Added Nine Nitroimidazole Drugs Corresponding to the Added Concentration Values in Lyophilized Royal Jelly Farina Food ($n = 10$)

Compound Name	Added Level (μg/kg)	Average Test Values (μg/kg)	Standard Deviation (SD; μg/kg)	Average Recovery Values (%)	Coefficient of Variation (CV; %)
Metronidazole-OH; MNZ-OH	0.5	0.403	0.052	80.6	12.9
	1.0	0.736	0.035	73.6	4.73
	2.0	1.641	0.112	82.1	6.82
Metronidazole; MNZ	0.5	0.374	0.034	74.7	9.04
	1.0	0.747	0.042	74.7	5.56
	2.0	1.800	0.073	90.0	4.05
2-Hydroxymethyl-1-methyl-5-nitro-imidazole; HMMNI	0.5	0.438	0.049	87.6	11.2
	1.0	0.824	0.038	82.4	4.55
	2.0	1.803	0.073	90.2	4.05
Ternidazole; TerNDZ	0.5	0.437	0.036	87.4	8.24
	1.0	0.839	0.065	83.9	7.74
	2.0	1.822	0.069	91.1	3.79
Ronidazole; RNZ	0.5	0.350	0.032	70.0	9.21
	1.0	0.712	0.044	71.2	6.25
	2.0	1.636	0.089	81.8	5.42

Dimetridazole; DMZ	0.5	0.353	0.036	70.6	10.2
	1.0	0.704	0.035	70.4	4.95
	2.0	1.675	0.068	83.8	4.04
Tindazole; TNZ	0.5	0.338	0.025	67.6	7.38
	1.0	0.728	0.055	72.8	7.52
	2.0	1.667	0.066	83.4	3.95
Ipronidazole-OH; IPZ-OH	0.5	0.355	0.032	71.0	9.05
	1.0	0.712	0.038	71.2	5.31
	2.0	1.682	0.058	84.1	3.48
Ipronidazole; IPZ	0.5	0.398	0.043	79.5	10.9
	1.0	0.718	0.048	71.8	6.65
	2.0	1.715	0.063	85.8	3.69

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Chapter 17

Benzimidazole

17.1

Determination of Thiabendazole, Albendazole, Fenbendazole, Oxfendazole, and Febantel Residues in Milk and Milk Powder—HPLC-MS-MS (GB/T 22972-2008)

17.1.1 SCOPE

This method is applicable to the determination of thiabendazole, albendazole, fenbendazole, oxfendazole, and febantel residues in milk and milk powder.

The limit of determination of this method: 0.01 mg/kg in milk, 0.08 mg/kg in milk powder.

17.1.2 PRINCIPLE

The test sample is extracted with acetonitrile and the extract is cleaned up by passing through a C_{18} SPE column. The determination is made by LC-MS-MS. An external standard method is used.

17.1.3 REAGENTS AND MATERIALS

Unless otherwise specified, all the reagents used should be analytical grade; "water" is of grade 1 water specified in GB/T6682.

Acetonitrile: HPLC grade.
Methanol: HPLC grade.
n-propanol.
Sodium bicarbonate ($NaHCO_3$).

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00017-8

Sodium carbonate (Na_2CO_3).
Sodium bicarbonate solution: 0.1 mol/L; weigh 8.4 g sodium bicarbonate, dissolve and make up to 1000 mL with water.
Sodium carbonate solution: 0.1 mol/L; weigh 1.06 g sodium carbonate, dissolve and make up to 100 mL with water.
Carbonate buffer solution (pH9.1): Mix 900 mL sodium bicarbonate solution and 100 mL sodium carbonate solution.
C_{18} SPE column: 500 mg, 6 mL. The column is conditioned by washing with 5 mL methanol, 5 mL water, and 2 mL carbonate buffer solution, keeping it wet before adding sample solution.
Membrane filter: 0.2 μm (organic).
Standards: Thiabendazole, CAS: 148-79-8; Albendazole CAS:54965-21-8; Fenbendazole, CAS:43210-67-9; Oxfendazole, CAS:53716-50-0; Febantel, CAS:58306-30-2; Purity ≥99%.
Stock standard solution: 1.0 mg/mL. Accurately weigh an appropriate amount of every benzimidazole standard and dissolve with methanol; the concentration is 1.0 mg/mL. It should be stored at 0–4°C in the refrigerator. The period of validity is 6 months.
Mixed standard stock solution: 100 μg/mL. Pipette a certain amount of stock standard solution, dissolve with methanol and the concentration is 100 μg/mL. It should be stored at 0–4°C in the refrigerator. The period of validity is 3 months.
Matrix mixed standard working solution: According to the sensitivity and the line scope of the instrument of every standard, pipette the appropriate amount of mixed standard stock solution and prepare standard working solution of appropriate concentration with blank sample solution; the solution should be prepared on the same day as use.

17.1.4 APPARATUS

Liquid chromatography–tandem mass spectrograph (LC-MS-MS): equipped with electrospray ion source;
Balance: with 0.01 g and 0.1 mg sensitivity;
Solid phase extraction (SPE) device;
Rotary evaporator;
Centrifuge: speed > 4000 rpm;
Vortex mixer;
Ultrasonic cleanser.

17.1.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Take representative portions from the whole primary sample, about 500 g; mix well and place in a clean container, which is sealed and labeled as the test sample.

The test samples should be stored at 0–4°C. In the course of sampling and sample preparation precautions must be taken to avoid contamination or any factors that may cause a change of residue content.

(2) Extraction

Milk sample

Weigh approximately 10 g of the milk sample (accurate to 0.01 g) into a 100-mL centrifuge tube; add 30 mL acetonitrile, vortex 3 min and then extract with ultrasonic cleanser for 30 min and centrifuge at 4000 rpm for 5 min. Transfer the supernatant liquid, add 10 mL n-propanol, evaporate organic solvent using a rotary evaporator with 40°C, and then make up to 10 mL with carbonate buffer solution.

Milk powder sample

Weigh approximately 12.5 g of the milk powder sample into a beaker and add an appropriate amount of water of 35–45°C to dissolve the sample adequately and cool the solution to ambient temperature. Then add water to 100 g and mix to homogeneity. Weigh approximately 10 g of the previous sample solution (accurate to 0.01 g) into a 100-mL centrifuge tube and according to the above-mentioned extraction steps of milk sample.

(3) Clean-up

Apply 5 mL of the extract to the C18 SPE column conditioned, adjust the flow rate to 1.0 mL/min, and wash the column with 5 mL water. Discard all the washing solution and evacuate the column to dryness. Elute the column with 6 mL acetonitrile and the flow rate is 1.0 mL/min. Evaporate the eluant to dryness at 40°C under nitrogen flow. Accurately add 1.0 mL methanol/water (2/8) to dissolve the residue. After being filtrated with a 0.2-μm filter, the final solution is ready for analysis by LC-MS-MS determination.

17.1.6 DETERMINATION

(1) LC operating condition

Column:BEH C_{18}, 50 mm × 2.1 mm (i.d.), 1.7 μm, or equivalent;
Column temperature: 40°C;
Injection volume: 10 μL;
Mobile phase and flow rate are shown in Table 17.1.
Ion source: electrospray ion source (ESI);
Scan mode: positive ion scan;
Determination mode: multiple reaction monitoring (MRM);
Ionization voltage: 2.0 kV;
Ion source temperature: 110°C;
Flow rate of cone gas: 80 L/h;
Desolvation temperature: 380°C;
Flow rate of desolvation gas: 700 L/h;
Qualitative ion pair, quantitative ion pair, cone energy and collision energy: see Table 17.2.

TABLE 17.1 Gradient Program of LC

Time (min)	Flow Rate (μL/min)	Methanol (%)	0.1% Formic acid (%)
0.0	200	5	95
12.0	200	90	10
12.1	200	5	95
14.0	200	5	95

TABLE 17.2 MS Parameters of Five Benzimidazole Drugs

Compounds	Parent Ions (*m*/*z*)	Daughter Ions (*m*/*z*)	Cone Energy (V)	Collision Energy (eV)
Thiabendazole	202.0	174.7[a]	46	25
		130.7	46	31
Albendazole	266.0	233.9[a]	34	19
		190.9	34	33
Fenbendazole	300.0	267.9[a]	34	21
		158.7	34	34
Oxfendazole	316.0	158.7[a]	38	34
		190.8	38	21
Febantel	447.0	382.9[a]	26	18
		414.9	26	14

[a]*Quantitative ion.*

(2) Confirmation

Choose one parent ion and two or more daughter ions for every one of the benzimidazole drugs. Under the same conditions, the ratio of the retention time of the analyte in the sample solution should correspond to that in the standard solution at a tolerance of ±2.5%, and the relative intensities of the detected ions of the analyte of the sample should correspond to those of the matrix standard solution at comparable concentrations; the deviation being within the tolerances listed in Table 17.3, then the corresponding analyte must be present in the sample.

TABLE 17.3 Maximum Permitted Tolerances for Relative Ion Intensities During Confirmation

Relative intensity (K)	$K > 50\%$	$20\% < K < 50\%$	$10\% < K < 20\%$	$K \leq 10\%$
Permitted tolerances	±20%	±25%	±30%	±50%

(3) Determination of quantitative

Inject the matrix mixed standard working solutions into the instrument. The standard working curve is drawn with the standard solution concentration as x-axis and the peak area as y-axis, and the quantification for the test sample is made according to the working curve described. The responses of the analyte in the sample solution should be within the linear range of the instrument detection (Figs. 17.1 and 17.2).

17.1.7 PRECISION

(1) General specification

The precision data of this standard are confirmed according to GB/T6379.1 and GB/T6379.2, and the values of repeatability and reproducibility are calculated according to a 95% confidence level.

(2) Repeatability

Under repeatability conditions, absolute differences between two test results should be within the repeatability limit. Fortifying concentrations and repeatability equations of five benzimidazole drugs in milk and milk powder are shown in Table 17.5. If the differences are greater than the

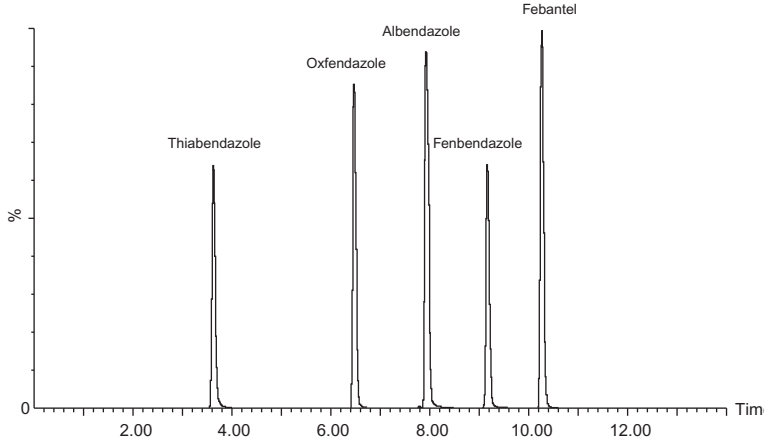

FIG. 17.1 LC-MS-MS chromatogram of five benzimidazole drugs.

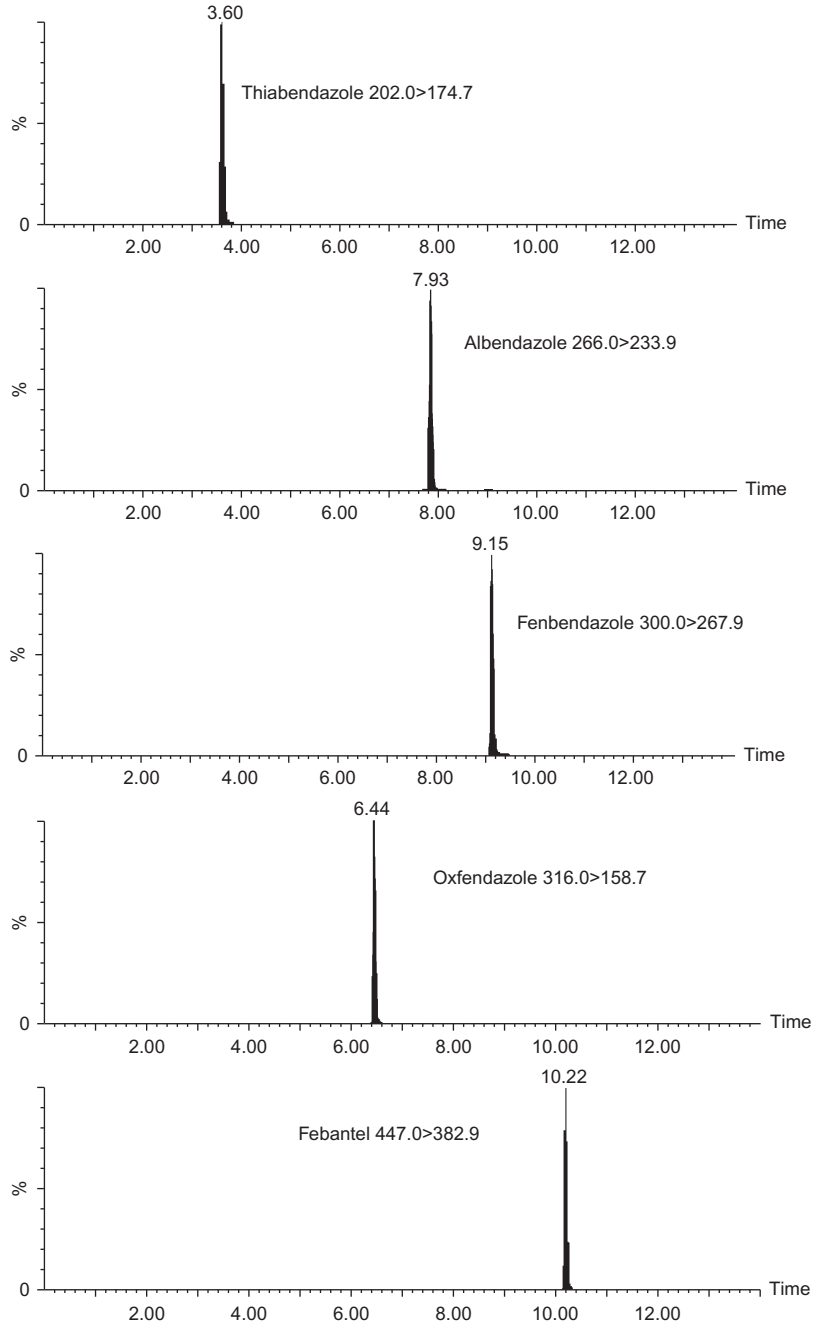

FIG. 17.2 MRM chromatograms of five benzimidazole drugs.

TABLE 17.4 Fortifying Concentration and Repeatability/Reproducibility Equations of Five Benzimidazole Drugs in Milk and Milk Powder (Unit: mg/kg)

Compound	Fortifying Concentration	Matrix	Repeatability Limit (r)	Reproducibility Limit (R)
Thiabendazole	0.01–0.50	Milk	$\lg r = 0.9199 \lg m - 1.1233$	$\lg R = 0.9995 \lg m - 0.8861$
		Milk powder	$\lg r = 1.1086 \lg m - 0.8097$	$\lg R = 1.0845 \lg m - 0.8136$
Albendazole	0.01–0.50	Milk	$\lg r = 1.0017 \lg m - 1.1712$	$\lg R = 0.9836 \lg m - 1.0378$
		Milk powder	$\lg r = 0.9819 \lg m - 1.1588$	$\lg R = 0.9537 \lg m - 1.1316$
Fenbendazole	0.01–0.50	Milk	$\lg r = 0.9508 \lg m - 1.1002$	$\lg R = 0.9474 \lg m - 1.0888$
		Milk powder	$\lg r = 1.1370 \lg m - 0.8373$	$\lg R = 1.0626 \lg m - 0.8758$
Oxfendazole	0.01–0.50	Milk	$\lg r = 0.9737 \lg m - 1.0712$	$\lg R = 1.0285 \lg m - 0.9771$
		Milk powder	$\lg r = 0.9493 \lg m - 1.0959$	$\lg R = 0.9309 \lg m - 1.0786$
Febantel	0.01–0.50	Milk	$\lg r = 0.9074 \lg m - 1.1761$	$\lg R = 0.8887 \lg m - 1.1820$
		Milk powder	$\lg r = 1.0003 \lg m - 1.0081$	$\lg R = 0.9771 \lg m - 0.9930$

Note: m *is the arithmetic mean of two results.*

TABLE 17.5 Recovery and Fortifying Concentration of Five Benzimidazole Drugs

Compound	Fortifying Concentration (mg/kg)	Average Recovery (%)	
		Milk	Milk Powder
Thiabendazole	0.01	72.5	71.4
	0.10	80.4	81.2
	0.50	82.4	83.3
Albendazole	0.01	80.2	82.6
	0.10	85.0	87.4
	0.50	92.7	93.6
Fenbendazole	0.01	81.0	79.1
	0.10	85.2	86.2
	0.50	83.0	90.2
Oxfendazole	0.01	78.5	75.2
	0.10	81.6	80.7
	0.50	83.6	83.9
Febantel	0.01	71.2	70.8
	0.10	80.1	80.5
	0.50	81.1	82.8

repeatability limit (r), the test results should be discarded and two new tests should be redone independently.

(3) Reproducibility

Under reproducibility conditions, absolute differences between two test results should be within the reproducibility limit (R). Fortifying concentration and reproducibility equations of five benzimidazole drugs in milk and milk powder are shown in Table 17.4.

17.1.8 RECOVERY

Under optimized condition, the recoveries of thiabendazole, albendazole, fenbendazole, oxfendazole, and febantel residues in milk and milk powder using this method are listed in Table 17.5.

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17.2

Determination of Benzimidazole Residues in Fugu, Eel, and Baked Eel—LC-MS-MS Method (GB/T 22955-2008)

17.2.1 SCOPE

This method is applicable to the determination of benzimidazole residues in balloonfish, eel, and roast eel. The benzimidazole residues include oxfendazole, fenbendazole and metabolite oxfendazole sulfone; albendazole and metabolites albendazole-2-aminosulfone, albendazole sulfoxide, albendazole sulfone; mebendazole and metabolites mebendazole-mine, 5-hydroxymebendazole; flubendazole and metabolite 2-aminoflubendazole; thiabendazole and metabolite thiabendazole-5-hydroxy; cambendazole and oxibendazole.

The limit of quantitation of the method for the benzimidazole residues in balloonfish, eel, and roast eel is 5 μg/kg.

17.2.2 PRINCIPLE

Benzimidazole residues are extracted from the sample with ethyl acetate in alkaline medium. The ethyl acetate extract is evaporated to dryness and dissolved with acetonitrile 0.1 mol/L hydrochloride. Then the extract is defatted by n-hexane and purified through an MCX SPE column. The residues are determined by LC-MS, quantified by peak area, and an external standard method is applied.

17.2.3 REAGENTS AND MATERIALS

Unless otherwise specified, all reagents are analytically pure; "water" is first-grade water prescribed by GB/T 6682.

Ethyl acetate.
N-hexane.
Acetonitrile, HPLC grade.

Methanol, HPLC grade.
Anhydrous sodium sulfate: ignited at 650°C for 4 h, stored in a desiccator.
Butylated hydroxytoluene (BHT).
Hydrochloride.
Potassium hydroxide.
25% ammonia.
Formic acid: guaranteed reagent.
Ammonium acetate.
1% BHT solution: weigh 1.0 g of BHT into a 150-mL conical flask with stopper, dissolve with ethyl acetate, and mix well. Prepare before use.
0.1 mol/L Hydrochloride aqueous solution: Transfer 9 mL of hydrochloride into a 1 L volumetric flask which filled 900 mL water, dilute to 1 L with water and mix well.
0.005 mol/L Formic acid: Transfer 188 μL of formic acid into a 1-L volumetric flask containing 900 mL water; dilute to 1 L with water and mix well.
50% Potassium hydroxide aqueous solution: Weigh 50 g potassium hydroxide, dissolved and diluted with water to 100 mL, and mix to homogeneity.
10% ammonium in acetonitrile: accurately draw 10 mL of 25% ammonia into a 100-mL volumetric flask, dilute with acetonitrile to 100 mL, and mix well. Prepare before use.
0.025 mmol/L ammonium acetate solution: Accurately weigh 1.93 g ammonium acetate, dissolved in 900 mL water, transfer to a 1000-mL volumetric flask, add water to 1000 mL, and mix well.
Standard: Oxfendazole (CAS№ 53716-50-0), Fenbendazole (CAS№ 43210-67-9), Oxfendazole sulfone (CAS№ 54029-20-8), Albendazole (CAS№ 54965218), Albendazole-2-aminosulfone (CAS№ 80983-34-2), Albendazole sulfoxide (CAS№ 54029-12-8), Albendazole sulfone (CAS № 75184-71-3), Mebendazole (CAS№ 14798000), Mebendazole-amine (CAS№ 52329-60-9), 5-Hydroxymebendazole (CAS№ 60254-95-7), Flubendazole (CAS№ 13678000), 2-Aminoflubendazole, Thiabendazole (CAS№ 148798), Cambendazole (CAS№ 26097-80-3), Oxibendazole (CAS№ 20559-55-1): purity $\geq$98%. The concentration of thiabendazole-5-hydroxy (CAS№ 17450500) is 10 mg/L.
Stock standard solution: 100 μg/mL.
Separately, accurately weigh 10 mg of each benzimidazole standard (accurately to 0.1 mg, calibrated by purity, and exclude thiabendazole-5-hydroxy); dissolve in methanol and dilute to 100 mL.
Mixed working standard solution: According to the requirement, accurately measure an adequate volume of stock standard solution of each benzimidazole (exclude thiabendazole-5-hydroxy) and a standard solution of thiabendazole-5-hydroxy, to the same black volumetric flask. Dilute with acetonitrile-water (2+8, v/v) solution just before use.
MCX SPE cartridge: 150 mg, 6 mL, or other equivalent. Pretreated the column with 5 mL methanol and 5 mL 0.1 mol/L hydrochloride aqueous solution just before use.
Membrane filter: 0.2 μm, for organic solvent.

17.2.4 APPARATUS

Liquid chromatography–tandem mass spectrometry, with ESI ion-source.

Tissue blender.
Homogenizer: ≥8000 rpm.
Vortex mixer.
Centrifuge: ≥4000 rpm.
Rotary evaporator.
Ultrasonic bath.
Electronic balance: 0.01 g and 0.001 g sensitivity.

17.2.5 SAMPLE PRETREATMENT

(1) Preparation of test sample

Sample, about 500 g, blended and homogenized thoroughly in a blender, placed in clean containers as the test sample, sealed and labeled. The test sample should be stored at –18°C.

In the course of sample preparation, precautions should be taken to avoid contamination or any factors that may cause the change of residue content.

(2) Extraction

Accurately weigh 2 g of the test sample (accurate to 0.01 g) into a 50-mL centrifuge tube. Add 20 mL of ethyl acetate, 0.15 mL of 50% potassium hydroxide aqueous solution, 1 mL of 1% BHT solution, mix by the ultrasonic bath for 5 min, and homogenize for 30 s at 8000 rpm. Add 1 g anhydrous sodium sulfate and mix well. Then centrifuge for 5 min at 4000 rpm. Transfer the supernatant into a 100-mL eggplant type flask. Add 20 mL of ethyl acetate, 0.15 mL of 50% potassium hydroxide aqueous solution, and 1 mL of 1% BHT solution to another 50 mL centrifuge tube; rinse the homogenize probe. Transfer the rinse solution to the first centrifuge tube and mash into pieces with glass rod. Then vortex to mix for 2 min and mix by the ultrasonic bath for 5 min and centrifuge at 4000 rpm for 5 min. Combine the supernatants in the eggplant type flask. Evaporate to dryness below 38°C by rotary evaporator under vacuum.

(3) Clean-up

Add 1.5 mL of acetonitrile into the eggplant type flask to dissolve residues, mix by vortex and ultrasonic bath for 5 min. Then add 1.5 mL of 0.1 mol/L hydrochloride aqueous solution, mix by vortex, and transfer into a 15-mL centrifuge tube. Wash the eggplant type flask with 5 mL n-hexane, and combine into the centrifuge tube. Mix by vortex, and centrifuge at 4000 rpm for 5 min. Discard upper n-hexane. Repeat operation with 3 mL n-hexane.

Add 3 mL of 0.1 mol/L hydrochloride aqueous solution to defatted extract. Mix well by vortex. Pass the extract through the MCX SPE cartridge at a rate of 1–2 mL/min. Rinse the column with 5 mL of 0.1 mol/L hydrochloride aqueous solution and 5 mL of methanol. Elute the benzimidazole residues with 15 mL 10% ammonium in acetonitrile. The eluted solution is then evaporated

to dryness at 38°C. Add 0.50 mL of acetonitrile to dissolve the residue and mix by the ultrasonic bath for 5 min. Add 1.50 mL of 0.025 mol/L ammonium acetate and mix well. Accurately draw 100 μL of the sample solution and mix with 1900 μL of acetonitrile-water (2+8, v/v). Filter the solution through a 0.2-μm organic filter for LC-MS-MS determination.

17.2.6 DETERMINATION

(1) HPLC conditions

Column: YMC C18, 3 μm, 150 mm × 2.1 mm (i.d.), or other equivalent;

Mobile phase: acetonitrile + 0.005 mol/L formic acid. The condition of gradient eluent is: acetonitrile 15%–80% within 7 min, keeping 2 min, 80%–15% within 0.01 min, keeping 11 min.

Flow rate: 0.25 mL/min.

Column temperature: 40°C.

Injection volume: 5 μL.

Ion source: ESI. Scan polarity: Positive mode. Scan mode is multiple reaction monitoring (MRM).

Nebulizer gas, curtain gas, heater gas, and collision gas are high purity nitrogen or other equal gas; optimize the flow rate of gas to reach the requirements of the sensitivity of mass spectrometry. DP, EP, CE should be optimized to the highest sensitivity. Reference MS parameters for praziquantel are listed in Table 17.6.

(2) Qualitative determination

The qualitative ions for each analyst include one precursor ion and two product ions at least. Under the same determination conditions, the ratio of the chromatographic retention time of the analyte shall correspond to that of the calibration solution at a tolerance of ±2.5%. Under these chromatography conditions, the reference retention times of 16 benzimidazoles

TABLE 17.6 Reference Retention Time and MS Parameters

Compound	Qualitative Ions *m/z*	Quantitative Ions *m/z*	DP *v*/v	CE v/v	Reference Retention Times t_r/*min*
Oxfendazole	316/159	316/159	38	55	7.9
	316/191			35	
Fenbendazole	300/159	300/268	37	50	10.3
	300/268			35	
Oxfendazole sulfone	332/159	332/159	31	60	8.8
	332/300			35	

TABLE 17.6 Reference Retention Time and MS Parameters—cont'd

Compound	Qualitative Ions *m/z*	Quantitative Ions *m/z*	DP *v*/v	CE v/v	Reference Retention Times t_r/*min*
Albendazole	266/191	266/234	32	50	9.2
	266/234			30	
Albendazole-2-aminosulfone	240/133	240/133	35	45	2.4
	240/198			35	
Albendazole sulfoxide	282/191	282/208	31	55	6.7
	282/208			40	
Albendazole sulfone	298/224	298/159	55	40	7.8
	298/159			30	
Mebendazole	296/105	296/264	40	50	9.1
	296/264			35	
Mebendazole-amine	238/105	238/105	70	37	6.8
	238/133			51	
5-Hydroxymebendazole	298/160	298/266	56	50	7.1
	298/266			31	
Thiabendazole	202/131	202/175	58	50	3.3
	202/175			40	
Thiabendazole-5-hydroxy	218/147	218/191	47	40	2.0
	218/191			30	
Flubendazole	314/123	314/123	43	55	9.4
	314/283			40	
2-Aminoflubendazole	256/95	256/95	65	58	7.0
	256/123			39	
Cambendazole	303/217	303/217	40	45	7.4
	303/261			30	
Oxibendazole	250/176	250/218	44	40	7.6
	250→218			30	

are listed in Table 17.6. Then if the relative intensities of the qualification ions of each analyte shall correspond to those of the calibration standard at comparable concentrations, within the tolerances shown in Table 17.3, then the corresponding analyte must be present in the sample.

(3) Quantitation determination

With optimal condition of the equipment, inject the mixed working standard solution. Using linear regression, construct a standard curve by plotting concentration in ng/mL (x) versus peak area (y). The response of the sample must be within the standard curve. A reconstituted ion chromatogram of 16 benzimidazoles is shown in Fig. 17.3.

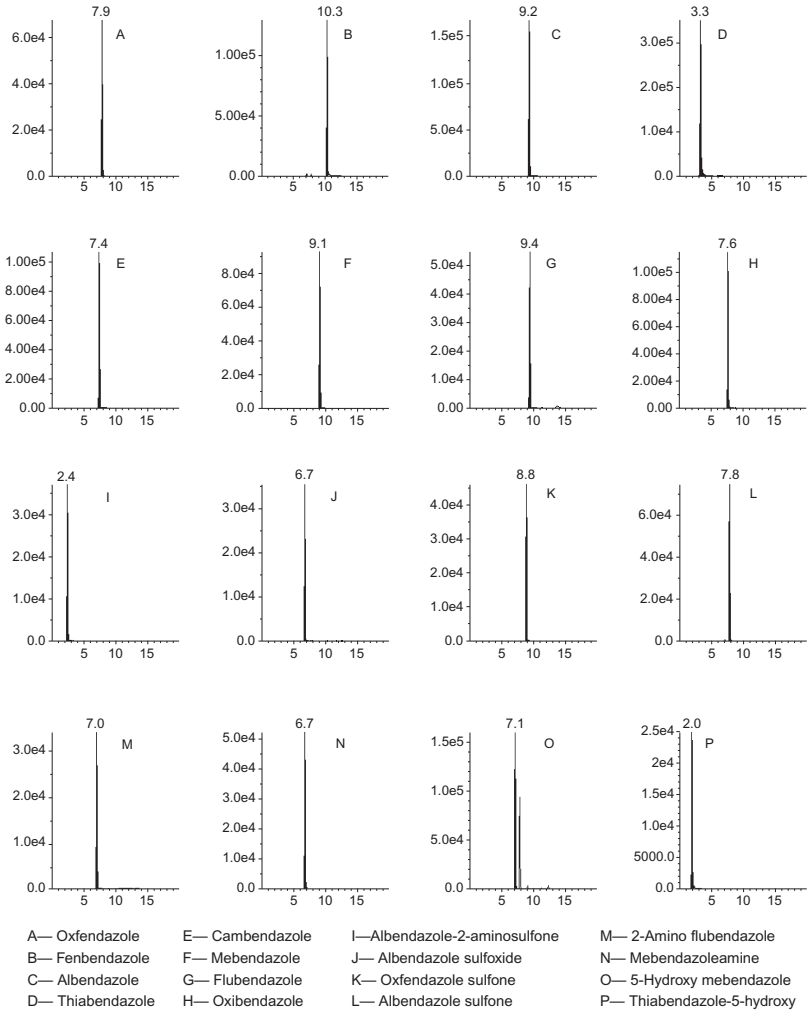

FIG. 17.3 Reconstituted ion chromatogram of 16 benzimidazoles.

17.2.7 PRECISION

The precision data are in accordance with GB/T 6379.1 and GB/T 6379.2. Repeatability and reproducibility are calculated according to the 95% confidence level.

(1) Repeatability

In repeatability conditions, the absolute difference between two independent test results does not exceed the repeatability limit (r). The scope of content and repeatability equations for the analytes is shown in Tables 17.7–17.9.

TABLE 17.7 The Scope of Content and Repeatability/Reproducibility Equations for Benzimidazole (Matrix: Muscle of Balloonfish)

Compound	Scope of Content (μg/kg)	Repeatability Limit r	Reproducibility Limit R
Albendazole-2-aminosulfone	10–100	$r=0.8329m-0.6193$	$R=0.8492m-0.4245$
2-Aminoflubendazole	10–100	$r=0.7803m-0.6827$	$R=0.9737m-0.6774$
Albendazole	10–100	$r=0.7525m-0.8178$	$R=0.5695m-0.1925$
Albendazole sulfone	10–100	$r=1.0899m-1.1719$	$R=1.0509m-0.7256$
Albendazole sulfoxide	10–100	$r=0.8330m-0.5811$	$R=0.9659m-0.5545$
Cambendazole	10–100	$r=0.9086m-1.0547$	$R=1.1101m-0.8576$
Fenbendazole	10–100	$r=1.1481m-1.4297$	$R=0.7007m-0.2852$
Flubendazole	10–100	$r=1.2024m-1.3592$	$R=0.9866m-0.7560$
Mebendazole-amine	10–100	$r=0.6880m-0.6037$	$R=0.5558m-0.0608$
Mebendazole	10–100	$r=1.0866m-1.155$	$R=1.0739m-0.8610$
Oxfendazole	10–100	$r=0.8728m-0.8434$	$R=0.7654m-0.3605$
Oxfendazole sulfone	10–100	$r=1.1447m-1.3226$	$R=1.1366m-0.9689$
Oxibendazole	10–100	$r=0.9103m-1.1427$	$R=0.7586m-0.4368$
5-Hydroxymebendazole	10–100	$r=0.9928m-1.1341$	$R=1.1255m-0.8329$
Thiabendazole	10–100	$r=1.2691m-1.5069$	$R=1.0470m-0.8076$
Thiabendazole-5-hydroxy	10–100	$r=1.1320m-1.0951$	$R=1.0769m-0.7882$

Remarks: m *is arithmetic average of two test.*

TABLE 17.8 The Scope of Content and Repeatability/Reproducibility Equations for Benzimidazole (Matrix: Muscle of Eel)

Compound	Scope of Content (μg/kg)	Repeatability Limit r	Reproducibility Limit R
Albendazole-2-aminosulfone	10–100	$r=0.8091m-0.5417$	$R=0.7975m-0.3126$
2-Aminoflubendazole	10–100	$r=0.9609m-0.8060$	$R=1.1055m-0.8544$
Albendazole	10–100	$r=0.9873m-1.0219$	$R=0.8939m-0.7496$
Albendazole sulfone	10–100	$r=1.1634m-1.1932$	$R=0.9510m-0.5360$
Albendazole sulfoxide	10–100	$r=0.8279m-0.5333$	$R=0.8965m-0.5186$
Cambendazole	10–100	$r=0.9017m-0.8756$	$R=0.7925m-0.6531$
Fenbendazole	10–100	$r=1.0009m-1.1233$	$R=0.9725m-0.8093$
Flubendazole	10–100	$r=1.0432m-0.9395$	$R=1.0924m-0.9387$
Mebendazole-amine	10–100	$r=0.7567m-0.5987$	$R=0.5169m-0.0400$
Mebendazole	10–100	$r=1.1505m-1.1776$	$R=1.0411m-0.7837$
Oxfendazole	10–100	$r=0.8963m-0.7079$	$R=0.7653m-0.3645$
Oxfendazole sulfone	10–100	$r=1.1808m-1.2341$	$R=0.9940m-0.6858$
Oxibendazole	10–100	$r=1.3296m-1.5650$	$R=0.9166m-0.6611$
5-Hydroxymebendazole	10–100	$r=1.0942m-1.0545$	$R=0.8541m-0.3941$
Thiabendazole	10–100	$r=1.3717m-1.5337$	$R=1.0299m-0.7676$
Thiabendazole-5-hydroxy	10–100	$r=1.2262m-1.2094$	$R=0.8472m-0.4269$

Remarks: m *is arithmetic average of two tests.*

If the absolute difference more than the margin threshold repeatability limit, test results should be discarded and re-completion of the determination of two single test.

(2) Reproducibility

In reproducibility conditions, the absolute difference between two independent test results does not exceed reproducibility limit (R). The scope of content and reproducibility equation for analytes shown in Tables 17.7–17.9.

17.2.8 RECOVERY

Under optimized condition, the recoveries of benzimidazole residues in balloonfish, eel, and roast eel are listed in Table 17.10.

TABLE 17.9 The Scope of Content and Repeatability/Reproducibility Equations for Benzimidazole (Matrix: Roast Eel)

Compound	Scope of Content (μg/kg)	Repeatability Limit r	Reproducibility Limit R
Albendazole-2-aminosulfone	10–100	$r=1.2481m-1.4254$	$R=1.0128m-0.8278$
2-Aminoflubendazole	10–100	$r=0.9616m-0.9686$	$R=0.7423m-0.4116$
Albendazole	10–100	$r=0.9571m-0.9049$	$R=0.9271m-0.5523$
Albendazole sulfone	10–100	$r=0.9906m-1.0955$	$R=1.0094m-0.9235$
Albendazole sulfoxide	10–100	$r=0.8921m-0.8058$	$R=0.8531m-0.5708$
Cambendazole	10–100	$r=1.1936m-1.2815$	$R=0.8004m-0.4719$
Fenbendazole	10–100	$r=1.2481m-1.4254$	$R=1.0128m-0.8278$
Flubendazole	10–100	$r=0.8573m-0.9145$	$R=0.8977m-0.7178$
Mebendazole-amine	10–100	$r=0.6628m-0.5212$	$R=0.7976m-0.5901$
Mebendazole	10–100	$r=0.8195m-0.7543$	$R=0.9955m-0.7825$
Oxfendazole	10–100	$r=1.2137m-1.3933$	$R=1.0636m-0.9992$
Oxfendazole sulfone	10–100	$r=0.9378m-0.9399$	$R=0.8649m-0.5510$
Oxibendazole	10–100	$r=0.6214m-0.5385$	$R=0.7547m-0.5536$
5-Hydroxymebendazole	10–100	$r=0.7681m-0.6829$	$R=0.7005m-0.4387$
Thiabendazole	10–100	$r=0.9745m-1.1009$	$R=0.7704m-0.6037$
Thiabendazole-5-hydroxy	10–100	$r=0.7981m-0.8052$	$R=0.9111m-0.8015$

Remarks: m *is arithmetic average of two tests.*

TABLE 17.10 The Spiking Level and Recovery of 16 Benzimidazoles

Compound	Spiking Level (μg/kg)	Average Recovery, %		
		Muscle of Balloonfish	Muscle of Eel	Roast Eel
Oxfendazole	10	108.9	99.1	106.0
	20	98.5	96.5	95.1
	50	98.7	96.8	94.7
	100	99.9	101.0	104.1
Fenbendazole	10	87.9	99.0	97.5
	20	78.7	91.4	89.1
	50	82.1	87.0	93.2
	100	85.6	90.8	90.9
Albendazole	10	92.6	96.5	95.7
	20	89.4	92.8	93.6
	50	90.9	93.6	94.7
	100	92.9	95.5	100.4
Thiabendazole	10	102.9	92.5	93.7
	20	94.7	95.5	96.2
	50	95.9	96.0	96.6
	100	98.6	99.7	100.6
Cambendazole	10	86.5	81.9	80.7
	20	82.8	81.3	79.7
	50	84.1	85.1	90.0
	100	87.5	89.4	83.4
Mebendazole	10	91.5	96.3	93.9
	20	87.6	90.5	91.5
	50	86.6	90.6	89.7
	100	86.9	92.5	90.8
Flubendazole	10	103.0	106.5	101.3
	20	94.5	98.1	98.5
	50	96.0	97.0	97.5
	100	96.2	99.5	97.8

TABLE 17.10 The Spiking Level and Recovery of 16 Benzimidazoles—cont'd

Compound	Spiking Level (μg/kg)	Average Recovery, %		
		Muscle of Balloonfish	Muscle of Eel	Roast Eel
Oxibendazole	10	98.5	104.0	102.5
	20	90.3	94.3	100.4
	50	90.6	91.0	93.1
	100	90.1	90.9	99.9
Thiabendazole-5-hydroxy	10	76.7	73.6	85.4
	20	98.3	80.4	77.6
	50	94.8	96.8	80.1
	100	116.0	94.5	110.8
Albendazole-2-aminosulfone	10	111.8	102.8	103.2
	20	92.8	97.3	103.6
	50	94.7	94.8	87.1
	100	102.9	106.5	104.1
Albendazole sulfoxide	10	93.4	99.3	98.8
	20	90.2	86.8	77.8
	50	91.3	92.5	81.8
	100	101.2	103.9	104.5
Oxfendazole sulfone	10	105.4	112.3	109.8
	20	97.9	105.4	104.4
	50	101.0	105.1	104.3
	100	102.3	105.6	110.5
Albendazole sulfone	10	99.7	114.4	108.6
	20	97.7	103.3	102.6
	50	99.7	100.1	101.5
	100	102.1	103.3	105.1
2-Aminoflubendazole	10	80.3	79.4	84.0
	20	93.6	87.4	93.2
	50	99.2	90.4	85.9
	100	97.3	99.3	93.2

Continued

TABLE 17.10 The Spiking Level and Recovery of 16 Benzimidazoles—cont'd

Compound	Spiking Level (μg/kg)	Average Recovery, %		
		Muscle of Balloonfish	Muscle of Eel	Roast Eel
Mebendazole-amine	10	92.8	90.0	99.0
	20	88.0	103.4	94.0
	50	91.3	90.2	93.5
	100	88.8	92.5	92.2
5-Hydroxymebendazole	10	106.7	88.6	95.6
	20	95.2	87.0	96.8
	50	94.7	100.6	99.6
	100	96.2	99.4	106.4

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Chapter 18

Levamisole

18.1 DETERMINATION OF LEVAMISOLE RESIDUES IN MILK AND MILK POWDER—LC-MS-MS METHOD (GB/T 22994-2008)

18.1.1 Scope

This method is applicable to the determination of levamisole residues in liquid milk (including raw milk, pure milk, and skim milk) and powdered milk (including pure milk powder, skim milk powder, and infant formula milk powder).

The limits of determination of this standard are 0.4 μg/kg in milk and 3.2 μg/kg in milk powder, respectively.

18.1.2 Principle

Levamisole in the test sample is extracted with ethyl acetate under alkaline conditions and then acidified by dilute hydrochloric acid and converted into levamisole hydrochloride. It is then transferred into a dilute hydrochloric acid layer. Clean-up is achieved by use of a strong cation exchange solid phase extraction (SPE) column. Determination is made by LC-MS-MS using an external standard method.

18.1.3 Reagents and Materials

Unless otherwise specified, all reagents used should be analytically pure; "water" is the first-degree water as GB/T6682 describes.

Ethyl acetate: HPLC grade.
Acetonitrile: HPLC grade.
Methanol: HPLC grade.
Sodium hydroxide.
Hydrochloride: 37%.
Ammonia: 28%.
Sodium chloride.
Anhydrous sodium sulfate: Ignite at 650°C for 4 h and keep in a desiccator.
Formic acid: Guaranteed reagent. Purity ≥99.9%.
0.1% Formic acid. Dissolve 1.0 mL formic acid in 1000 mL water. Prepare it just before use.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00018-X

Hydrochloric acid: 0.2 mol/L. Dissolve 18 mL hydrochloric acid in 1000 mL water.
NaOH solution: 10 mol/L. Dissolve 40 g sodium hydroxide in 100 mL water.
Ammonia-methanol (1 + 3): Dissolve 50 mL ammonia in 150 mL methanol.
Strong cation exchange (SCX) SPE column: 3 mL, 500 mg. Before using, the column should be conditioned with 3 mL methanol, 3 mL water, and 1 mL 0.2 mol/mL hydrochloric acid, and kept wet until use.
Filter membrane: 0.2 μm (amphibious: for water and organic reagents).
Levamisole (formula $C_{11}H_{12}N_2S$, molecular weight 204.29, CAS No. 16595-80-5) hydrochloride standard: Purity ≥98%.
Standard stock solution: Accurately weigh an adequate amount of levamisole hydrochloride standard (equivalent 50.0 mg levamisole), dissolve in methanol and dilute to scale in a 50-mL umber volumetric flask. Concentration of the solution is 1 mg/mL. Store at −18°C in a refrigerator. The solution can be stored for 6 months.
Standard medium solution: Pipette 1.00 mL standard stock solution into a 100-mL umber volumetric flask, dilute with methanol to the scale, and mix well. Concentration of the solution is 10 μg/mL. Store at 4°C in a refrigerator; the solution can be stored for 2 weeks.
Standard working solution: According to the requirement, prepare a standard working solution of appropriate concentrations by diluting the medium solution with mobile phase.

18.1.4 Apparatus

LC-MS-MS equipment with electronic spray ionization (ESI) source.

SPE purification equipment.
Analytical balance: With sensitivity of 0.1 mg and 0.01 g, respectively.
Vortex oscillator.
Ultrasonic cleaner.
Polypropylene centrifuge tube: 50 mL.
Pipettor: 1 mL, 2 mL.
Nitrogen evaporator.
Vial: 2 mL.

18.1.5 Sample Pretreatment

(1) Preparation of test sample

A representative sample of 1 kg is taken from the primary sample, mixed, and divided into two equal portions. Each portion is placed in a clean vessel as the test sample, which is sealed and labeled. In the operation of sampling and sample preparation, precaution should be taken to prevent the samples being contaminated or the residue content changing.

The test samples should be stored at –18°C.

(2) Extraction

For liquid milk

Take 10.0 mL of test sample into a 50-mL polypropylene centrifuge tube. Add 10 mol/mL NaOH solution 0.1 mL, 5 g sodium chloride, and 20 mL ethyl acetate; vortex mix for 1 min and ultrasonic extract for 10 min. Centrifuge for 10 min at 4000 rpm; decant supernatant liquid 10 mL into another 50-mL tube. Add 0.2 mol/L hydrochloric acid 20 mL, shake 10 min, and centrifuge for 5 min at 2000 rpm. Take the underlayer hydrochloric acid 10 mL and prepare for clean-up.

For milk powder

Weigh 12.5 g of test sample into a beaker, dissolve with an adequate volume of water at 35–50°C, and dilute to 100.0 mL after the solution cools to room temperature. Take 10.0 mL of this solution into a 50-mL centrifuge tube and then operate the same as the extraction step of liquid milk sample.

(3) Clean-up

Load the hydrochloric acid extract into the SCX column and wash the column with 3 mL water, 1 mL 0.2 mol/mL hydrochloric acid and 3 mL methanol, one after another. Elute with 4 mL ammonia-methanol. Evaporate the eluate to nearly dry using a nitrogen evaporator. Dissolve the residue in 1.0 mL mobile phase and put through the filter. Collect the filtrate and prepare for injection.

18.1.6 Determination

(1) Operating condition

Column: BEH C_{18}, 1.7 μm, 50 mm × 2.1 mm, or equivalent.
Mobile phase: Acetonitrile +0.1% formic acid (15 + 85, v/v). Flow rate: 0.25 mL/min.
Column temperature: 35°C.
Injection volume: 5.0 μL.
Ionization mode: Electronic spray ionization, positive ion scan (ESI+).
Determination mode: Multiple reaction monitoring (MRM).
Capillary voltage: 2.5 kV.
Ion source temperature: 110°C.
Desolvation gas temperature: 380°C.
Desolvation gas (N2) flow: 600 L/h.
Cone gas (N2) flow: 50 L/h.
Collision-induced dissociation gas (Ar) flow: 0.1 L/h.
Cone voltage, collision-induced dissociation energy, dwell time, parent ion and daughter ions: see Table 18.1.

(2) LC-MS-MS confirmation

Under the preceding LC-MS-MS operating conditions, the retention time of levamisole is about 1.2 min. A chromatogram of standards is shown in Fig. 18.1.

TABLE 18.1 Diagnostic Ions, Quantitative Ions, and Collision Energy

Diagnostic Ions (*m*/*z*)	Quantitative Ions (*m*/*z*)	Collision Energy (V)	Dwell Time (ms)	Cone Voltage (V)
205.5/91.9	205.5/91.9	38	300	40
205.5/178.9		20	300	40

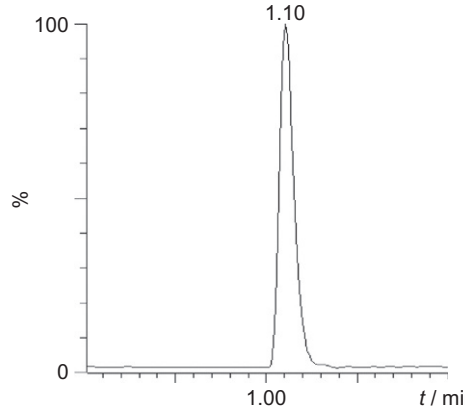

FIG. 18.1 The MRM chromatogram of levamisole standard.

If the retention times of the sample chromatogram peak are consistent with the standard, and if after background compensation all the diagnostic ions are present, the relative ion intensity corresponds to that of the levamisole calibration standard, at comparable concentration, measured under the same conditions, within the tolerances listed in Table 18.2, then levamisole must be present in the sample.

(3) LC-MS-MS quantified determination

Inject series of levamisole standard working solutions separately and make the standard working curve. Check the capability of the instrument,

TABLE 18.2 The Tolerance Deviation of Relative Ion Intensity in Confirmation (unit: %)

Relative ion intensity *K*	$K > 50$	$20 < K < 50$	$10 < K < 20$	$K \leq 10$
Tolerances deviation	±20	±25	±30	±50

determining the linear range. Determine quantitatively the samples through the instrument workstation or through the curve of the external standard. The responses of levamisole in the sample solutions should be within the linear range of the instrumental detection. The standard working solution and the sample solution should be injected alternatively.

18.1.7 Precision

The precision data of this part is confirmed according to GB/T 6379.1 and GB/T 6379.2. The values of repeatability and reproducibility are calculated at the 95% confidence level.

(1) Repeatability

Under the repeatability condition, the difference of absolute value of two independent results is not above the repeatability limit (r). The content ranges and the repeatability equation of levamisole in milk and milk powder are shown in Table 18.3.

(2) Reproducibility

Under the reproducibility condition, the difference of absolute value of two independent results is not above the reproducibility limit (R). The content ranges and the reproducibility equations of levamisole in milk and milk powder are shown in Table 18.3.

18.1.8 Recovery

Under optimized condition, the recoveries of levamisole in milk and milk powder using this method are listed in Table 18.4.

TABLE 18.3 Content Ranges and the Repeatability/Reproducibility Equations (Unit: μg/kg)

Analyte	Matrix	Content Range (μg/kg)	Repeatability Limit r	Reproducibility Limit R
Levamisole	Milk	0.4–20	$\lg r = 1.1339 \lg m - 0.5680$	$\lg R = 1.5791 \lg m - 0.8882$
	Milk powder	3.2–160		

Note: m *is average result of two determinations.*

TABLE 18.4 The Data of Spiked Level and Recoveries of Levamisole ($n = 10$)

Matrix	Spiked Level (μg/kg)	Recovery Range (%)	Mean Recovery (%)	RSD (%)
Milk	2.0	65.5–103.6	78.2	17.8
	10	75.2–95.1	80.5	10.2
	20	79.3–96.8	86.0	8.6
	100	78.7–98.9	89.1	9.01
Milk powder	5.0	64.6–105.0	87.3	18.3
	10	71.3–96.4	83.5	12.5
	20	79.0–97.6	88.5	8.8
	100	83.1–99.2	93.4	6.4

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Chapter 19

Thiourea

19.1

Curative Effects and Side Effects of Thiouracil

Thiouracil derivatives have medical and biological properties. They have been applied illegally to obtain a higher live weight gain. This gain was mainly due to higher water retention in edible tissue and filling of the gastrointestinal tract. The first effect gives a fraudulous higher weight ("water instead of meat") which in turn leads to a reduction of the meat quality. Thiouracil derivatives inhibit nucleic acid metabolism. Moreover, they also inhibit the formation of thyroid hormones and are used for the treatment of chromatoghyperthyroidism and Basedow's disease.

The use of thyreostatic drugs (i.e., thiouracils) for promoting animal growth is prohibited in European Union (EU) member states because not only could the thyreostatic drug be harmful to human health but also the meat derived from animals treated with such drugs is of lower quality.

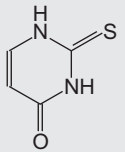
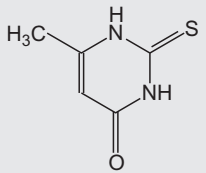
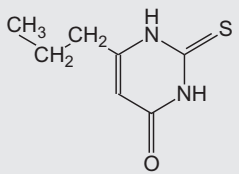
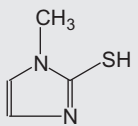
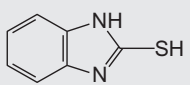
19.2

Chemical Character and Maximum Residue Limit of 2-Thiouracil, Methyl Thiouracil, Propyl Thiouracil, Tapazole, and 2-Mercaptobenzimidazole

Table 19.1 contains chemical structures and maximum residue limits (MRLs).

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00019-1

TABLE 19.1 Chemical Structures and MRLs

Compound Names	Molecular Structure	Molecular Weight	Cas. No.	MRLs (μg/kg)
2-Thiouracil		128.15	141-90-2	EU:banned
Methyl thiouracil		142.18	56-04-2	EU:banned
Propyl thiouracil		170.23	2954-52-1	EU:banned
Tapazole		114.02	60-56-0	EU:banned
2-Mercaptobenzimidazole		150.20	583-39-1	EU:banned

19.3

Determination of 2-Thiouracil, Methyl Thiouracil, Propyl Thiouracil, Tapazole and 2-Mercaptobenzimidazole Residues in Bovine Thyroid and Muscles—LC-MS-MS Method GB/T 20742-2006)

19.3.1 SCOPE

This method is applicable to the determination of 2-thiouracil, methyl thiouracil, propyl thiouracil, tapazole, and 2-mercaptobenzimidazole residues in bovine thyroid and muscle tissues.

The limit of determination of this method for 2-thiouracil, methyl thiouracil, propyl thiouracil, tapazole and 2-mercaptobenzimidazole is 2 μg/kg.

19.3.2 PRINCIPLE

The residues of 2-thiouracil, methyl thiouracil, propyl thiouracil, tapazole and 2-mercaptobenzimidazole are extracted from samples with ethyl acetate and cleaned up on an Amino Bond Elut (NH_2) SPE column, and derivatized with 4-Chloro-7-nitrobenzo-2-furazan. The derivatized product is cleaned up on an Oasis HLB cartridge and analyzed by LC-MS-MS.

19.3.3 REAGENTS AND MATERIALS

Methanol, acetonitrile, and ethyl acetate are all of HPLC grade; Disodium hydrogen phosphate, $Na_2HPO_4{\cdot}12H_2O$, potassium dihydrogen phosphate, KH_2PO_4, acetate acid, concentrate hydrochloric acid, sodium hydroxide, chloroform, and hexane are all of G.R. grade; 4-Chloro-7-nitrobenzo-2-furazan, $C_6H_2ClN_3O_3$: purity $\geq$99%;

Phosphate buffer solution: pH = 8, 0.2 mol/L. Place 67.7 g disodium hydrogen phosphate and 1.5 g potassium dihydrogen phosphate into 1000-mL volumetric flask and dissolve in water. Dilute solution to volume with water. Hydrochloric acid solution: 0.2 mol/L. Dilute 17 mL concentrate hydrochloric acid to 1000 mL with water.

Sodium hydroxide solution: 1 mol/L, place 40 g sodium hydroxide into 1000-mL volumetric flask and dissolve in water. Dilute solution to volume with water; Agent of derivation: 5 mg/mL. Place 0.05 g 4-Chloro-7-nitrobenzo-2-furazan in 10 mL methanol. Freshly prepare each day.

Mercaptoethanol: Analytically pure; Na_2-EDTA solution: 0.1 mol/L. Place 37.2 g $C_{10}H_{14}N_2O_8Na_2{\cdot}2H_2O$ in 1000 mL water;
Sep-Park Amino Propyl solid extraction columns or equivalent extraction: 500 mg, 3 mL. Condition each column with 20 mL hexane before use; Oasis HLB solid extraction columns or equivalent extraction: 60 mg, 3 mL. Condition each column with 5 mL methanol followed by 10 mL water before use.
Standard stock solutions of 2-thiouracil, methyl thiouracil, propyl thiouracil, tapazole and 2-mercaptobenzimidazole: 1.0 mg/mL. Accurately weigh appropriate amount of each drug standard, dissolve in a small volume of methanol, and then dilute with methanol separately to prepare the standard stock solutions of 1.0 mg/mL in concentration. Keep at −18°C. Storage life is 6 months.
Internal standard: 2-methoxethoxymercaptobenzimidazole, purity ≥99%.
Standard stock solutions of internal standards: 1.0 mg/mL. Accurately weigh appropriate amount of internal standard, dissolve in a small volume of methanol, and then dilute with methanol to prepare the internal standard stock solutions of 1.0 mg/mL in concentration. Keep at −18°C. Storage life is 6 months.
Standard working solutions of internal standards: 1.0 μg/mL. Accurately pipette appropriate amount of internal standard stock solutions. Dilute with methanol to prepare the internal standard working solutions of 0.1 μg/mL in concentration. Keep at −18°C. Storage life is 3 months. Return to room temperature before use.

19.3.4 APPARATUS

LC-MS-MS: Equipped with ESI source; Analytical balance: Capable weighing to 0.1 mg, 0.01 g; Solid phase extraction (SPE) vacuum apparatus; Nitrogen evaporator; Incubator; Vacuum pump: Vacuum to 80 kPa; Microsyringes: 25 μL, 100 μL; Brown centrifuge tubes: 25 mL, 50 mL; pH meter: Capable of measuring ±0.02 unit; Reservoirs and adapters to fit SPE columns: 50 mL; Centrifugal machine: Rotate speed exceeded 4000 rpm.

19.3.5 SAMPLE PRETREATMENT

(1) Sample preparation

Add three times weight of dry ice to 50–100 g negative samples. Smash with the tissue-smashing machine and then evaporate the dry ice to room temperature. Take 0.5 kg as the test sample. The test samples should be stored at −18°C.

(2) Extraction and initial clean-up

Weigh 5 × 1 g samples (accurate to 0.01 g) into 50-mL brown centrifuge tubes, Fortify five blank samples respectively with appropriate amount of mixed standard working solution. Form 1.0, 2.0, 5.0, 10, and 20 ng/mL of the five analytes. Add internal standard as 2.0 ng/mL.

Weigh 1 g unknown sample (accurate to 0.01 g) into 50-mL brown centrifuge tubes and add internal standard as 2.0 ng/mL.

Add 10 mL ethyl acetate, 3 g anhydrous sodium sulfate, 20 μL mercaptoethanol, 30 μL 0.1 mol/L Na_2-EDTA solution to the above centrifuge tubes, and homogenize for 15 s. Then wash the homogenizer with 5 mL ethyl acetate. Mix in the centrifuge tubes. Centrifuge at 4000 rpm for 5 min. Evaporate the supernatant solution to dryness on a nitrogen evaporator at 50°C. Dissolve the residue with acetonitrile, add 3 mL hexane, and shake for 1 min. Centrifuge at 4000 rpm for 5 min and dump the hexane. Repeat the approach with 3 mL hexane. Evaporate the solution to dryness on nitrogen evaporator at 50°C. Dissolve the remnants with 0.5 mL chloroform and 3 mL hexane. Solution is decanted into preconditioned Sep-Park Amino Propyl solid extraction column with reservoir, and the flow rate is adjusted to $\leq$2 mL/min. Let the solution pass through the Sep-Park Amino Propyl solid extraction column. Wait until the solution has thoroughly drained and then rinse the evaporate tube with 10 mL hexane and pass through the solid extraction column. Discard all the effluents. Finally, elute with 5 mL Elution solution into 25-mL brown centrifuge tubes. Evaporate the elute solution to dryness on a nitrogen evaporator at 50°C.

(3) Derivatization and clean-up

Dissolve the remnants with 5 mL phosphate buffer solution (0.1 mol/L pH = 8). Add 0.3 mL derivatizate solution to the samples and and mix for 15 s. Keep the reaction mixture for 3 h at 50°C. Return to room temperature and adjust pH to 3–4 with 0.2 mol/L hydrochloric acid solution. Solution is decanted into preconditioned Oasis HLB solid extraction column with reservoir, and the flow rate is adjusted to $\leq$2 mL/min. Let solution pass through the Oasis HLB solid extraction column. Wait until the solution has thoroughly drained and then rinse evaporate tube with 10 mL water and pass through the Oasis HLB solid extraction column. Discard all the effluents. Dry the cartridge by drawing air through it for 10 min under 65-kPa Vacuum. Finally, elute derivatives with 5 mL ethyl acetate into 25-mL brown centrifuge tubes. Evaporate the elute solution to dryness on a nitrogen evaporator at 50°C. Add accurately 0.3 mL anhydrous ethanol and 0.7 mL dilute solution. Filter with 0.2-μm syringe filter and determine with LC-MS-MS.

19.3.6 DETERMINATION

(1) Operating conditions

LC column: Atlantis C18, 3.5 μm, 150 mm × 2.1 mm (i.d.), or equivalent; Column temperature: 45°C; Injection volumes: 20 μL. Mobile phase and flow rate: see Table 19.2.

(2) Qualitative analysis

Select one precursor and more than two product ions. Under the same conditions, compare the ratio of the chromatographic retention time of the analyte and the internal standard. If the ratio error is between 2.5% compared with the matrix standard solutions, and the relative ion

TABLE 19.2 Mobile Phase and Flow Rate

Time (min)	Flow Rate (μL/min)	0.3% Acetate Acid Solution (%)	0.3% Acetate Acid Acetonitrile (%)
0.00	200	85	15
1.00	200	85	15
1.01	200	50	50
12.00	200	10	90
12.01	200	85	15
20.00	200	85	15

TABLE 19.3 Maximum Permitted Tolerances for Relative Ion Intensities While Confirmation

Relative Intensity (k)	$k > 50$	$20 < k \leq 50$	$10 < k \leq 20$	$k \leq 10$
Permitted tolerance	±20	±25	±30	±50

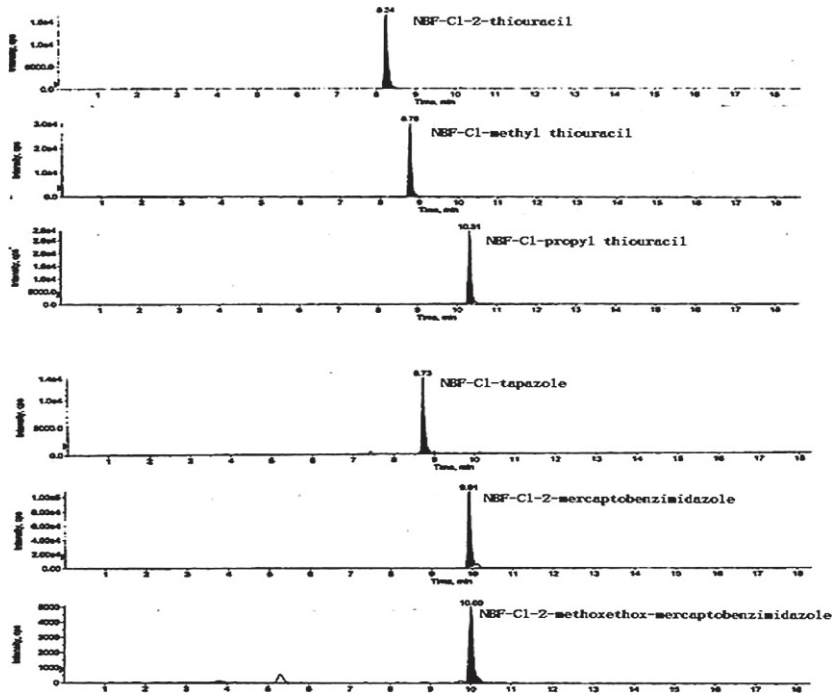


FIG. 19.1 MRM chromatogram of the analyte standards and internal standard.

TABLE 19.4 Precursor/Product Ion Combinations, Collision Energy, and Declustering Potential

Analytes	Qualifying Parent/ Production (*m*/*z*)	Quantifying Parent/ Production (*m*/*z*)	Time (ms)	Declustering Potential (V)	Collision Energy (V)
2-Thiouracil	292/229	292/229	100	55	29
	292/216				29
Methyl thiouracil	306/243	306/243	100	55	29
	306/230				31
Propyl thiouracil	334/271	334/271	100	55	30
	334/258				31
Tapazole	278/202	278/202	100	50	32
	278/232				26
2-Mercaptobenzimidazole	314/238	314/238	100	52	36
	314/268				28
2-Methoxethox-mercaptobenzimidazole (internal standard)	344/268	344/268	100	55	34
	344/281				35

intensity of 2-thiouracil, methyl thiouracil, propyl thiouracil, tapazole, and 2-mercaptobenzimidazole in the sample and matrix standard solutions is in correspondence with the data in Table 19.3, this sample is confirmed to contain this compound.

(3) Quantitative analysis

Internal standard method: Use the software with the instrument.

External standard method: Inject the different concentration mixed matrix calibration standard solutions of 2-thiouracil, methyl thiouracil, propyl thiouracil, tapazole and 2-mercaptobenzimidazole, respectively, in duplication under LC and MS conditions. Draw these standard curves of 2-thiouracil, methyl thiouracil, propyl thiouracil, tapazole and 2-mercaptobenzimidazole (concentration vs. peak area). Calculate the concentrations of the corresponding content from the working curve. The responses of the analyte in the sample solutions should be in the linear range of the instrumental detection. For an MRM chromatogram of the analyte standard and internal standards see Fig. 19.1.

Ion source: ESI source; Scan mode: Positive scan; Monitor mode: Multiple reaction monitor (MRM); Ion spray voltage: 4500 V; Turbo ion spray gas rate: 7 L/min; Source temperature: 450°C; Focusing potential: 140 V; Collision cell exit potential: 12 V; Precursor/product ion combinations, collision energy, and declustering potential: see Table 19.4.

TABLE 19.5 Analytical Range, Repeatability, and Reproducibility

Analytes	Content Range (μg/kg)	Limit of Repeatability (r)	Limit of Reproducibility (R)
2-Thiouracil	2-20	lg r=0.8797 lg m−0.8542	lg R=0.7701 lg m−0.6505
Methyl thiouracil	2-20	lg r=0.8320 lg m−0.8572	lg R=1.0239 lg m−0.8446
Propyl thiouracil	2-20	lg r=1.1093 lg m−1.0710	lg R=1.0348 lg m−0.8900
Tapazole	2-20	R=0.1327 m−0.0009	lg R=1.0130 lg m−0.7885
2-Mercaptobenzimidazole	2-20	lg r=1.0599 lg m−0.9321	lg R=1.0539 lg m−0.8078

Note: m *is the average value obtained from two independent determination results.*

TABLE 19.6 The Fortifying Concentrations and Recoveries

Analytes	Fortifying Concentration and Average Recovery (%)							
	2 µg/kg		5 µg/kg		10 µg/kg		20 µg/kg	
	Bovine-thyroid	Beef	Bovine-thyroid	Beef	Bovine-thyroid	Beef	Bovine-thyroid	Beef
NBF-Cl-2-thiouracil	86.6	88.9	87.1	89.9	82.1	86.5	83.2	87.8
NBF-Cl-methyl thiouracil	87.8	90.2	89.2	91.2	84.2	85.4	85.4	90.1
NBF-Cl-propyl thiouracil	85.9	91.3	82.8	90.5	87.3	81.5	83.7	88.2
NBF-Cl-tapazole	83.8	86.7	87.9	89.1	85.2	88.3	82.9	83.2
NBF-Cl-2-mercaptobenzimidazole	85.6	88.4	84.6	87.2	87.1	82.1	81.7	85.4

19.3.7 PRECISION

The precision data of the method has been determined in accordance with the stipulations of GB/T6379. The values of repeatability and reproducibility are obtained and calculated at the 95% confidence level.

(1) Repeatability

Under the condition of repeatability, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of repeatability (r); repeatability and content range of this method are shown in Table 19.5.

If the difference of values exceeds the limit of repeatability, the testing results should be discarded and two individual testing determinations shall be re-conducted, and completed.

(2) Reproducibility

Under the condition of reproducibility, the difference of the absolute values obtained from two independent determination results shall not exceed the limit of reproducibility (R), and reproducibility and content range of this method are listed in Table 19.5.

If the difference of values exceeds the limit of reproducibility, the testing results should be discarded and two individual testing determinations shall be reconducted and completed.

19.3.8 RECOVERY

Under optimized conditions, the recoveries of 2-thiouracil, methyl thiouracil, propyl thiouracil, tapazole and 2-mercaptobenzimidazole using this method are listed in Table 19.6.

RESEARCHERS

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Chapter 20

Polyether

20.1 DETERMINATION OF SIX POLYETHER ANTIBIOTIC RESIDUES IN MILK AND MILK POWDER—LC-MS-MS METHOD (GB/T 22983-2008)

20.1.1 Scope

This method is applicable to the determination of lasalocid, monensin, nigericin, salinomycin, narasin, and madubamycin ammonium in liquid milk (including raw milk, pure milk, and skim milk) and powdered milk (including pure milk powder, skim milk powder, and infant formula milk powder).

The limits of determination of lasalocid, monensin, nigericin, salinomycin, narasin, and madubamycin ammonium are 0.2 μg/L in milk and 1.6 μg/kg in milk powder, respectively.

20.1.2 Principle

Polyether antibiotics in the test samples are extracted with acetonitrile. The extract is cleaned up by SPE column. Determination is made by LC-MS-MS using an external standard method.

20.1.3 Reagents and Materials

Unless otherwise specified, all reagents used should be analytically pure; "water" is the first–degree water as GB/T 6682 describes.

Acetonitrile: HPLC grade.
Methanol: HPLC grade.
n-Hexane: HPLC grade.
n-Hexane saturated by acetonitrile: Add 2 mL acetonitrile into 100 mL n-hexane, mix well and keep quiescent; transfer supernatant liquid to use.
Formic acid: Guaranteed reagent. Purity ≥99.9%.
Ammonium acetate.
Anhydrous sodium sulfate: Ignite at 650°C for 4 h and keep in a desiccator.
Aqueous mobile phase: Dissolve 0.358 g ammonium acetate and 1.0 mL formic acid in 1000 mL water. Prepare it at time of use.

Analytical Methods for Food Safety by Mass Spectrometry. https://doi.org/10.1016/B978-0-12-814165-6.00020-8

Methanol-water (50+50): Mix methanol and water by volume ratio 1:1.
SPE column: 3 mL, 60 mg, Oasis HLB or equivalent. The SPE column should be conditioned with 3 mL methanol and 5 mL water, and keep it wet until using.
Filter: 0.22 μm, for organic solvent.
Standards: Lasalocid, monensin, nigericin, salinomycin, narasin and madubamycin ammonium: Purity ≥95%.
Stock standard solution: Accurately weigh an adequate amount of each standard and separately dissolve in methanol to prepare a solution of 1 mg/mL in concentration as the standard stock solution. Store at −18°C; the solution can be stored for 2 months.
Working standard solution: Pipette 1.00 mL standard stock solution into a 100-mL umber volumetric flask, dilute with methanol to the scale, and mix well. Concentration of the solution is 10 μg/mL. Store at 4°C in a refrigerator; the solution can be stored for 1 week.
Matrix adjusted standard working solutions: Mix suitable adequate amount of the working standard solution with 10-g blank samples, according to the sample extraction and clean-up process; prepare matrix adjusted standard working solutions with series concentrations of 1.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/mL.

20.1.4 Apparatus

LC-MS-MS: Equipped with electronic spray ionization source (ESI).
Analytical balance: With sensitivity of 0.1 mg and 0.01 g, respectively.
Vortex mixer.
Oscillator.
Centrifuge: Rotate speed > 5000 rpm.
Pipette: 1 mL.
Rotary vacuum evaporator.
Heart-shaped flask: 50 mL.
SPE system.
Nitrogen evaporator.

20.1.5 Sample Pretreatment

(1) Preparation of test sample

Take 1 kg representative sample from the primary sample, mix, and divide into two equal portions. Each portion is placed in a clean vessel as the test sample, which is sealed and labeled. In the operation of sampling and sample preparation, precaution should be taken to prevent the samples from being contaminated or the residue content changing.

The test samples should be stored at −18°C.

(2) Extraction

For liquid milk

Take 10.0 mL of test sample into a 50-mL polypropylene centrifuge tube. Add 2 × 20 mL of acetonitrile and 10 g anhydrous sodium sulfate. Mix by vortex mixer and homogenize for 1 min at 10,000 rpm. Centrifuge for 3 min at 3000 rpm, combine the supernatant liquid into another 50-mL tube, add 10 mL hexane saturated by acetonitrile, and vortex for 1 min. Discard the hexane layer and decant the acetonitrile extract to a heart-shaped flask; evaporate to near dryness at 40°C using a rotary vacuum evaporator. Dissolve the residue in 4 mL methanol-water and prepare for clean-up.

For milk powder

Weigh 12.5 g of test sample into a beaker, dissolve with an adequate volume of water at 35–50°C and dilute to 100.0 mL after the solution cools to room temperature. Take 10.0 mL of this solution into a 50-mL centrifuge tube, and then follow the same procedure as the above-mentioned extraction steps of liquid milk sample.

(3) Clean-up

Load the extract into the SPE column, rinse the heart-shaped flask with 2 mL methanol-water, and load into the SPE column. Wash the column with 5 mL water and 3 mL methanol-water. Elute the column with 4 mL methanol. Evaporate the eluant to dryness at 40°C under nitrogen flow. Dissolve the residue in 1.0 mL methanol and put through the filter. Collect the filtrate and prepare for injection.

20.1.6 Determination

(1) HPLC operating condition

Column: BEH C_{18}, 1.7 μm, 50 mm × 2.1 mm, or equivalent.
Mobile phase: A: Methanol. B: Aqueous mobile phase. For the flow rate and the wash program, see Table 20.1.
Column temperature: 40°C.
Injection volume: 5.0 μL.

TABLE 20.1 Mobile Phase Flow Rate and the Wash Program

Time (min)	Flow Rate (μL/min)	A (%)	B (%)
0.00	250	90.0	10.0
2.00	250	70.0	30.0
2.01	250	90	10.0
2.50	250	90	10.0

Ionization mode: Electronic spray ionization.
Scan mode: Positive ion scan.
Determination mode: Multiple reaction monitoring (MRM).
Capillary voltage: 2.8 kV.
Ion source temperature: 110°C.
Desolvation gas temperature: 380°C.
Desolvation gas (N2) flow: 600 L/h.
Cone gas (N2) flow: 50 L/h.
Collision-induced dissociation gas (Ar) flow: 0.1 L/h.
Cone voltage, collision-induced dissociation energy, dwell time, parent ion and daughter ions: see Table 20.2.

(2) HPLC-MS-MS determination

Inject the series of matrix adjusted standard working solutions (4.15) separately and make the standard working curve. Check the capability of the instrument and determine the linear range. Determine the samples quantitatively through the instrument workstation or through the curve of the external standard. The responses of polyether antibiotics in the sample solutions should be within the linear range of the instrumental detection. The standard solution and the sample solution should be injected alternatively. Under the preceding LC-MS-MS operating conditions, the retention times of six polyether antibiotics are shown in Table 20.2. Chromatograms of the standards are shown in Fig. 20.1–20.6.

If the retention times of sample chromatogram peaks are consistent with the standards, and if after background compensations all the diagnostic ions are present, the relative ion intensities correspond to those of the calibration standard, at comparable concentrations, measured under the same conditions, within the tolerances listed in Table 20.3, then the corresponding analyte must be present in the sample.

(4) Parallel test

The operation of the parallel test is the same as that described in the method of determination.

(5) Blank test

The operation of the blank test is the same as that described in the method of determination but with the omission of the sample addition.

20.1.7 Precision

The precision data of this part is confirmed according to GB/T 6379.1 and GB/T 6379.2. The values of repeatability and reproducibility are calculated at a 95% confidence level.

(1) Repeatability

Under the repeatability condition, the difference of the absolute value of two independent results is not above the repeatability limit (r). The

TABLE 20.2 Mass Parameters and Retention Times of Six Polyether Antibiotics

Compound Name	Retention Time (min)	Quantitative Ions (*m*/*z*)	Diagnostic Ions (*m*/*z*)	Collision Energy (V)	Dwell Time (ms)	Cone Voltage (V)
Lasalocid	1.48	613.5/377.1	613.5/377.1 613.5/595.3	28 38	50	72
Monensin	1.23	693.5/675.6	693.5/675.6 693.5/479.3	51 40	50	65
Nigericin	1.76	742.7/657.6	742.7/657.6 742.7/461.4	30 25	50	42
Salinomycin	1.55	773.6/431.1	773.6/773.6 773.6/531.5	50 48	50	65
Narasin	1.85	787.6/431.5	787.6/787.6 787.6/531.3	45 48	50	70
Madubamycin ammonium	1.38	939.6/877.4	939.6/877.4 939.6/897.3	30 30	50	38

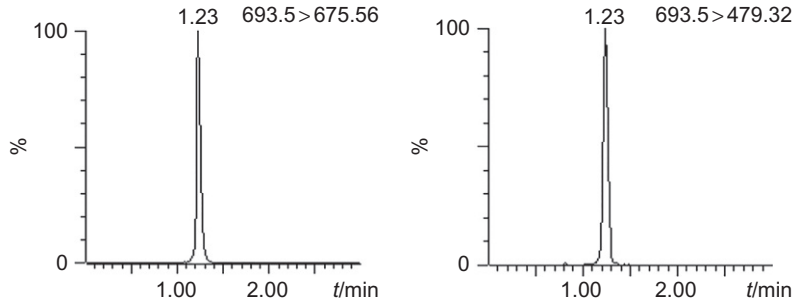

FIG. 20.1 The MRM chromatograms of monensin.

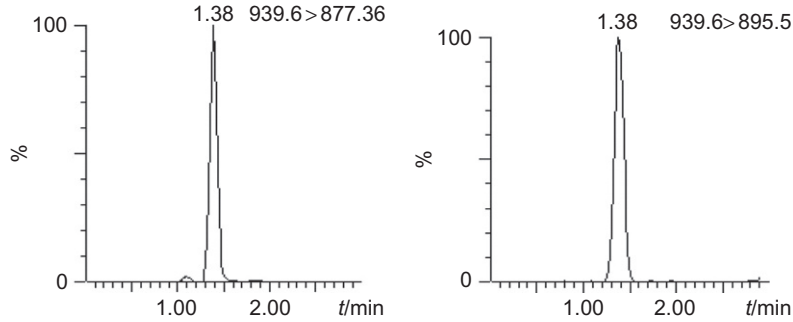

FIG. 20.2 The MRM chromatograms of madubamycin ammonium.

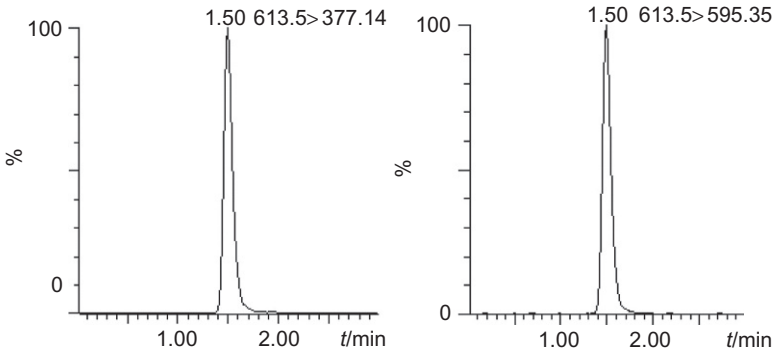

FIG. 20.3 The MRM chromatograms of lasalocid.

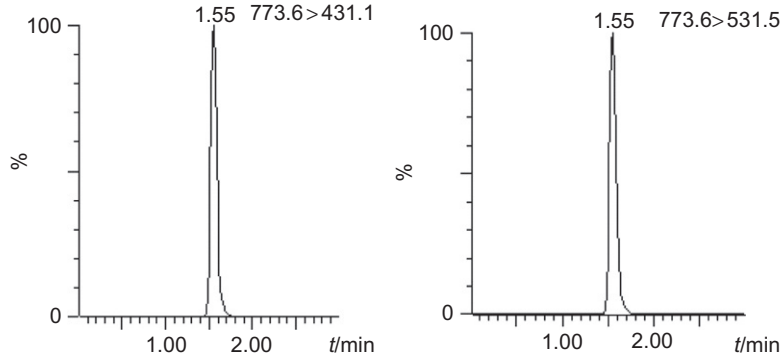

FIG. 20.4 The MRM chromatograms of salinomycin.

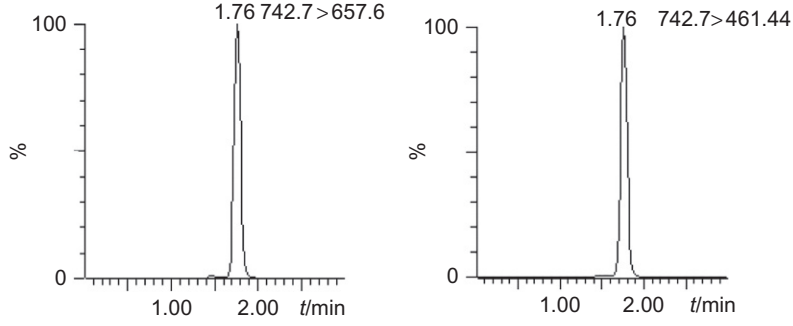

FIG. 20.5 The MRM chromatograms of nigericin.

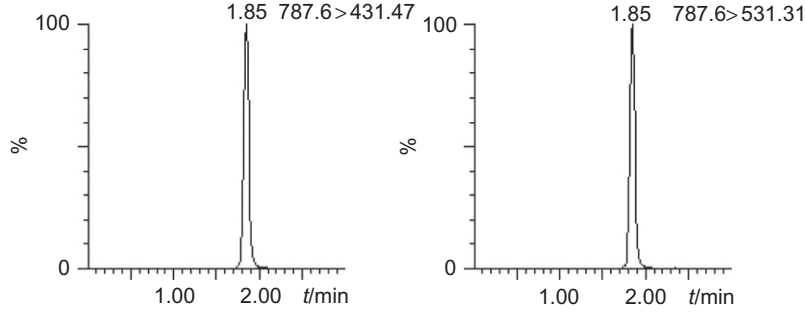

FIG. 20.6 The MRM chromatograms of narasin.

TABLE 20.3 Maximum Allowed Deviation of the Ion Ratio by Qualitative Analysis (unit: %)

Ion ratio k	$K > 50$	$20 < K < 50$	$10 < K < 20$	$K \leq 10$
Tolerance deviation	±20	±25	±30	±50

TABLE 20.4 Content Ranges and the Repeatability and Reproducibility Equations (Unit: μg/kg)

Analyte	Matrix	Content Range (μg/kg)	Repeatability Limit r	Reproducibility Limit R
Lasalocid	Milk	0.2–4	$\lg r = 1.0148 \lg m - 0.5230$	$\lg R = 0.9584 \lg m - 0.5858$
	Milk powder	1.6–32		
Monensin	Milk	0.2–4	$\lg r = 1.1903 \lg m - 0.5961$	$\lg R = 1.3408 \lg m - 0.5902$
	Milk powder	1.6–32		
Nigericin	Milk	0.2–4	$\lg r = 0.9507 \lg m - 0.5201$	$\lg R = 0.8905 \lg m - 0.5728$
	Milk powder	1.6–32		
Salinomycin	Milk	0.2–4	$\lg r = 0.9679 \lg m - 0.5258$	$\lg R = 0.9683 \lg m - 0.9527$
	Milk powder	1.6–32		
Narasin	Milk	0.2–4	$\lg r = 1.0899 \lg m - 0.5782$	$\lg R = 1.0254 \lg m - 0.6140$
	Milk powder	1.6–32		
Madubamycin ammonium	Milk	0.2–4	$\lg r = 1.0860 \lg m - 0.6474$	$\lg R = 1.0523 \lg m - 0.6765$
	Milk powder	1.6–32		

Note: m *equals the average of two results.*

content ranges and the repeatability equations of the six polyether antibiotics in milk and milk powder are shown in Table 20.4.

If the difference is above the repeatability limit (r), the result of the experiment should be abandoned and two independent experiments should be redone.

(2) Reproducibility

Under the reproducibility condition, the difference of absolute value of two independent results is not above the reproducibility limit (R). The content ranges and the reproducibility equations of the six polyether antibiotics in milk and milk powder are shown in Table 20.4.

20.1.8 Recovery

Under optimized condition, the recoveries of **six polyether antibiotics** in milk and milk powder using this method are listed in Table 20.5 and 20.6.

TABLE 20.5 The Mean Recoveries in Milk of Six Polyether Antibiotics ($n = 6$)

No.	Analyte	Spiked Level (μg/kg)	Recovery Range (%)	Mean Recovery (%)	RSD (%)
1	Lasalocid	1	81.0–106.0	97.6	16.6
		2	78.0–102.7	92.3	12.3
		5	87.0–104.4	95.8	6.9
		10	80.1–100.5	87.6	9.0
2	Monensin	0.5	90.0–108.0	94.0	15.3
		2	82.5–103.0	91.7	11.2
		5	83.0–107.0	92.4	9.8
		10	79.2–101.0	89.9	8.7
3	Nigericin	0.5	86.0–108.0	88.3	12.5
		2	88.0–105.0	94.4	13.0
		5	86.8–103.9	97.0	9.7
		10	81.0–104.3	95.5	8.5
4	Salinomycin	1	79.0–104.0	87.8	13.4
		2	79.5–105.0	92.3	10.1
		5	91.2–101.4	85.8	9.9
		10	79.0–100.9	97.5	9.4
5	Narasin	0.5	82.0–104.0	86.6	10.3
		2	88.0–104.1	91.7	11.3
		5	81.6–103.6	92.4	7.9
		10	83.4–100.6	89.8	8.5

Continued

TABLE 20.5 The Mean Recoveries in Milk of Six Polyether Antibiotics ($n = 6$)—cont'd

No.	Analyte	Spiked Level (μg/kg)	Recovery Range (%)	Mean Recovery (%)	RSD (%)
6	Madubamycin ammonium	1	83.0–111.0	88.9	13.1
		2	90.5–101.5	94.8	10.9
		5	93.4–115.6	97.0	10.2
		10	89.0–103.4	98.5	5.1

TABLE 20.6 The Mean Recoveries in Milk of Six Polyether Antibiotics ($n = 6$)

No.	Analyte	Spiked Level (μg/kg)	Recovery Range (%)	Mean Recovery (%)	RSD (%)
1	Lasalocid	2	84.5–105.0	87.6	15.0
		4	83.7–103.2	92.3	12.2
		10	75.6–105.3	85.8	8.9
		20	86.0–105.2	95.5	9.2
2	Monensin	1	93.2–110.1	86.0	11.3
		2	89.1–105.0	91.7	11.7
		5	81.0–107.30	92.4	6.9
		10	95.4–105.4	89.9	8.7
3	Nigericin	1	95.9–106.0	88.3	13.5
		2	89.0–102.5	94.4	10.0
		5	81.2–106.8	97.0	7.9
		10	84.5–104.3	87.5	4.8
4	Salinomycin	2	79.5–105.0	87.6	11.6
		4	79.7–104.7	92.3	11.1
		10	80.1–104.8	85.8	8.3
		20	81.4–101.9	95.5	8.5

TABLE 20.6 The Mean Recoveries in Milk of Six Polyether Antibiotics ($n = 6$)—cont'd

No.	Analyte	Spiked Level (μg/kg)	Recovery Range (%)	Mean Recovery (%)	RSD (%)
5	Narasin	1	86.0–104.0	86.0	6.3
		2	89.1–104.5	91.7	11.2
		5	81.0–102.0	92.4	9.0
		10	80.9–105.6	89.9	8.7
6	Madubamycin ammonium	2	83.5–105.5	88.3	17.2
		4	88.3–106.2	94.4	10.4
		10	83.4–101.4	97.0	7.4
		20	79.9–104.5	87.5	5.5

TABLE 20.A.1 Molecular Formula, Molecular Weight, and CAS No. of Six Polyether Antibiotics

No.	Name	Molecular Formula	Molecular Weight	CAS No.
1	Lasalocid	$C_{34}H_{53}NaO_8$	612.86	25999-20-6
2	Monensin	$C_{36}H_{61}O_{11}Na$	692.96	22373-78-0
3	Nigericin	$C_{40}H_{68}O_{11}Na_5$	748.07	28643-80-3
4	Salinomycin	$C_{42}H_{69}O_{11}Na$	818.02	55721-31-8
5	Narasin	$C_{43}H_{72}O_{11}$	765.15	55134-13-9
6	Madubamycin ammonium	$C_{47}H_{79}O_{17}NH_4$	934.17	84878-61-5

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ANALYTICAL METHODS FOR FOOD SAFETY BY MASS SPECTROMETRY

VOLUME II VETERINARY DRUGS

GUO-FANG PANG
Academician, Chinese Academy of Engineering, Beijing, China
Chairman of the academic committee and a chief scientist of Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing, China

Analytical Methods for Food Safety by Mass Spectrometry, Volume II Veterinary Drugs introduces the methods for the analysis of 20 veterinary drugs residues in animal tissues (as muscle, liver, kidney and fat), diaries (as milk and milk powder), bee products (as honey, royal jelly and its lyophilized powder), aquatic products (as fugu, eel and roasted eel) and urine of animal are selected as main subject of study. The 65 analytical methods for the analysis of multi veterinary drugs residues are described in different chapter by category (sulfonamides, β-adrenergic agonists, aminoglycosides, chloramphenicols, β-lactams, macrolides, nitrofurans, anabolic steroids, non steroidal anabolic steroids, glucocorticoids, fluoroquinolones, tetracyclines, sedatives, pyrazolones, quinoxalines, nitromidazoles, benzimidazoles, levamisole, thiourea pyrimidines and polyethers), and 95% of the methods are LC-MS-MS. Meanwhile the physical and chemical properties, efficacy side effect and maximum allowable residual limit of all the compounds are also provided. This book is a valuable reference for not only university students, but also technical personnel of different specialties engaged with technical study and applications such as food safety, agricultural environment protection and pesticide development and utilization in scientific research units, institutions, and quality inspection organizations.

Key Features

- A method system consisting of 65 national standards were formulated for simultaneous determination of 200 veterinary drug residues using LC-MS-MS.
- Sample pretreatment techniques like SPE, GPC, IEC, IAC, derivatisation, etc. were integrated and optimized.
- Broad applicability: animal tissues, milk powders, fugu and eels, honeys and royal jelly.
- the sensitivity of the method for the target matter is less than 10 μg/kg, with recoveries between 60%-120% and less than 20% RSD accounting for over 80%, has satisfied the limit requirements prescribed by CAC,EU, Japan and America, etc..

About the author

Professor Pang has worked at the forefront of the inspection and quarantine work over the past 30 years. He has devoted himself to the research on the theory and practice of food scientific analysis and conducting the pioneering research work in the field of trace element analytical techniques of pesticide and veterinary drug residues. He has made many achievements in the standardization of analytical techniques and engineering and has focused his study on the high through-put techniques of the trace elements of over 1000 pesticides and veterinary drug residues and established 139 China National Standards and 3 AOAC Official Methods, improving the quality of relative products and giving an impetus to the development of foreign trade.

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