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Pesticide Toxicology

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Pesticide Toxicology

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Chapter 1

Advanced and Recent Approaches for Laboratory Methods of Pesticide Residues and Their Metabolites by Mass Spectrometry Techniques

Patrizia Stefanelli and Danilo Attard Barbini

Abstract

Several sectors like food safety, environmental exposure assessment, and lastly the biomonitoring have focused recently on the analysis of pesticide residues by mass spectrometric techniques. MS detectors such as ion trap, triple quadrupole, and quadrupole-linear ion trap tandem have made surprisingly good progress, largely as a result of the replacement of classical sample preparation by innovative, dynamic and modular dispersive solid-phase extraction (QuEChERS). The last years have seen a growing interest for QuEChERS approach, since fully miniaturizing sample preparation is technically possible, relatively cheap, and potentially of major benefit in terms of time of analysis. This approach is hugely versatile to provide good efficiency and robustness for target list of hundreds of compounds in several matrices. Moreover, introducing high-resolving power and mass accuracy has resolved complex analytical problems involved in untargeted and unknown pesticide analysis.

The MS ionization efficiency of analytes is usually influenced by matrix components. Changing MS conditions, optimizing chromatographic separations, and improving cleanups are basic strategies to minimize up to suppress the matrix effects. Several detection methods and compensation strategies of matrix effects are available, offering simple tools to mitigate the negative effects.

Key words Pesticides, Gas and liquid chromatography, Mass spectrometry, Target analysis, Multi-analyte methods, Dispersive solid-phase extraction, Matrix effect

1 Introduction

Pesticides are biological active compounds used intentionally to protect crops from fungal diseases, insects, and competitive plants and to guarantee the quantity and quality of consumer goods [1]. These substances are primarily passed on to humans when the pesticide residues enter the food chain [2, 3]. Therefore, European and international authorities ensure that appropriate monitoring programs are in place in order to minimize the exposure based on the dietary intake [4–7].

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Under authorized conditions of human exposure to pesticide residues in food, finite doses are encountered. In fact, to protect the consumers as well to promote international trading, the European Union and the Codex Alimentarius Commission have established threshold values of pesticide residues in foodstuffs and feed, expressed in mg/kg (MRL—maximum residue limit) [8, 9].

Usually, the classical evaluation of the human exposure to pesticides was focused to the monitoring of foods and environmental matrices. Recently a new interest in pesticide sector has been noted in the use of biomonitoring data, integrating all sources and routes of exposure [10–12].

These data are complementary and integral to the food monitoring data; in fact, the food control to pesticide residues was still considered an essential key of the human chemical risk assessment plan [13, 14].

In recent years, main international bodies and agencies (CEN, FDA, EPA, pesticide EURLs) have released analytical procedures intended to be used for food, environmental, and biological controls [15–18].

Numerous methods have developed recently to increase the residue number rate for each target analysis at once and reduce significantly the time of analysis. In addition, the extremely wide list of pesticides, including metabolites and transformation products, demands sensitive, selective, and precise methods.

Current trend on pesticide residues is to develop multi-analyte methods and to prefer those methods of analysis, which are uniformly applicable to more categories of products than to individual products. Recently, the European Standard based on QuEChERS (acronym for Quick, Easy, Cheap, Effective, Rugged, and Safe) procedure has responded to this need with the further advantage of being able to analyze simultaneously a target list of hundreds of pesticide residues in all vegetable commodities. The acronym explains all the advantages of this procedure, which allows preparing several samples in short time and extracting a large number of different compounds with good efficiency and robustness [19, 20]. This advanced approach uses dispersive-phase sorbents as cleanup after a buffered extraction/partition water–acetonitrile.

The innovative and versatile analytics platform, with a few minor adjustments, is also suitable for different matrices such as soils and biological fluids [21–23].

In exceptional circumstances, the application of this very popular fast and simple multi-residue method produced negative results for single pesticides with remarkable polarity and ionic structure. Strictly, in these cases, it is still necessary to use single residue methods for the analysis of one or more pesticides from similar chemical families [24, 25].

Techniques and models from mass spectrometry form the mainstay of pesticide residue determination in many areas of both

research and official controls, in combination with gas (GC) and liquid (LC) chromatography. Moreover, the low levels of detection require MS instruments that provide high sensitivity and high selectivity as well as fragmentation for a confident confirmation. In the last years, tandem mass spectrometry with triple quadrupole (QQQ) ion trap (IT) and quadrupole-linear ion trap tandem (Q-Trap) analyzers is becoming more and more popular in the target confirmation and quantification analysis of pesticide residues in food and environmental matrices. LC-MS/MS detection technique prevails in the analysis of the new active substances due to the marketing authorizations of highly degradable and polar pesticides [26]. However, organochlorines, pyrethroids, and high fat content commodities still need GC-MS/MS as tool for the analysis [27]. Several authors pinpointed electron impact (EI) for gas chromatography mass spectrometry and electrospray (ESI) for liquid chromatography mass spectrometry as the ionization techniques most popular in pesticide residue analysis and in routine by now [28, 29].

Advances in the official confirmation controls provide the HRMS such as Orbitrap and TOF-MS instrumentations. Currently, these systems have already been widespread in the screening of samples, untargeted analysis of metabolites [30, 31], unknown analysis of adjuvants or additives present in commercial formulations, and recently for the identification of potential emerging chemical contaminants of the food chains [32, 33].

In Europe, to support the quality of data obtained during enforcement actions as well assessments of consumer exposure to pesticides or research tasks, a detailed technical document lays down the rules concerning the quality control and validation procedures exclusive for official pesticide residue analysis, setting out the European Commission's harmonized vision of the validity of analytical results [34]. In addition, the US legislation has established standards covering different areas of quality control data (chemical methods, mass spectrometry identification, validation procedures), nonexclusive to pesticide residues [35].

The European document is a landmark for pesticide residue laboratories operating in conformity with ISO/IEC 17025. Moreover, the guidance focuses the identification and quantification on the mass spectrometry, identifying it as one of the primary determination tools since specific detectors combined with different polarity columns do not provide unambiguous determination.

Another primary strand is the topic of the matrix effects, occurring in both GC and LC mass spectrometry methods. It was noted that in GC the matrix effects have been prevalent in injector and column systems whereas in LC occur during the electrospray ionization, especially with complex matrices. Several detection methods and reduction/compensation strategies of matrix effects are available, offering simple tools to mitigate the negative effects [36–38].

This chapter describes practical protocols and topical strategies for the determination of pesticide residues in routine analysis based on mass spectrometry, discussing several aspects and its potentiality in the pesticide residue analysis.

2 Materials

Reference materials were used with a 95.0% certified purity. Stock solutions of standard were prepared at 1 mg/mL in acetonitrile or toluene, stored in darkness at −20 °C, the working solution was further diluted to 10 μg/mL in acetonitrile, and stored at −20 °C. Sodium chloride was purified from interfering substances by 3 h heating at 600 °C in a muffle. The following buffers have been used: sodium citrate dibasic sesquihydrate (>99%), sodium citrate tribasic dehydrate; magnesium sulfate anhydrous (>97%). QuEChERS extract tube Dispersive SPE, Method EN (15 mL, 150 mg primary secondary amine sorbent—PSA, 900 mg magnesium sulfate) was employed. Centrifuge tube (50 mL) made of polypropylene with screw caps was used. Formic acid, acetonitrile, and water were used pure for analysis.

A vortex mixer to mix the samples before the extraction, and a centrifuge after the extraction together with a mechanical shaker was used to shake the samples.

A Ultra High Performance Liquid Chromatography (UHPLC), equipped with quadrupole-linear ion trap tandem (Q-Trap), was used for. The liquid chromatography was equipped with an autosampler. A valve of injection with a loop of 20 μL was used. The injection volume was 5 μL. The detection was performed by mass spectrometry equipped with electrospray ionization interface (ESI positive and negative mode). Typical source parameters were as follows: ion spray voltage 4500 V; temperature 550 °C; ion source gases at 55 and 60 psi, the collision gas medium. Data processing was performed using professional software.

A gas chromatograph equipped with a triple quad detector was employed. The gas chromatograph was equipped with an autosampler, and a PTV injector was used. Sample injection was carried out using the PTV solvent vent mode.

The mass spectrometer was used in electron ionization (EI) operating in multiple reaction monitoring (MRM) acquisition mode. The transfer line temperature was kept at 280 °C. Data processing was performed using professional software.

A portion of 10 g of homogenized foodstuff was weighed into a 50 mL polypropylene centrifuge tube. In case of cereal samples, a portion of 5 g of milled grains was weighed into a 50 mL polypropylene centrifuge tube, and volumes of 10 mL of cold water are added to the homogeneous sample before the extraction procedure.

The homogenized sample was extracted with 10 mL of acetonitrile after vortex for 1 min. In case of the multi-residue method EN 15662: 2018, a mixture of 4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 1 g of tri-sodium citrate dihydrate, and 0.5 g of disodium citrate sesquihydrate were added to the sample into a centrifuge tube. Commercially available salt mixtures of identical composition may be used. The tube was capped well and shaken vigorously by vortex for 1 min. Then, the homogenate was centrifuged at >3000 rpm for 5 min. An aliquot of 6 mL of the acetonitrile phase was transferred into a centrifuge tube with 150 mg PSA and 900 mg of anhydrous magnesium sulfate, and in case of cereals and cereal products, the recommended cleanup module uses more C_{18} reversed-phase sorbent. The tube was closed and shaken vigorously for 2 min, and after was centrifuged (e.g., for 5 min at >3000 rpm). A 5 mL aliquot of cleanup extract was isolated and slightly acidified by adding 50 μL of a 5% formic acid solution in acetonitrile. This extract was measured by LC–MS/MS and GC–MS/MS.

In case of the QuEChERS-based method for the analysis of acidic flonicamid metabolites, TFNA and TFNG in vegetable products were used. Acidification is achieved by using acetonitrile (10 mL) containing 1% formic acid, shaking 20 min using a mechanical shaker. No buffering salts were used but only a mixture of 4 g $MgSO_4$ and 1 g NaCl. The final extract was directly measured by LC–MS/MS. The transitions were: 230.2/203.1 *m/z* and 230.2/148.1 *m/z* for flonicamid, 192.2/98.1 *m/z* and 192.2/148.1 *m/z* for TFNA (IUPAC name: 4-(trifluoromethyl) pyridine-3-carboxylic acid), and 248.2/148.0 *m/z* and 249.2/203.1 *m/z* for TFNG (IUPAC name: 2-({hydroxy [4-(trifluoromethyl)-pyridin-3-yl] methylidene} amino) acetic acid).

The QuPPe-plant origin method was used to determine chlormequat residue in vegetable products. Acidification is achieved by using methanol (10 mL) containing 1% formic acid, adding isotopically labeled internal standard (ILIS) chlormequat D_4, and shaking manually. The final extract after centrifugation was directly measured by LC–MS/MS. In case of liquid samples (e.g., wine), *dilute and shoot* methods were applied.

The methods have been accredited according to the ISO/IEC 17025 standard. Internal matrix calibration, using a single representative matrix or a mixture, was used to quantify the compounds. The employed instruments were calibrated and their performance checked, the personnel were well-trained, and all data were obtained and properly documented following written standard operating procedures (SOPs). Recovery control charts were used as monitoring tools for IQC, collecting total pesticide residue data at different concentration levels. When available, analysis of certified reference materials (CRMs) was selected as preferable option to provide evidence of method performance. Usually, as an alternative, in-house quality control samples were employed.

Moreover, the laboratory participates regularly in proficiency test schemes.

3 Methods

3.1 Methods of Sample Preparation for Multi and Single Residue Analysis

The QuEChERS method is a modular procedure, which is suitable for the analysis of compounds with a very wide polarity range for a wide group of matrices. The extraction was focused to pesticide multi-residue analysis in food matrices. The first publication was in 2003, and after several preparation protocols were based on QuEChERS approach in certain instances with limited modifications. The acronym explains exactly all the advantages, which allows preparing many samples in short time and extracting a large number of different compounds with good efficiencies.

The importance of this procedure in the field of pesticides is evident from the fact that in 2009 the European Committee for Standardization has issued it as a standard, hence an official method, and updated in 2018.

The homogeneous samples are extracted with the help of acetonitrile by a partition water–solvent. To help the dehydration of organic phase, a mixture of anhydrous $MgSO_4$ and NaCl is originally used. Samples with low water content require the addition of water before the initial extraction with different combination of sample amount/water volume/commodity as summarized in Table 1.

In the original version of QuEChERS, the pH control was not performed, thus the scope was limited to pesticide classes with no intrinsic acidity/basicity. Later, a specific citrate buffer was introduced in the procedure to maintain pH range ongoing around the value of 5, for pesticides particularly susceptible at pH values that might encounter serious degradation at both high and low pH values.

Moreover, in individual cases, quantitative recoveries could be achieved using an acidic version of the QuEChERS method by using acetonitrile containing 1% formic acid. A typical example is flonicamid and its metabolites TFNA and TFNG in food commodities. Figure 1 shows its application in different matrices (beans, spinach, and cabbage), in term of single recoveries of all components included in the MRL definition (so-called residue definition: *sum of flonicamid*, *TFNA*, *and TFNG expressed as flonicamid*). No buffering salts are used. This pH value allows for achieving satisfactory recoveries for metabolites of flonicamid. The parent compounds showed good recovery results by either the normal or the acidified QuEChERS procedure; whereas using the normal procedure (EN-15662), the recoveries of TFNA and TFNG were less than 70% [39].

In the QuEChERS method, a revolutionary cleanup step replaced the traditional SPE cartridge approach; the PSA primary

Table 1
Typical water addition to matrix in the QuEChERS method (UNI EN 15662: 2018)

Commodity grouping	Typical water content	Matrix amount	Water addition	Example
Vegetables with high water content	>80%	10 g	–	Apple, lettuce, ...
Vegetables with intermediate water content	40–80%	10 g	2.5–4.5 mL	Banana, potato, ...
Vegetables with low water content	15–40%	Homogenize 500 g vegetable with 850 mL water and analyze 13.5 g aliquots (equivalent to 5 g of vegetable)		Dried fruits and similar commodities
Vegetables with very low water content	<15%	2–5 g	10 mL	Cereals, coffee, honey, lentils, tobacco, tea, ...

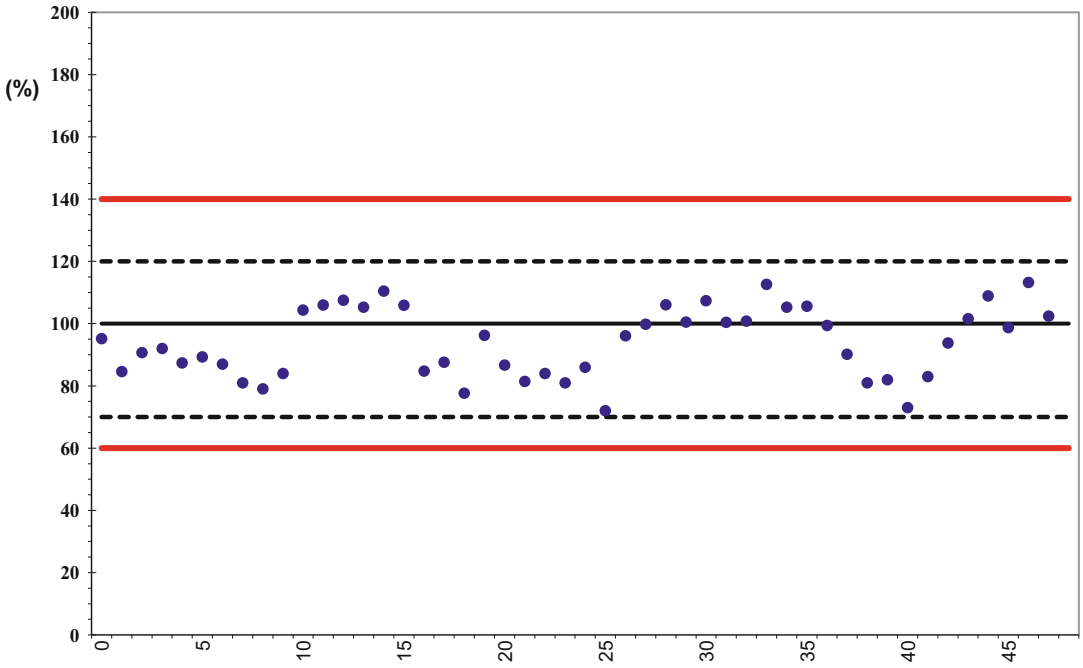


Fig. 1 Control chart of single recoveries for *flonicamid* and its *TFNA* and *TFNG* metabolites. Red lines: action limits—dashed lines: warming limits

secondary amine sorbent was added as dispersive phase with a minimum amount of anhydrous $MgSO_4$ to the acetonitrile extract. This cleanup module is used for the determination of neutral and alkaline pesticides in all commodities with high water content. It is also used for citrus fruits extracts (Table 2 reports the commodity

Table 2
Vegetable and fruits, cereals commodity groups, and representative commodities. (Annex A. SANTE/12684/2019)

Commodity groups	Typical commodity categories within the group	Typical representative commodities within the category
High water content		
	Pome fruits	Apples, pears
	Stone fruits	Apricots, cherries, peaches
	Other fruits	Bananas
	Alliums	Onions, leeks
	Fruiting vegetables/ cucurbits	Tomatoes, peppers, cucumbers, melons
	Brassica vegetables	Cauliflowers, brussels-sprouts, cabbages, broccoli
	Leafy vegetables and fresh herbs	Lettuce, spinach, basil
	Stem and stalk vegetables	Celery, asparagus
	Fresh legume vegetables	Fresh peas with pods, peas, mange tout, broad beans, runner beans, French beans
	Fresh fungi	Champignons, chanterelles
	Root and tuber vegetables	Sugar beet, carrots, potatoes, sweet potatoes
High acid content and high water content		
	Citrus fruits	Lemons, mandarins, tangerines, oranges
	Small fruits and berries	Strawberries, blueberries, raspberries, black currants, red currants, white currants, grapes
High sugar and low water content		
	Honey, dried fruit	Honey, raisins, dried apricots, dried plums, fruit jams
High oil content and very low water content		
	Tree nuts	Walnuts, hazelnuts, chestnuts
	Oil seeds	Oilseed rape, sunflower, cotton-seed, soybeans, peanuts, sesame, etc.
	Pastes of tree nuts and oil seeds	Peanut butter, tahina, hazelnut paste
High oil content and intermediate water content		
	Oily fruits and products	Olives, avocados, and pastes thereof

(continued)

Table 2
(continued)

Commodity groups	Typical commodity categories within the group	Typical representative commodities within the category
High starch and/or protein content and low water and fat content		
	Dry legume vegetables/pulses	Field beans, dried broad beans, dried haricot beans (yellow, white/navy, brown, speckled), lentils
	Cereal grain and products thereof	Wheat, rye, barley and oat grains; maize, rice, whole-meal bread, white bread, crackers, breakfast cereals, pasta, flour
"Difficult or unique commodities"		Hops cocoa beans and products thereof, coffee, tea spices

group and representative commodities for fruit and vegetables as defined in the Annex A of the document SANTE/12682/2019).

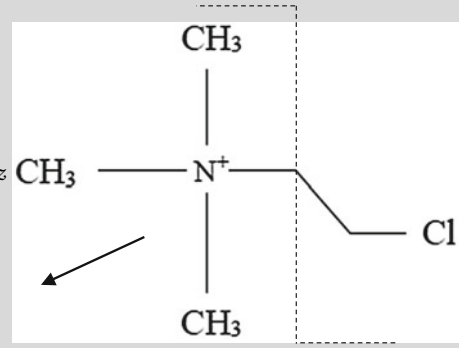
The addition of silica-based reverse-phase sorbent (C_{18}) to PSA sorbent is used to reduce fat and cleanup in one step for dried fruits, cereals, and processed products thereof, avocado and olives. When the extracts of samples have incredibly high contents of carotenoids and/or chlorophyll (such as spinach or strawberries), the recommended cleanup module includes the use of graphitized carbon black (GCB). Inacceptable losses of planar compounds occurred during cleanup with GCB, so it is not appropriate to use PCB 18, PCB 28, and PCB 52 as procedural internal standards.

Some complex matrices (e.g., fatty food matrices, spices, essential oils, soils, and sediments) require removing a higher amount of matrix components; consequently, the traditional SPE approach by itself [40] or in addition to dispersive PSA could make more efficient the cleanup.

One or more polar pesticides from similar chemical families (e.g., phenoxy acid herbicides) are strongly caught by PSA, and these specific cases need to be analyzed by single residue methods.

In the last years, a quick polar pesticide method (QuPPE) was published by the EURL–SRM [41] to analyze highly polar pesticides in food of animal and plant origin including honey. This dynamic approach involves a simple extraction with acidified methanol and LC–MS/MS measurement. In the last version of QuPPE method, an update for cereal matrices involved the addition of EDTA during the extraction step for complexation of metal ions such as calcium and magnesium that may interfere with analysis of difficult compounds such as glyphosate and AMPA metabolite. Table 3 reports information of the QuPPE application to

Table 3
Information of the QuPPE application to Chlormequat in vegetable products and wine

product ion = 58.2 m/z 		
Exact mass	122.1 *m/z*	
Molecular formula	$C_5H_{13}ClN$	
Transitions	*Quantifier*	*Qualifier*
Chlormequat	122.1 > 58.2	124.1 > 58.2
Chlormequat D4 (ILIS)	126.3 > 58.2	128.3 > 58.2
Validation/verification data	*Vegetable products*	*Wine*
Mean recovery % (n = 6)		
LOQ 0.01 mg/kg	101	104
High level 0.1 mg/kg	94	100
RSD_r % (n = 6)		
LOQ 0.01 mg/kg	5.6	3.6
High level 0.1 mg/kg	3.5	2.2
Linearity		
Residual max %	14	9
Statistical verification precision data		
S pool	4.64	F_{calc} *1.27* ($p = 0.05$) < F_{tab} *Pass*
S method (RSD% 4%; Rec mean 103%, $n = 6$)	4.12	F_{tab} 4.74

chlormequat in vegetable products and wine. Figure 2 shows the UHPLC–MS/MS (Q–Trap) chromatogram for chlormequat and its own isotope D4.

Isotope-labeled analogues of compounds are used as internal standard (ILIS) and added directly to the test portion at the beginning of the procedure to compensate for any factors having an influence on the recovery rates such as volume deviations, analyte losses during sample preparation as well as matrix effects during measurement. Where isotopically labeled standards are not available or are too costly, it is possible to use the procedural internal

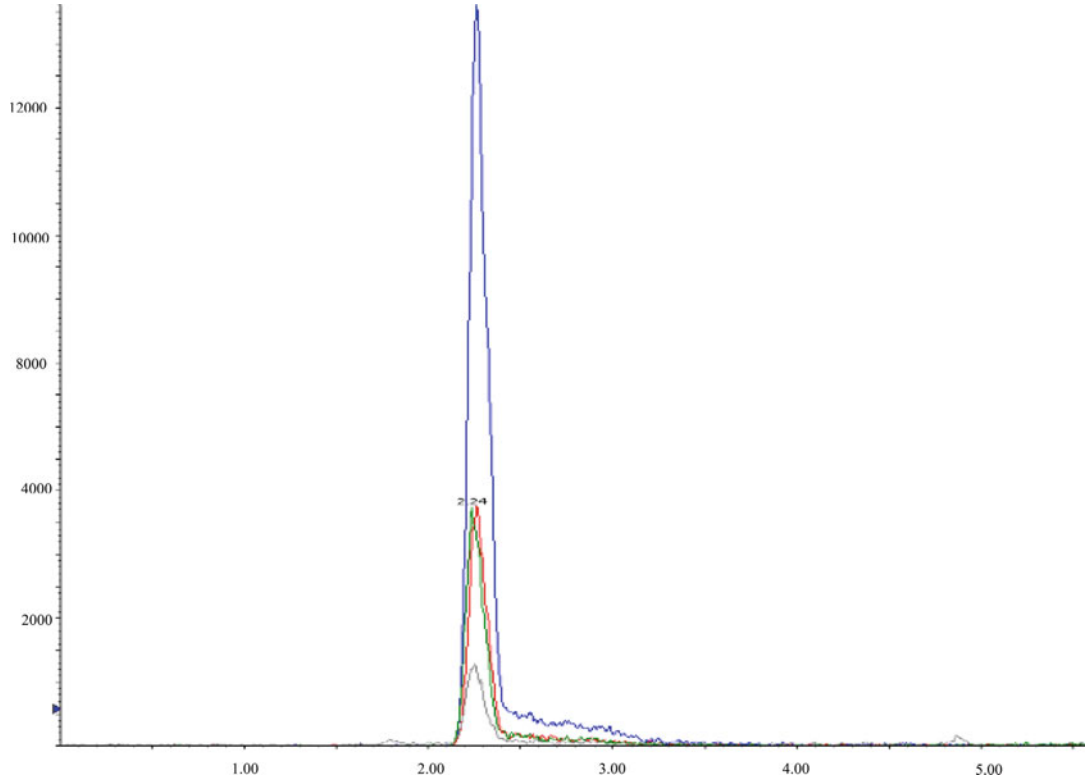


Fig. 2 Overlay of UHPLC-Q trap-MS/MS signals obtained for *chlormequat* and isotope-labeled standards *chlormequat D4* in vegetable products

standard (P-IS); in Table 4 are summarized potential internal standards.

Often the high lipid fat, wax, and sugar content commodity requires an extra cleanup step by freezing out overnight and consequently to be centrifuged frozen, but making sure to filter the supernatant quickly to avoid redissolution of certain matrix components (e.g., fat). In this case, the laboratory should use a refrigerated high-speed centrifugation.

Finally, mini QuEChERS methods were also optimized by using different extraction solvents (acetonitrile or ethyl acetate) in combinations of salts for the analysis of a wide range of pesticides in a small volume of clinical samples (blood or urine) to support biomonitoring [42].

Figure 3 shows the LC–MS/MS linearity in urine matrix for AMPA–fluazinam and DAPA–fluazinam, human metabolites of Fluazinam pesticide applied intensively in viticulture.

Calibration curves for the metabolites were obtained by plotting the analyte concentration against the peak area. Linear relationship gets in electrospray ionization (ESI, positive mode)

Table 4
Potential procedural internal standards (P-ISs)

Compound	Log P	Chlorine atoms	GC	LC	
			MS/MS or MSD EI	MS/MS ESI (+)	MS/MS ESI (−)
PCB 18	5.55	3	+ +	−	−
PCB28	5.62	3	+ +	−	−
PCB 52	6.09	4	+ +	−	−
Triphenyl phosphate (TPP)	4.59	−	+ + +	+ + +	−
Tris-(1,3-dichlorisopropyl)-phosphate	3.65	6	+ + +	+ + +	+
Triphenyl methane	5.37	−	+ + +	−	−
2,4-D ^{13}C6 (ring)	Depends on pH	2	−		
Chlorpyrifos D10	4.7	3	+ + +	−	+ + +
Diuron D6	2.9	2	−	+ + +	−
Diazinon D10	3.8	−	+ + +	+ + +	−
Metalaxyl D6	1.65	−	+ + +	+ + +	−
N,N′-Bis-4-nitrphenyl urea	3.76	−	−	−	+ + +

+ + + very good detectable
+ + good detectable
+ poor detectable
− not applicable

between the analyte concentration and the detector response in urine matrix, extracted by a modified QuEChERS strategy [43].

Viscous and liquid matrices such as milk, fruit juices, and vegetable oils show an important emulsion formation during the application of QuEChERS dispersive purification/extraction. This effect could be substantially reduced with the application of MSPD (matrix solid-phase dispersion) technique [44].

In MSPD, almost 0.5–1 g of sample is mixed with a similar amount of adsorbent solid phase (e.g., C_{18}, florisil, silica) until a homogeneous mixture is obtained. Then, an extraction tube was filled with the mixture and eluted with suitable organic solvent. The combination of solid/liquid solvent phase is a compromise between the polarity of analytes and the type of matrix samples. This approach has proven difficult to automate; therefore, the analysis times are considerably longer than QuEChERS.

Also, the *dilute and shoot* approach is often applied for aqueous liquid samples (e.g., wine) by a tenfold dilution with appropriate

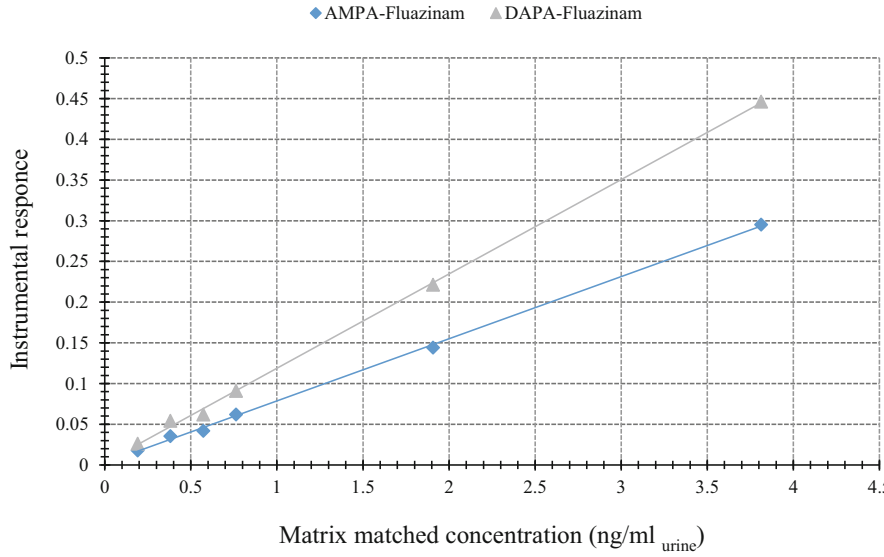


Fig. 3 Linear regression of the matrix-matched calibration for human metabolites of *Fluazinam* pesticide (*AMPA* and *DAPA*)

solvent, usually the same composition of mobile phase. This technique offers a valid and simple alternative to an intensive sample cleanup also in terms of shortening of analysis times. The only requirement is an LC–MS/MS instrument able to achieve sufficiently low limits of quantification.

3.2 Mass Spectrometry Methods for Identifying and Quantifying Pesticide Residues

Multi-residue methods determine a large number of pesticides in a single run fulfilling specific criteria as compromise on different physicochemical properties of the target compounds compared to common analyzers in the case of mass spectrometry (triple quadrupole (QQQ), ion trap (IT), and quadrupole-linear ion trap tandem (Q-Trap)). In the last years, the pesticide residue analysis has increased exponentially the use of LC–MS/MS with excellent results in several matrices (soil, food, human fluids, or tissues) for almost 500 pesticides. Nevertheless, gas chromatography–mass spectrometry (GC–MS) methods continue to play a key role for the analysis of pesticides that are not sensitive to ionization mode or for certain complex matrices such as high fat content commodities incurred lipophilic organochlorines and pyrethrins particularly suitable to its application [45].

The triple quadrupole methods in multi-reaction monitoring mode (MRM) are the most widely employed for the target analysis of pesticide residues in food and environmental matrices. Their use ensures good selectivity, linear dynamic range, and sensitivity decreasing the possibility of spectral interferences. Usually, a minimum of two product ions are required for identification according to criteria reported in guidance document SANTE/12682/2019.

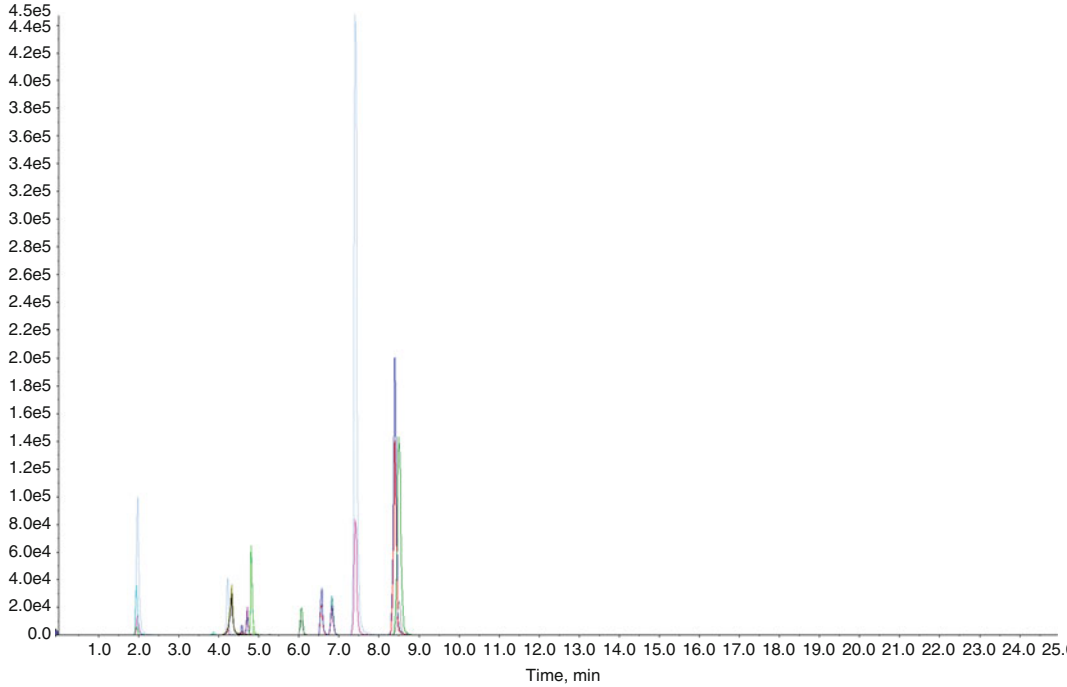


Fig. 4 LC–MS/MS multi residue chromatogram where compounds are identified at specific transitions in the EUPT-FV20 Green Beans with Pods homogenate sample

The most abundant transitions are selected with molecular ions as precursor ions; in LC–MS/MS, the adduct ions are even selected as precursors (e.g., in ESI positive mode, $[M + H^+]$ and $[M + NH4^+]$) [46]. Figures 4 and 5 show the multi-residue chromatogram where compounds are identified at specific transitions by UHPLC–MS/MS (Q–Trap) and EI GC-MS/MS.

The number of time segments per chromatographic run is normally 20, each with almost 50–100 transitions; but these agreements are also conditional on the instrumental models, the acquisition speeds, and the dwell times allowing fast data acquisition. Modern triple quadrupoles offer quick dwell times particularly suitable for interfacing with the new ultra-performance liquid chromatographic (UPLC and UHPLC) instruments and enable to switch between positive and negative ionization in the same run in support to the multi-residue strategies.

In advanced models, a Q–Trap system can be operated as a conventional triple quadrupole instrument with all the scan functions such as MRM, and it provides the same sensitivity as its equivalent QQQ model. Nevertheless, in a Q–Trap device, the third quadrupole can be configured as a linear ion trap (IT) to provide additional powerful qualitative scan functions to enhance the performance and flexibility. These hybrid instruments improve

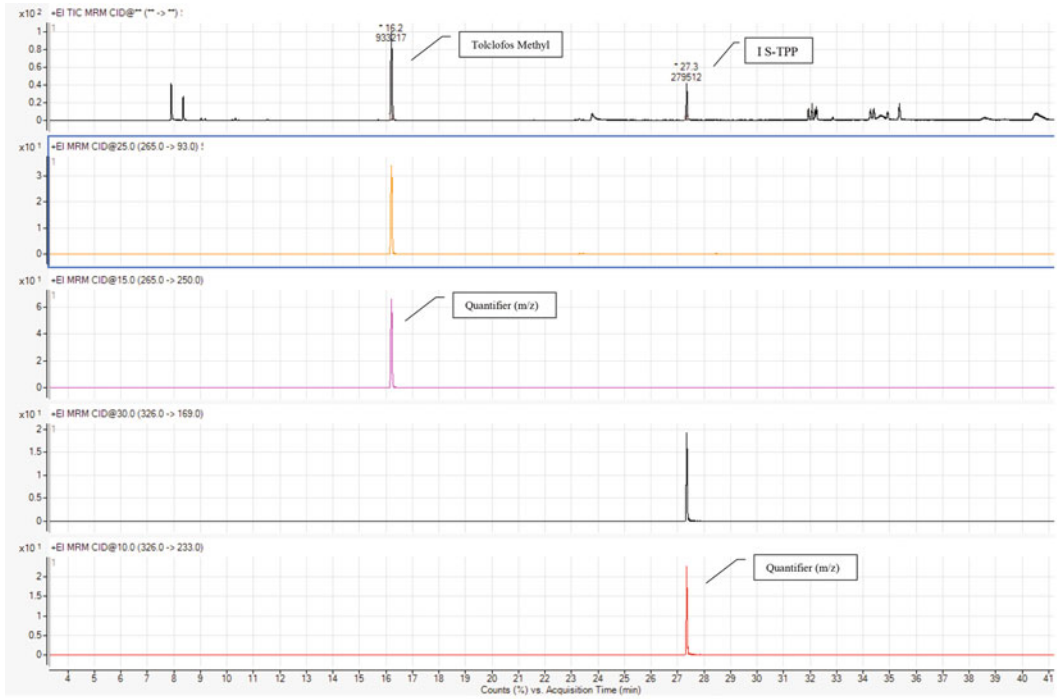


Fig. 5 Example extracted ion chromatograms (EI GC–MS/MS) diagnostic ions of selected *Tolclofos Methyl* spiked to cabbage at 0.01 mg/kg

considerably the IT technology typified by limited linear dynamic range and lower sensitivity than QQQ systems [47].

Target methods require additional time to set up because a preventive list of all MRM transitions (precursor/product ions of target compounds) with possible false negatives if not all transitions are followed and false positives due to the presence of other compounds (interferences) with the same transitions is needed. Moreover, the time segmentation involves the risk of losing analytes that elute near or between time segments. In support of analyst, pesticide platforms/databases are available, reporting typical precursor/product ions and maximal sensitivity with either GC or LC [48]. In addition to the targeted quantification, software and spectra database allows for untargeted analysis of samples that were acquired.

The NIST library is particularly widespread in GC-MS with the use of EI (Electron Impact) at high-energy electrons (70 eV). This technique generates reproducible fragmentation types so that they can be compared to the spectra collected in dedicated databases. These tools such as NIST library enable us to confirm the identity of the target compounds and to identify the unknowns.

In addition, unambiguous determination of the target pesticides could be performed by the application of full survey scan MS, starting mass at 50 m/z and ending mass at 500 m/z [49].

A very recent option for full-scan screening is the application of the Orbitrap technology, which combines mass accuracy capability and high resolution (up to 100,000 FWHM at m/z 200). The low mass errors observed with the Orbitrap system in both techniques (GC and LC) are enabled through the high-mass resolving power that is able to discriminate between matrix interferences and target analyte ions. This technology offers numerous workflows that can be used for pesticide residue analysis, but in the same time, the selection of the most appropriate workflows requires well-trained and experienced personnel. When the resolution is insufficient, the mass profile of two ions overlap, which results in the incorrect assignment of the mass of the target compound. In fact, the main limitations of QQQ techniques are the lack of sufficient mass accuracy which prevents the determination of compounds at very low concentration levels and also the slow scan speeds reducing the scans that could be simultaneously acquired.

After the implementation of QuEChERS strategies in the analysis of pesticide residues, recent advances have appeared in mass spectrometry coupled to liquid/gas chromatography.

The QuEChERS extracts in acetonitrile are not compatible with relatively nonpolar GC stationary phases, and the type of solvent could bring to a large expansion volume during the GC injection, and consequently some technical optimizations are introduced in injection and column systems.

Ideally, the use of PTV (programmable temperature vaporizing inlet) is implemented in solvent vent mode to eliminate acetonitrile. PTV inlets combine the benefits of split, splitless, and on column inlets. The sample extract is usually injected into a cool liner, so syringe needle discrimination does not occur. Then, the inlet temperature is increased to vaporize the sample. If vent times and temperature are scheduled, the transfer of sample to the column will achieve the equivalent of split or splitless. Then, the implementation of analyte protectants helps to deactivate GC system in every injection. Finally, the new application of column backflushing improves the mitigation of less volatile matrix components from instrumental system, improving ruggedness of the technique, increasing column lifetime, and preventing the MS source contamination. The backflushing can eliminate less volatile matrix components from the GC column by reversing the column flow at a pressure junction point.

The commonest organic solvents in LC–MS pesticide residue applications are acetonitrile and methanol in accordance with the extracts of QuEChERS and QuPPE procedures, respectively, with the addition of polar additives such as formic acid, acetic acid, ammonium formate, and ammonium acetate. The adjustment of the organic phase polarity, where elutes the majority of compounds, increases the ionization efficiency and consequently the sensitivity.

The molecules basically ionize in methanol much better than in acetonitrile but with poor peak shape compared to acetonitrile.

Another significant stepping in the development of LC–MS techniques was the introduction of hydrophilic interaction liquid chromatography (HILIC) stationary phases, which contain aminopropyl (NH_2) or cyanopropyl (CN) groups particularly suitable for polar pesticides.

HILIC increases the sensitivity in MS detection as compared to conventional reverse-phase (RP) LC stationary phase [50]. An important hint is to check the pH and ionic strength of the mobile phase in order to avoid unexpected shifts in retention times. One more hint is to condition carefully the columns before the use.

The RP–LC (C_{18} or C_8 bonded materials on silica stationary phase) is the most common type of separation mode in multi-residue methods. Meanwhile, the use in routine of HILIC columns in mass spectrometry triple quadrupole analyzer has accelerated the analysis of polar compounds by single residue methods; otherwise, the laboratory should have included the ion-pairing chromatography, allowing longer production times. For this point of view, the introduction of ultra-performance liquid chromatography (UPLC) has greatly accelerated response times (more than 50%), in particular for multi-residue analysis.

The UPLC uses innovative stationary column phases in term of particle size (up to 2 μm diameter), greater resolution, and sensitivity of the analytical response.

The smaller particles require a higher pressure to work with, so UPLC systems need to be capable of operating up to 6000–15,000 psi pressure, which is typically the upper limit of conventional HPLC. Then, the only drawback is to have special HPLC pumps able to withstand this very high pressure.

3.3 Method Validation and Quality Control Procedures for Pesticide Residues Analysis

Method validation is the process of demonstrating or confirming that a method is suitable for its intended purposes. The purpose of these methods may include but is not limited to qualitative and quantitative analysis, screening/confirmatory analysis, matrix/platform extensions, and alert and no programmable operations.

Usually, the method validation is required for submission of a new and original method, the expansion of the scope of an existing method to include additional analytes and matrices; the modification to a method that may alter its performance specification, for example, affects significantly the precision and trueness.

Performance characteristics that should be evaluated in order to validate a method depend on the intended use of method, the type of method, and the degree to which it has been previously validated. Usually, a quantitative method validation should include at a minimum the following parameters: trueness (recovery), precision, limit of quantification, linearity, selectivity, robustness, and uncertainty measurement [51].

A method that has been previously validated elsewhere such as a standard method, before its application, should be verified that the laboratory could properly perform it, in particular in term of precision. If the standard method procedure reports the precision data (including the number of the replicates), the laboratory should demonstrate no significant differences occurred between the own precision data and the published data by comparing the standard deviations (σ). The statistical F-test is performed at 95% confidence level ($F_{calc} = \sigma^2/\sigma^2_{std\ mtd} > 1$; $F_{calc} < F_{tab}$). The F_{tab} can be found in statistical tables or by using the FINV function in Excel (FINV (0.05; ν) with ν degrees of freedom). In the official controls of pesticide residues in food and feed also, the EURL methods are considered official, if reporting complete validation data.

A single matrix can be selected even if the original method is applicable to multiple matrices. The selected spiking concentration should ensure that the method meets the requirements, for example, in enforcement action relevant regulatory limits. Figures 6 and 7 report the explicative workflow of the validation/verification procedure (e.g., internal method or standard) in according to the requirements of ISO/IEC 17025: 2018. In general, the technical supervisor in collaboration with the quality manager should approve method performance results.

The method validation and quality control procedures for pesticide residues in food and feed are laid down in guidance document published by the European Commission. This document is

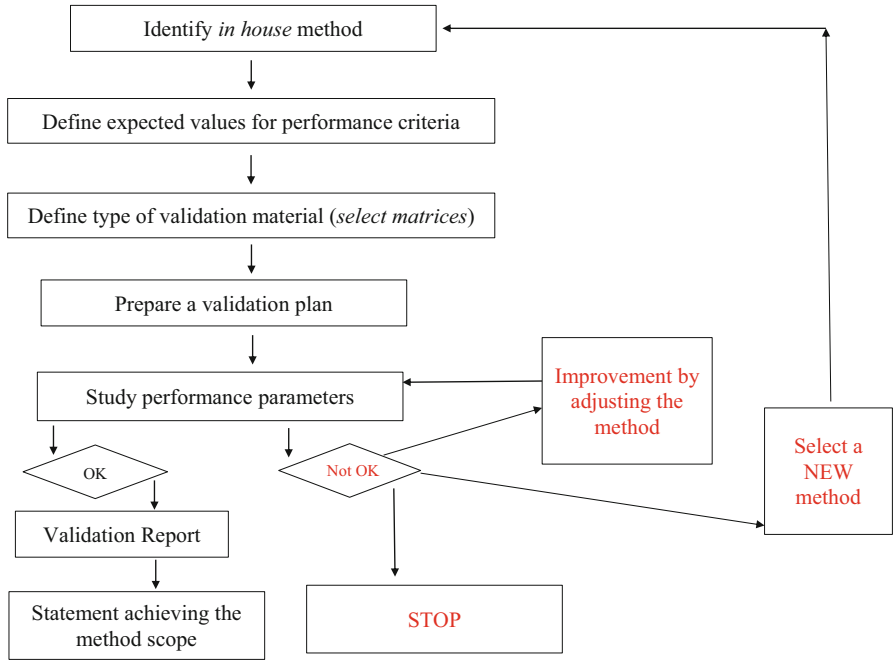


Fig. 6 Optimization strategy in the validation procedure of the *internal* method

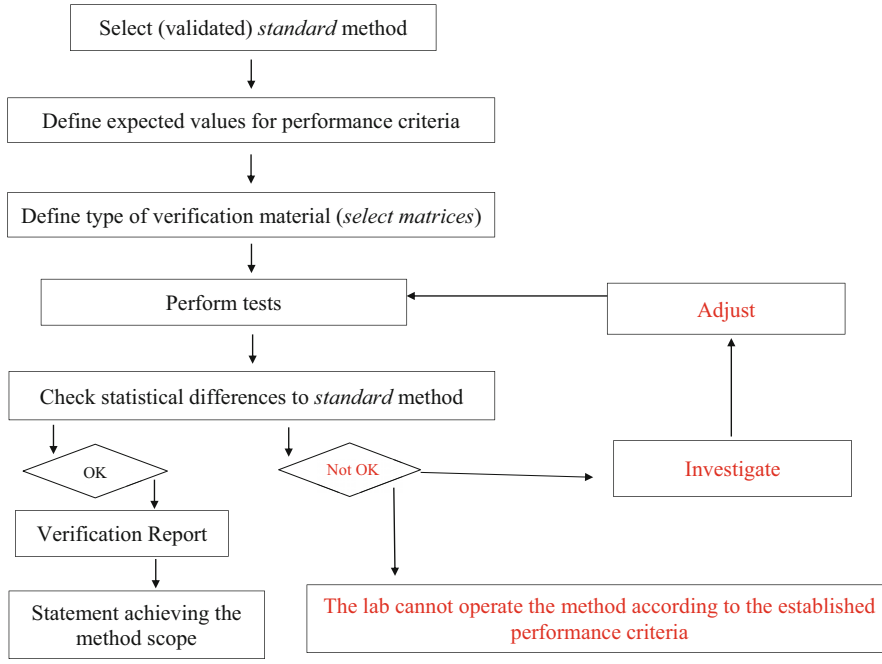


Fig. 7 Optimization strategy in the verification procedure of the *standard* method

complementary and an integral part of the requirements of the standard ISO/IEC 17025: 2018.

This guide is very popular and chosen as reference in sectors where a specific and detailed document for pesticide has not been available yet. Table 5 indicates the recommended validation parameters and acceptance criteria in the current SANTE document.

A quantitative analytical method should be demonstrated at both initial and extended validation stages, as being capable of providing acceptable mean recovery values at each spiking level investigated and for at least one representative commodity from each of the relevant commodity groups. Mean recoveries from initial validation should be within the range 70–120% with an associated repeatability RSD $\leq$20%. It is recognized that for some situations such as with difficult matrices, extremely low analyte concentrations, multi-residue methods and with emergencies such as rapid alerts, these general acceptability range may not be achievable or required but the mean recovery should not be above 140%.

Also, the ion ratio is listed between the validation parameters in order to check the ability of the method to identify the analyte with the mass spectrometry technique. The relative intensities or ratios of selective ions expressed as a ratio relative to the most intensive product ion should correspond to those calibration standards. The matrix-matched calibration may need to be used. In Europe, for pesticide residues, the fixed generic criteria given for ion ratio is equal or less than 30% (*see* Table 6).

Table 5
Validation requirements and criteria (SANTE/12684/2019) for vegetable and fruits, cereals, and food of animal origin quantitative methods

Parameter	What/how	Criterion
Sensitivity/ linearity	Linearity check from five levels	Deviation of back-calculated concentration from true concentration $\leq \pm 20$
LOQ	Lowest spike level meeting the identification and method performance criteria for recovery and precision	$\leq$ MRL
Specificity	Response in reagent blank and blank control samples	$\leq$30% of RL
Recovery	Average recovery for each spike level tested	70–120%
Precision (RSD_r)	Repeatability RSD_r for each spike level tested	$\leq$20%
Precision (RSD_{wR})	Within-laboratory reproducibility, derived from on-going method validation/verification	$\leq$20%
Robustness	Average recovery and RSD_{wR}, derived from on-going method validation/verification	*See* above
Ion ratio	Check compliance with identification requirements for MS techniques	

In certain cases, individual performance-based criteria could be used rather than the generic value of the ion ratio. These individual criteria are determined during initial validation and subsequently on-going quality control performed during routine analysis. Factors that affect ion ratio variations include the stability of the instrument electronics, conditions in the collision cell, the linear range of the detector, peak integration, and signal contributions from interfering compounds. Furthermore, it has been shown that in the ionization source certain analytes can be protonated at different positions within the molecule, a process that can be influenced by matrix, resulting in different fragmentation and response variation for the product ions [52]. Nevertheless, the risk of false negative or positive increased when the tolerances are either too narrow or too wide compare to the acceptance criteria of 30%.

Improving the confidence of identification means adding extra information such as additional MS/MS transitions, accurate mass product ions, and finally a full-scan spectra assessment. In support to full-scan analysis, specific algorithms such as the deconvolution are available in order to provide the arising spectrum of the chromatographic peak is representative. Finally, the extracted ion chromatograms of sample and reference material should overlap with each other in term of retention time, peak shape, and response factor.

Table 6
Quality control identification requirements for different MS techniques (SANTE/12684/2019)

MS detector/characteristics			Requirements for identification	
Resolution	**Typical systems (examples)**	**Acquisition**	**Minimum number of ions**	**Other**
Unit mass resolution	Single MS Quadrupole Ion trap, TOF MS/MS Triple quadrupole, ion trap, Q-trap, Q-TOF, Q-Orbitrap	Full-scan, limited *m/z* range, SIM Selected or multiple reaction monitoring (SRM, MRM), mass resolution for precursor ion isolation equal to or better than unit mass resolution	3 ions 2 product ions	S/N $\geq$ 3 Analyte peaks from both product ions in the extracted ion chromatograms must fully overlap Ion ratio from sample extracts should be within $\pm$30% (relative) Of overage of calibration standards from same sequence
Accurate mass measurement	High-resolution MS: (Q-) TOF (Q-) Orbitrap Sector MS	Full-scan, limited *m/z* range, SIM, fragmentation with or without precursor-ion selection, or combinations thereof	2 ions with mass accuracy $\leq$5 ppm	S/N $\geq$ 3 Analyte peaks from precursor and/or product ion(s) in the extracted ion chromatograms must fully overlap

Routine laboratory Quality Control (QC) should mark similar ways for improving instrumental performance. Anyhow, specific characteristics of the method are unchangeable in order not to constitute a new testing procedure, which should undergo a new validation process: mass spectrometry resolution, mass spectrometry source conditions, ionization polarity, dwell times, data collection mode (e.g., MRM), injection type, column stationary phase chemistry, mobile phase, in term of composition, and gradient.

Basic tool of quality control for the analytical result is the participation of proficiency test (PT). A laboratory should define its own level and frequency of PT participation after a careful analysis of its other quality assurance measures such as regular use of reference material (RM) or certified material (CRM). These tools are complementary but not entirely guarantee the quality results.

Usually, the planning PT participation starts by listing the areas of technical competence, defined in term of three parameters: product, property, and instrumental technique. Often legislation defines a minimum frequency of PT participation in certain areas; for all official control laboratories in food and feed, it is mandatory to participate regularly in proficiency test schemes, particularly

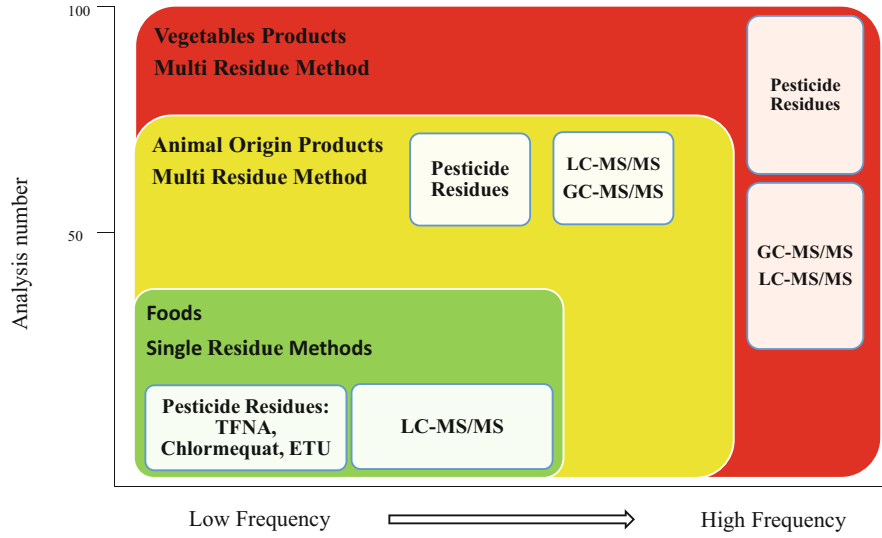


Fig. 8 PT participation plan frequency compared to number of analyzed samples

those organized yearly by the EURLs. In other sector to decide on suitable level and frequency of PT participation, the laboratory should conduct a simple risk assessment by considering: limitations in methodology, experience of technical staff, and complexity of the test procedure and how the results will be used, e.g., enforcement actions [53].

Figure 8 reports an example of PT participation plan of a laboratory, performing mainly analysis on vegetable products with multi-residue methods. Pesticide multi-residue and matrix methods use typically two instrumental techniques, LC–MS and GC–MS. Therefore, the laboratory should divide the PT participation in several areas and select with more frequency the PT scheme that is close to the routine analysis.

4 Notes

The influence of one or more undetected components from the sample on the instrumental response of the analyte concentration defines the matrix effect occurring in both LC–MS and GC–MS [54]. The detection and subsequently the reduction and compensation of the matrix effects are demanding goals in the analysis of mass spectrometry techniques. It was noted that in GC the matrix effects have been prevalent in injector and column systems due to active sites whereas in LC occur during the electrospray ionization, especially with complex matrices.

Several authors suggest as matrix effect's detection way the comparison of response from solvent and matrix-matched standards [55, 56].

These comparisons should be made with caution in a wide concentration range by carrying out calibration graphs. The ratio between the slopes of calibration curves in matrix-matched standard and in solvent corresponds to the matrix effect.

Both graphically and quantitatively (with a ratio equal or less of the Fig. 1), it is possible to make a distinction between a suppression effect of instrumental signal (with or without saturation) and interference effect. Positive matrix effects can be attributed to overlapped signals of coeluting components. Negative matrix effects could be attributed to overload of the MS source due to coextracted matrix constituents. The graphical estimation is an easy and robust method and therefore is popular and widely spread.

Changing MS conditions, optimizing chromatographic separations, and improving cleanups are basic strategies to minimize or suppress the matrix effects. Recently, the latest available technology has allowed us to implement most straightforward reduction strategies of matrix effects, for example, with a single tenfold dilution of samples with mobile phase or with new commercial switching device which allows the use of backflush of analytical columns in order to avoid an accumulation of some late eluting components of difficult matrices.

In support to reduction strategies of matrix effects, the laboratory has compensation tools for matrix effects, among which the use of isotope-labeled standards (ILISs), matrix-matched standards, and lastly the standard addition method.

The method of standard addition is the best approach to compensate for matrix effects, even if it has been laborious and took a long time. The standard addition approach is the method of choice where no appropriate ILIS is available. This approach typically compensates matrix effect better than the matrix-matched calibrations. The mass fraction of the pesticide in the sample is calculated via linear regression using a graphical presentation. This technique assumes some knowledge of the likely residue level of the analyte in the sample. The SANTE document recommends that the amount of added analyte is similar to that already present in the sample. In particular, it is recommended that standard addition is used for confirmatory quantitative analyses in cases of enforcement actions and/or when no suitable blank materials are available for the preparation of matrix-matched standard solutions. In the standard addition, a test sample is divided into three (or preferably more) test portions. One portion is analyzed directly, and increasing amounts of the analyte are added to the other test portions immediately prior to extraction. In the standard addition approach, the concentration of the analyte in the test sample extract is derived by extrapolation, thus a linear response in the appropriate concentration range is essential for achieving accurate results.

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Chapter 2

Determination of Neonicotinoid Insecticides in Bee Products by Using Ultra-High-Performance Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

As a result of growing concern regarding the disappearance of pollinators, numerous studies have been carried out in different countries in an attempt to find the causes of this phenomenon. A large part of this study is aimed at justifying this concern due to the presence of pesticide residues from the crops visited by bees. In particular, neonicotinoid insecticides have been identified as one potential cause, and three of these (thiamethoxam, clothianidin, and imidacloprid) have been banned by the European Authorities. Therefore, the determination of these insecticides in different matrices, whether they be food, environmental, etc., is an important current object of study. In this chapter, we summarize the analytical methods and the most relevant findings of four recent publications devoted to developing and validating specific analytical methods for determining neonicotinoid insecticides in different bee products, namely, honey, beeswax, bee pollen, and royal jelly. In all cases, ultra-high-pressure liquid chromatography methods coupled with tandem mass spectrometry were employed, and different sample treatments were proposed depending on the bee matrix studied. These included solid-phase extraction (honey and royal jelly), Quick, Easy, Cheap, Effective, Rugged, and Safe based-methodologies (bee pollen and honey), solvent extraction combined with dispersive solid-phase extraction (beeswax), and dispersive liquid–liquid microextraction (royal jelly). All the methods were fully validated and applied to an analysis of samples from different origins, namely, experimental and commercial apiaries.

Key words Analytical methods, Bee pollen, Bee products, Beeswax, Honey, Insecticides, Mass spectrometry, Neonicotinoids, Royal jelly, Sample treatment, Ultra-high-performance liquid chromatography, Validation

Abbreviations

ACET	Acetamiprid
ACN	Acetonitrile
AF	Samples spiked after sample treatment
BF	Samples spiked before sample treatment
CAR	*Centro Apícola Regional*

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CLO	Clothianidin
DAD	Diode array detector
DLLME	Dispersive liquid-liquid microextraction
DN	Dinetofuran
dSPE	Dispersive solid-phase extraction
EIC	Extracted ion chromatogram
EMR-lipid	Enhanced matrix removal-lipid
ESI	Electrospray ionization
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IMI	Imidacloprid
IS	Internal standard
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NEOs	Neonicotinoids
NT	Nitenpyram
PSA	Primary secondary amine
QC	Quality control
QTOF	Quadrupole time-of-flight
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
SE	Solvent extraction
THIA	Thiacloprid
TMX	Thiamethoxam
UHPLC	Ultra-high-performance liquid chromatography

1 Introduction

The main challenge of modern agriculture is to improve the quality of food production while avoiding adverse effects on the environment. However, the pests that attack crops have caused enormous concern due to notable losses in final production. The most widely used procedure for controlling these pests is to apply seeds coated with insecticides, since most of these pests are present in the soil when sowing is carried out [1]. These insecticides, and particularly neonicotinoids (NEOs), are rapidly absorbed and distributed throughout the plant, acting as repellants against insects that attack plants (or even by direct contact), causing interference in nerve transmission by binding to receptors [2, 3]. They can become more toxic metabolites during their translocation within the plant, as is the case of thiamethoxam (TMX) and its subsequent transformation to clothianidin (CLO). The appearance of these compounds in the pollen or nectar of crops from fields treated with NEOs has been related to the poisoning of bees [4, 5]. They cause bees to change their daily behavior, leading to depopulation of the hives and their gradual disappearance. This phenomenon is known in the

US as colony collapse disorder syndrome [6, 7]. As a result of growing concern regarding the disappearance of pollinators, numerous studies have been carried out in different countries in an attempt to find the causes of this phenomenon [8, 9]. A large part of this study is aimed at justifying this concern due to the presence of pesticide residues from the crops visited by bees. In fact, since the identification of exposure to NEOs as one of the factors involved in the sudden decline in the bee population, numerous studies have been published in the last few years relating to extracting and determining these insecticides in different bee products, such as honey, beeswax, bee pollen, royal jelly, nectar, or bee bread [10–14]. A discussion of the publications devoted to investigating the presence of NEOs in bee products is provided in another chapter of this book; therefore, the present study will focus on describing the more relevant findings of four publications in which these insecticides were determined in honey, bee pollen, beeswax, and royal jelly [15–18]. In view of the physicochemical properties of such insecticides, their low volatility and high polarity, liquid chromatography (LC) is the most suitable technique for carrying out separation and analysis, since the use of gas chromatography (GC) involves an additional derivatization step to transform analytes into volatiles. Since these compounds are usually found at the trace level, sensitive detectors such as mass spectrometry (MS) or tandem MS (MS MS) are required [19–21]. In all the publications summarized in this manuscript, ultra-high-performance liquid chromatography (UHPLC) was used instead of conventional LC, as this could secure better resolution and sensitivity, as well as shorter running times and thereby lower solvent consumption [22, 23]. In addition, we decided to use a quadrupole time-of-flight mass detector (QTOF), as its rapid acquisition rate makes it an ideal detector in combination with UHPLC [24], and the dynamic range of the QTOF is extended. Extracting NEOs in bee pollen was performed with Quick, Easy, Cheap, Effective, Rugged, and Safe methodology (QuEChERS), which consists of two stages: the first involves extraction with salts and the second is a cleaning stage, that is, liquid–liquid extraction [15]. This extraction procedure proposed for NEOs in beeswax was based on solvent extraction (SE) followed by a freezing process and a cleaning step with a recently commercialized sorbent (Enhanced Matrix Removal-Lipid; EMR-Lipid; [16]). In the case of honey [17], two different methodologies have been proposed in view of botanical origin and coloration. For dark honeys, the proposed method is a solid-phase extraction (SPE) with polymeric-type cartridges (Strata® X); while for light honeys, a modified QuEChERS method has been proposed with a buffered medium. Finally, two new methodologies have been developed for analyzing NEOs in products based on royal jelly [18], fresh royal jelly (FRJ), and liquid dietary supplement (LDS), that is, lyophilized royal jelly in the form

of ampoules. In order to extract these compounds in FRJ, a dispersive liquid–liquid microextraction (DLLME) has been proposed, while an SPE (Strata® X cartridges) has been proposed for LDS. In all cases, further aims of these studies involved validating the proposed methods in accordance with current European legislation [25] and internationally recognized guidelines [26], and analyzing samples from different origins, namely, experimental and commercial apiaries.

The current chapter summarizes the experimental conditions and the most relevant findings of four different publications in which different analytical methodologies were developed and validated for determining NEOs in different bee products (honey, beeswax, bee pollen, and royal jelly). The chapter is in the form of a conventional research article, but with the difference that each section has specific subsections according to the bee product in question. As previously mentioned, those readers who are interested in more specific aspects relating to the toxicity of NEOs, or extraction/separation methods in other matrices or from previous years, can consult some of the aforementioned texts and other chapters of this book.

2 Materials and Methods

2.1 Reagents and Materials

Fluka-Pestanal analytical standards of acetamiprid (ACET; Det. Purity 99.9%), CLO (Det. Purity 99.9%), dinetofuran (DN; Det. Purity 98.8%), IMI (Det. Purity 99.9%), nitenpyram (NT; Det. Purity 99.8%), thiacloprid (THIA; Det. Purity 99.9%), TMX (Det. Purity 99.6%), and TMX-d3 (Det. Purity ≥98%) were purchased from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). An isotope-labeled standard (TMX-d3) was chosen as internal standard (IS), since it has the same physical and chemical properties as the unlabeled analyte. It was required in the analysis of honey, bee pollen, and royal jelly. LC-grade ethyl acetate, acetone, methanol, ethanol, dichloromethane, and acetonitrile (ACN) were supplied by Lab Scan Ltd. (Dublin, Ireland). Chloroform (LC grade) was supplied by Scharlab S. L. (Barcelona, Spain); while, formic acid (98–100% pure), ammonium formate, and magnesium sulfate anhydrous were obtained from Sigma–Aldrich Chemie GbmH (Steinheim, Germany). Sodium chloride, sodium acetate, trisodium citrate dihydrate, and disodium hydrogen citrate sesquihydrate were supplied by Panreac (Barcelona, Spain); while, primary secondary amine (PSA) and C_{18} were purchased from Supelco (Bellefonte, PA, USA). Strata® X (6 mL with 200 mg of sorbent) and Strata® C_{18}-E (3 mL with 500 mg of sorbent) cartridges (Phenomenex, Torrance, CA, USA), Isolute® HM-N diatomaceous earth packed (5 mL sample) cartridges (Biotage, Uppsala, Sweden), and a 10-port Visiprep vacuum manifold

(Supelco, Bellefonte, PA, USA) were used for SPE procedures. A vibromatic mechanical shaker, a drying oven, and an ultrasonic bath all from J.P. Selecta S.A. (Barcelona, Spain), a vortex mechanical mixer from Heidolph (Schwabach, Germany), a Moulinette chopper device from Moulinex (Paris, France), a 5810 R refrigerated bench-top Eppendorf centrifuge (Hamburg, Germany), and an R-210/215 rotary evaporator from Buchi (Flawil, Switzerland) were employed for the extractions. Nylon syringe filters (17 mm, 0.45 μm) were from Nalgene (Rochester, NY, USA), and ultrapure water was obtained using Millipore Milli-RO plus and Milli-Q systems (Bedford, MA, USA).

2.2 Standards

Stock standard solutions of each neonicotinoid insecticide, at a concentration of 1000 mg/L, were prepared in methanol. These solutions were further diluted with water and methanol mixtures (honey, beeswax, and royal jelly, 80:20, v/v; bee pollen, 60:40, v/v) in order to prepare the working solutions. Bee product samples were spiked before (BF samples) or after (AF samples) sample treatment with different amounts of the neonicotinoid insecticides and with the IS in some cases to prepare the matrix-matched standards. Those samples were employed for validation (quality control (QC) samples and calibration curves), matrix effect, and treatment studies. Each QC (low, medium, and high) sample was prepared with a different amount of each bee product spiked with three different concentrations of neonicotinoids within the linear range. These were summarized in Table 1. The stock solutions were stored in glass containers in darkness at −20 °C; working and matrix-matched solutions were stored in glass containers and kept in the dark at 4 °C. All solutions remained stable for over 2 weeks.

2.3 Sample Procurement and Treatment

Samples were in all cases from different Spanish regions in which an insecticide treatment with neonicotinoids has been applied. All of them underwent preliminary analysis by UHPLC–MS/MS to check for the presence of neonicotinoids. Once it was confirmed

Table 1
QC levels for the different bee products

	Bee pollen	Beeswax	Honey	Royal jelly
Low QC	5 μg/kg	LOQ (1.5–5.1 μg/kg)	LOQ (0.3–6.8 μg/kg)	LOQ (1.1–7.5 μg/kg)
Medium QC	63 μg/kg	32 μg/kg	50 μg/kg	83 μg/kg (LDS) 50 μg/kg (FRJ)
High QC	500 μg/kg	250 μg/kg	300 μg/kg	333 μg/kg (LDS) 250 μg/kg (FRJ)

QC quality control, *LOQ* limit of quantification, *LDS* liquid dietary supplement, *FRJ* fresh royal jelly

that there was no residual trace of the studied compounds, subsamples of these bee products were used as blanks to prepare matrix-matched standards.

2.3.1 Bee Products

Commercial bee pollen samples ($n = 20$) were purchased in local markets (Valladolid, Spain). They were mixed and dried at +45 °C in an oven, ground and pooled for optimum sample homogeneity, and subsequently stored in darkness at +4 °C until analysis.

Bee Pollen

Beeswax

Beeswax samples ($n = 21$) were collected from apiaries in different Spanish regions. Samples were mixed and grounded with dry ice to keep them cool for obtaining optimum sample homogeneity; subsequently, they were stored in the dark at −20 °C until analysis.

Honey

Samples from different regions of Spain were kindly donated by the "*Centro Apícola Regional*-CAR" at Marchamalo (Guadalajara, Spain). Their botanical origin was confirmed by melissopalynological analysis and corresponded to: rosemary, *Rosmarinus officinalis* ($n = 6$); multifloral ($n = 6$); and heather, *Erica* spp. ($n = 6$). In addition, multifloral honey samples ($n = 10$) collected from controlled apiaries were also supplied by CAR. Apiaries were located close to experimental crops, previously treated with TMX-dressed rapeseeds.

Royal Jelly

Two different types of royal jelly-based products (fresh royal jelly—FRJ and liquid dietary supplement—LDS) were analyzed. FRJ samples ($n = 7$) were obtained from local beekeepers or markets (Valladolid, Spain); meanwhile, LDS samples ($n = 5$), which contained freeze-dried royal jelly, fructose, and water as main constituents, were also purchased in local markets. All samples were stored at 4 °C before analysis.

2.3.2 Sample Treatment

Specific sample treatments were developed for each bee product, and all the steps are detailed in the corresponding publications [15–18]. However, the most relevant conditions (sample size, overall time, shaking method, clean up…) for each product are summarized in Table 2.

Efficient sample treatments involving optimized QuEChERS protocols were proposed for the analysis of bee pollen and light-color honeys, SPE with a polymeric sorbent (Strata® X) is recommended for analyzing dark honeys, and LDS, an SE followed by a dispersive solid-phase extraction (dSPE) was selected for the analysis of beeswax, while a DLLME was the best option when determining NEOs in FRJ.

2.4 UHPLC–MS/MS System

An Acquity™ UHPLC system (ACQUITY; Waters, Milford, MA) and a QTOF mass spectrometer (maXis impact; Bruker Daltonik, Bremen, Germany) were coupled through an electrospray (ESI) interface, which was operated in the positive ionization mode.

Table 2
Summary of the sample treatments selected for determining NEOs in the different bee products

Bee product	Sample size	Procedure	Reagents	Instrumentation	Overall time
Bee pollen	1 g	QuEChERS	Water (2.2 mL), ACN (6 mL), $MgSO_4$ (1 g), sodium chloride (0.5 g), magnesium sulfate (0.5 g), TC (0.8 g), PSA (25 mg), C_{18} (25 mg), methanol (0.8 mL)	Vortex (30 s) Vibromatic (5 min) Centrifuge 2 × (5 min, 10 °C, 10,000 rpm) Freezing (2 min) Rotary evaporator (60 °C)	30 min
Beeswax	1 g	SE + dSPE	Methanol (7.8 mL), ethyl acetate (3 mL), water (0.2 mL), EMR-lipid tube	Vibromatic (5 min) Centrifuge 2 × (3 min, 5 °C, 10,000 rpm) Freezing (3 min) Rotary evaporator (60 °C)	20 min
Honey Light honeys	5 g	QuEChERS	Water (10.2 mL), ACN (7 mL), ethyl acetate (3 mL), methanol (0.8 mL), magnesium sulfate (2 g), sodium acetate (1 g), TCD (1.5 g), TCS (0.5 g)	Vortex (30 s) Ultrasound (30 °C, 5 min) Centrifuge (3 min, 5 °C, 5,000 rpm) Rotary evaporator (60 °C)	15 min
Dark honeys		SPE	Strata® X cartridge, water (5.2 mL), Ammonium formate 10 mM (10 mL), ACN (3.2 mL), ethyl acetate (0.8 mL), methanol (5.8 mL)	Vacuum manifold Rotary evaporator (60 °C)	20 min

(continued)

Table 2 (continued)

Bee product		Sample size	Procedure	Reagents	Instrumentation	Overall time
Royal jelly	LDS	3 g	SPE	Strata® X cartridge, water (5.2 mL), ammonium formate 10 mM (10 mL), ethyl acetate (0.6 mL), methanol (7.2 mL)	Vacuum manifold Rotary evaporator (60 °C)	20 min
	FRJ	0.1 g	DLLME	Chloroform (0.25 mL), ACN (1 mL), methanol (0.08 mL), water (0.02 mL)	Vortex 2 × (30 s) Ultrasound 2 × (40 °C, 10 min) Centrifuge 2 × (3 min, 5 °C, 2,500 rpm) Nitrogen stream	30 min

ACN acetonitrile, *DLLME* dispersive liquid–liquid microextraction, *dSPE* dispersive SPE, *EMR* enhanced matrix removal, *FRJ* fresh royal jelly, *LDS* liquid dietary supplement, *PSA* primary secondary amine, *QuEChERS* quick, easy, cheap, effective, rugged, and safe, *SE* solvent extraction, *SPE* solid-phase extraction, *TC* trisodium citrate, *TCD* TC dihydrate, *TCS* TC sesquihydrate

The UHPLC instrument was equipped with a vacuum degasser, a binary solvent pump, an autosampler, and a thermostated column compartment. Data were acquired and processed with software Data Analysis 4.1 and Qualitative Analysis from Bruker Daltonik.

2.4.1 UHPLC Conditions

A Kinetex® EVO fused-core type column (C_{18}, 50 × 2.1 mm, 1.7 μm, 100 Å) was employed for UHPLC analysis, and this was protected by a Kinetex® EVO C_{18} guard column. Both were acquired from Phenomenex (Torrance, CA, USA). After optimization studies, the mobile phases were selected, and they were always composed by 0.1% (v/v) formic acid in acetonitrile (solvent A) and 0.1% (v/v) formic acid in water (solvent B) applied at a flow rate of 0.3 mL/min in the different gradient modes (*see* Table 3). Injection volume was set at 5 μL (beeswax, honey, and royal jelly) or 10 μL (bee pollen) depending on the bee product; while 30 °C was chosen as working temperature in all cases but in one, beeswax, it was selected 35 °C as optimal value.

2.4.2 QTOF Conditions

The optimal conditions were set after several experiments (flow injection analysis (FIA) in infusion mode, 80 μL/min) were conducted (*see* Table 4).

Spectra were acquired in a mass range of mass/charge (m/z) 50–350 or 50–400. The m/z scale of the mass spectra was calibrated daily by infusing a sodium formate and sodium acetate

Table 3
Gradient elution programs. Mobile phase was composed of 0.1% formic acid in ACN (solvent A) and 0.1% formic acid in water (solvent B)

Bee product	Gradient elution program
Bee pollen	(1) 0.0–1.8 min (A–B, 6:94, v/v); (2) 1.8–3.0 min (A–B, 15:85, v/v); (3) 3.0–3.5 min (A–B, 30:70, v/v); (4) 3.5–4.5 min (A–B, 15:85, v/v); (5) 4.5–5.0 min (A–B, 6:94, v/v); (6) 5.0–6.5 min (A–B, 6:94, v/v)
Beeswax	(1) 0.0–1.5 min (A–B, 5:95, v/v); (2) 1.5–3.0 min (A–B, 33:67, v/v); (3) 3.0–4.5 min (A–B, 70:30, v/v); (4) 4.5–7.5 min (A–B, 90:10, v/v); (5) 7.5–8.5 min (A–B, 70:30, v/v); (6) 8.5–9.0 min (A–B, 33:67, v/v); (7) 9.0–10.5 min (A–B, 5:95, v/v)
Honey	(1) 0.0–1.0 min (A–B, 10:90, v/v); (2) 1.0–1.5 min (A–B, 60:40, v/v); (3) 1.5–2.5 min (A–B, 90:10, v/v); (4) 2.5–3.5 min (A–B, 90:10, v/v); (5) 3.5–4.0 min (A–B, 60:40, v/v); (6) 4.0–4.5 min (A–B, 10:90, v/v); (7) 4.5–6.0 min (A–B, 10:90, v/v)
Royal jelly	(1) 0.0–1.5 min (A–B, 10:90, v/v); (2) 1.5–2.5 min (A–B, 80:20, v/v); (3) 2.5–3.5 min (A–B, 80:20, v/v); (4) 3.5–4.0 min (A–B, 90:10, v/v); (5) 4.0–4.5 min (A–B, 90:10, v/v); (6) 4.5–5.0 min (A–B, 20:80, v/v); (7) 5.0–6.0 min (A–B, 10:90, v/v); (8) 6.0–8.0 min (A–B, 10:90, v/v)

Table 4
Optimal MS/MS parameters

Bee product	Capillary voltage (V)	Drying gas (N_2) flow (L/min)	Drying gas (N_2) temperature (°C)	Nebulizer pressure (bar)	Mass range (*m/z*)
Bee pollen	3500	12	220	2	50–400
Beeswax	3500	12	220	2	50–400
Honey	3500	12	220	2	50–400
Royal jelly	4000	12	250	2	50–350

mixture. Compounds showed in all cases an intense $[M + H]^+$ (precursor ions) on their full-scan spectra, which was selected as a precursor to obtain product ions for MS/MS analyses, which were carried out by using an isolation width of 10 *m/z* and variable collision energies (10–30 eV; *see* Table 5). A window of ±0.01 *m/z* for the extracted ion chromatograms (EIC) was used, in order to extract the exact mass.

2.5 Method Validation

Validation of the methods was based on the current European legislation for pesticide residues analysis in foods [25] and the Eurachem Guidelines [26]. Reference standards were prepared in solvent as well as in matrix (i.e., matrix-matched calibration) and

Table 5
Ions and collision energies selected for QTOF analyses in each bee product

Compound	Precursor ion (*m/z*)	Product ions (*m/z*)	CE (eV)
Dinetofuran	203.1163[A]	113.1039[A], 113.1033[A,BP] 129.0908[B], 129.0904[B,BP]	15 15
Nitenpyram	271.0988[A]	99.0920[A], 99.0925[A,BP] 225.1059[B], 225.1056[B,BP]	15 15
Thiamethoxam	292.0296[A]	131.9675[A], 131.9678[A,BP] 211.0678[B], 211.0677[B,BP]	15 15
Thiamethoxam-d3 (IS)	295.0396[A]	131.9675[A], 131.9677[A,BP] 214.0687[B]	15 15
Clothianidin	250.0187[A]	134.9677[A], 134.9685[A,BP] 169.0566[B,BP,RJ],169.0495[B,H,BW]	15 15
Imidacloprid	256.0623[A]	175.0999[B], 175.0996[B,BP] 209.0614[A], 209.0618[A,BP]	25 25
Acetamiprid	223.0780[A]	56.1002[A], 56.1009[A,BP] 126.0117[B], 126.0114[B,BP]	30 25, 30[BW]
Thiacloprid	253.0342[A]	126.0118[B], 126.0113[B,BP] 186.0154[A], 186.0156[A,BP]	20 20

[A], Confirmation ions; [B], Quantification ions; [BP], bee pollen; [BW], beeswax, [H], honey; [RJ], royal jelly; CE, collision energy

treated with the selected procedure. Basic but efficient chemometric statistical tools from Excel (Microsoft Office 2010, Microsoft Corporation, Redmond, WA), Data Analysis 4.1 and Qualitative Analysis both from Bruker Daltonik were employed to acquire, process, and analyze the data in order to validate the methods.

2.5.1 Selectivity

To determine the selectivity of the proposed methods, a set of extracts from non-spiked samples ($n = 6$) was injected onto the chromatographic system, and the results were compared with those obtained for spiked samples.

2.5.2 Limits of Detection and Quantification

The limits of detection (LODs) and quantification (LOQs) were experimentally determined by the injection of a number of blank samples ($n = 6$), and the magnitude of background analytical response at elution time in each sample was measured. The LODs and LOQs were estimated to be three and ten times the signal-to-noise ratio, respectively.

2.5.3 Matrix Effect

In order to ascertain how the matrix influenced ESI ionization, a comparison was made of the results (analyte peak area or analyte peak area/IS area) with standard working solutions and spiked at

Table 6
Calibration curve data, LOD, and LOQ values

Bee product		Analytical range (μg/kg)	R^{2*}	LOD* (μg/kg)	LOQ* (μg/kg)
Bee pollen		LOQ–500	0.993–0.999	2.1–3.9	5.3–12.0
Beeswax		LOQ–250	0.9879–0.9999	0.4–1.4	1.5–5.1
Honey	MF	LOQ–300	0.991–0.999	0.1–0.6	0.3–2.2
	RM	LOQ–300	0.991–0.999	0.4–1.8	1.5–4.4
	HT	LOQ–300	0.991–0.999	0.7–2.0	3.3–6.8
Royal jelly	LDS	LOQ–333	0.991–0.999	0.8–2.4	2.5–7.5
	FRJ	LOQ–250	0.991–0.999	1.1–3.0	3.7–9.4

*, minimum and maximum values; MF, multifloral; RM, Rosemary; HT, heather; LDS, liquid dietary supplement; FRJ, fresh royal jelly; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification

three different concentrations (QC levels) following sample treatment (AF samples).

2.5.4 Linearity Studies

Different calibration curves (solvent-based or matrix-matched standards) were used to quantify NEOs in accordance to the bee product and the influence of the matrix effect onto the analyte ionization (*see* Table 6). Calibration curves ($n = 6$) were constructed by plotting the signal on the *y*-axis (analyte peak area or analyte peak area/IS area) against the analyte concentration on the *x*-axis.

2.5.5 Precision

Intraday precision experiments were performed concurrently by repeated sample analysis using blank samples spiked before sample treatment (BF samples) at three different concentrations (low, medium, and high QC levels), either on the same day ($n = 6$; intraday precision), or over three consecutive days ($n = 6$; interday precision).

2.5.6 Trueness

This was evaluated by the mean recoveries (as a measure of trueness), calculated by comparing the results (analyte peak area/IS area) obtained from blank samples spiked at three different concentrations (QC levels), either prior to (BF samples) or following (AF samples) sample treatment.

3 Results and Discussion

3.1 Optimization of the Sample Treatments

This section collates and provides an overview of the most relevant tests and results that were obtained when the specific sample treatments were developed for each bee product. The experiments are described in detail in the corresponding publications [15–18]. The process to optimize sample treatment is similar in all cases. First of

all, a bibliographic search is carried out where the most promising option or options are selected, and subsequently, the most relevant parameters of each one of the processes are studied sequentially, such as the amount of the sample, the type and amount of extractant, suitable instruments, the SPE sorbent, and the cleanup stage. As mentioned in Subheading 2.2, solvent and matrix standards have been used to optimize sample treatment. Each of the beekeeping matrices studied will be the object of specific discussion.

3.1.1 *Bee Pollen*

The bibliographic study carried out on the analysis of NEOs in pollen showed that nowadays the most common sample treatment for this purpose is QuEChERS [3, 5, 6, 27–30]. This is fundamentally associated with the prevailing current trend in this area, which implies a reduction in volumes, quantities, and stages. This explains why methods that do not meet these characteristics, such as solvent extraction or SPE, are becoming increasingly less popular. As mentioned above, the optimization process was carried out sequentially and consisted of various stages concerned with choosing optimal conditions in terms of recovery percentage, cleaning of the samples, and reducing as far as possible overall sample treatment time. These were related, among other parameters, to the amount of the sample, the nature and volume of the extractant, selecting the amount of salts needed for the first stage of the QuEChERS method, extraction and centrifugation times, and the components of the dSPE stage. ACN was chosen as the extractant solvent; this is a usual choice for the QuEChERS method because it is able to precipitate proteins and limit lipid solubility, which could facilitate cleaning the samples [6, 15, 31]. As for QuEChERS salts, different compounds were employed according to their function: (1) magnesium sulfate served to partition water from the sample; (2) sodium chloride was used to reduce polar co-extractives; and (3) trisodium citrate dihydrate was employed to buffer the liquid–liquid extraction and provide a suitable medium. Optimization of the other conventional QuEChERs parameters implied no significant difference regarding previous approaches in terms of difficulty. Details are given in Table 2. The results obtained when applying these conditions were acceptable in terms of recovery, but were unsuitable as regards removing matrix constituents from the extracts, and for that reason it was decided that a freezing step should be introduced to remove lipids, as this has shown good results in previous publications [6, 15]. After optimization of this step, the samples were cleaner and the recoveries were not affected. Both the effectiveness and usefulness of the sample treatment were demonstrated not only by the excellent results obtained (recovery, matrix effect, and precision; *see* Subheading 3.3 and Table 7) but also by comparison with previous publications. For example, the recovery values are similar to or better than those in the literature, but in many of these studies, the matrix effect was very significant, which is not the case with the proposed method. In addition, the freezing step has

Table 7
Summary of the studies devoted to evaluate efficiency (recoveries; mean values-%) of the sample treatment and the matrix effect (comparison of responses; mean values-%)

Bee product		Evaluation of the efficiency*			Evaluation of the matrix effect*		
		Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
Bee pollen		91–99	93–104	93–105	91–104	93–104	90–102
Beeswax		93–106	94–104	96–106	81–106	83–102	81–104
Honey	MF	87–102	90–107	92–103	71–105^{MMF}	73–107^{MMF}	75–102^{MMF}
	RM	90–109	87–105	85–99	81–103	86–104	83–107
	HT	80–108	82–102	83–101	45–105^{MH}	47–102^{MH}	45–108^{MH}
Royal jelly	LDS	94–109	92–105	90–106	68–101^{ML}	68–104^{ML}	70–99^{ML}
	FRJ	83–107	85–105	86–104	54–97^{MF}	48–101^{MF}	45–104^{MF}

*, minimum and maximum mean values; ^{MF}, matrix effect in fresh royal jelly: dinetofuran, clothianidin and thiacloprid; ^{ML}, matrix effect in liquid dietary supplements: dinetofuran, clothianidin and acetamiprid; ^{MMF}, matrix effect in multifloral honeys (MF): acetamiprid; ^{MH}, matrix effect in heather honeys (HT): dinetofuran, nitenpyram, clothianidin and imidacloprid; RM, rosemary

proven to be effective, as it avoids the use of other complex alternatives, such as the use of other solvents or a second extraction.

3.1.2 Beeswax

As in the case of the analysis of bee pollen samples, the most common option in sample treatment for NEOs and pesticide analysis is the QuEChERs methodology, replacing the more conventional solvent extraction combined with SPE [7, 16, 32–34]. However, due to the nature of beeswax, it was necessary to melt or dissolve the sample prior to treatment. Nevertheless, we decided to ascertain the suitability of a solvent extraction, instead of a QuEChERS method, in order to provide an alternative to the existing methods and because of its simplicity. The optimization procedure was performed similarly to the one described for bee pollen. Once the amount of beeswax had been selected, several solvent mixtures were chosen on the strength of several preliminary experiments (methanol and ethyl acetate; acetonitrile and ethyl acetate; methanol and water; ethanol and water; acetonitrile and water). The best results in terms of recoveries (>80%) were obtained when a methanol and ethyl acetate mixture (70:30, v/v) was used. Next, the influence of certain extraction parameters, such as volume, extraction time, and centrifugation time, was sequentially tested in order to obtain optimal conditions (*see* Table 2). Following this, the mixture was left to cool in a polystyrene box filled with dry ice, as in the case of bee pollen. However, this step was insufficient as regards removing certain matrix components which affected ionization of the NEOs, such as lipids and, in particular, two of these (NT and DN). Thus, we decided to include an additional cleanup step by using a recently commercialized

sorbent (EMR-lipid), which was chosen as it has shown promising results when analyzing pesticides, including NEOs [35]. This material is a sorbent, containing C_{18} and some special types of polymers, with pores that selectively bind long-unbranched hydrocarbon chains, while the analytes do not interact with the sorbent and remain in the solution [16]. Results showed that the use of this new sorbent was a success, since recoveries were higher than 80% and the matrix effect was minimized for all the NEOs (*see* Table 7). In this case, comparison with previous publications was also positive, as the recovery values were similar or better, and the absence of a matrix influence on the analyte signals was not achieved in several of these studies. Additionally, this was the first time that an EMR-lipid sorbent was employed in this matrix.

3.1.3 Honey

Optimization of the sample treatment was performed in a different way from that of the other bee matrices, as in this case we optimized and compared two different alternatives based on a review of the literature in order to select the most suitable for the different types of honey analyzed. As with other bee products, QuEChERS has usually been employed to determine NEOs in honey as a replacement for SPE [20, 36, 37]. However, in most of the publications, the optimization and validation studies were not compared for honey of different botanical origins. From our experience, this is unsatisfactory, as the matrix constituents could be quite different depending on the origin of the honey, and these could have a strong influence, for example, on the matrix effect. Consequently, we decided to optimize two different treatments (QuEChERS and SPE) for honey samples from three different botanical origins in order to select the most appropriate for each type. In the optimization experiments for QuEChERS, the three types of honeys were employed, while for the SPE experiments only heather honey was used, as this is the botanical origin which is mostly influenced by the matrix effect. Regarding optimization of each sample treatment, the QuEChERS approach was similar to that described for bee pollen. In this case, the selected extractant was a mixture of ACN and ethyl acetate (80:20, v/v), while trisodium citrate dihydrate and disodium hydrogen citrate sesquihydrate were used to buffer the liquid–liquid extraction, and different agitation sources (vibromatic, vortex, and ultrasound) were assayed in order to facilitate the extraction of NEOs; ultrasound was selected due to the higher recovery rate obtained with this method. The influence of the dSPE step on the recoveries and matrix effect for NEOs was also examined, and it was found that this step did not significantly minimize the matrix effect. The result was significant signal suppression for ACET in multifloral honey and for all the NEOs in heather honey, which negatively affected the recovery of the analytes, especially for DN and NT. Therefore, it was decided that a dSPE step should not be performed (*see* final conditions in Table 5).

The SPE procedure was optimized on the basis of our previous experience [38]. Consequently, an SPE cartridge (Strata® X) was selected, and apart from optimizing the different steps of the SPE procedure, it was also necessary to determine the amount of honey and the most suitable solvent and volume to dilute the honey (10 mM ammonium formate in water). It should be noted that the same two NEOs that were negatively affected in the dSPE step of the QuEChERS procedure showed a similar behavior in the washing step, as they disappeared when this was performed. Therefore, this step was removed for the final SPE procedure (*see* final conditions in Table 2). Under the optimal conditions, recoveries were above 75% for all the NEOs, and the matrix effect caused signal suppression in four of them (DN, NT, IMI, and CLO; *see* Table 7). Following optimization of the methods, performance between these and those of previous publications was compared. Firstly, it can be concluded that the best choice for light honeys (multifloral and rosemary) is QuEChERS, as better recoveries were obtained with a simpler procedure that also minimizes the matrix effect for all NEOs with the exception of one (ACET in multifloral honey). Meanwhile, SPE is the most appropriate choice for dark honeys (heather), as it reduces the matrix effect for several of the NEOs. The proposed methods displayed a similar or better overall performance than those of previous publications, but with the advantage of minimizing the matrix effect in most cases.

3.1.4 Royal Jelly

An analysis of NEOs in royal jelly has been less extensive than in the other bee matrices, and, in fact, at the time of this study, there was no publication specifically devoted to determining NEOs in royal jelly. Therefore, we decided to begin optimizing the QuEChERS procedure as it has provided good results in the case of other bee products [20, 27–30, 32–34, 36, 37]. In this regard, our intention was to propose specific methods for two different royal jelly-based products (FRJ and LDS). Experiments started with LDS, initially employing the optimized conditions for bee pollen analysis [15]. After some preliminary experiments were performed, it was found that the recoveries were satisfactory (70% and 93%) for most of the NEOs, but it was also observed that an interface was formed following centrifugation, which made it difficult to collect the supernatant and subsequently affected the reproducibility of the results. It was not possible to remove this interface, and consequently the decision was taken to change the sample treatment to an SPE (Strata® X), as this has been seen to perform satisfactorily in honey samples [17]. The optimization procedure was quite similar to that already explained for honey analysis, and it was also found that the washing step was unnecessary as DN and NT were again lost. The most relevant SPE parameters are summarized in Table 2. Subsequently, the study continued with the other royal jelly product, FRJ. As good results were obtained with SPE, the suitability of

this procedure was tested with FRJ. Unfortunately, it was not appropriate for this matrix, as once the sample was mixed with ammonium formate, a viscous solution was produced which caused the obstruction of the SPE cartridges regardless of the volume of solvent and amount of sample. Thus, the QuEChERS approach was assayed, but in this case, the interface was even thicker than in that of the LDS. Revising the related literature, we found a study in which NEOs were determined in honey-liqueur by DLLME [39], and consequently this option was selected to continue with the experiments. The optimization procedure began with the selection of the sample amount and continued with a study of the extraction and dispersive solvents (nature and volume). Different extraction (acetone, chloroform, and dichloromethane) and dispersive (acetonitrile, methanol, and ethanol) solvents were examined, and the best results in terms of extraction efficiency were obtained with chloroform and acetonitrile, respectively. Next, the influence of certain extraction parameters, such as agitation source (vortex and/or ultrasound) and extraction time, was also evaluated; the result was that the best performance came with a short agitation time in a vortex, followed by an ultrasound bath step. We also considered whether the number of extractions (one or two), with the same final volume, affected extraction efficiency (*see* final conditions in Table 2). Here the results showed that recoveries significantly improved with two extractions rather than one. Once the DLLME procedure was fully optimized for FRJ, its suitability was ascertained for LDS; it was seen that this was not appropriate, since an emulsion was formed when the extraction and dispersive solvents were added. The performance of both procedures was compared with that of previous studies, and the findings demonstrated that both proposals were an efficient, shorter, and greener alternative to those methods. In all cases, recoveries represented between 83% and 109%, and the matrix did not affect analyte signals for several of the NEOs (4 of 7; *see* Table 7). Finally, it has also been shown that different methodologies should be employed as sample treatments in accordance with the type of royal jelly-based products.

3.2 Optimization of UHPLC–MS/MS Conditions

3.2.1 UHPLC

First of all, it should be mentioned that our research group had previously carried out the neonicotinoid analysis, and in both cases the use of fused-core columns produced very good results. These columns have increasingly been used in the last few years, as they provide highly efficient separation with relatively low back pressure [15, 40]. Consequently, as we mentioned in the Introduction that what was intended in this series of studies was to develop the fastest and most efficient methods possible in terms of separation, we decided on UHPLC. Moreover, an additional purpose of these studies was to propose UHPLC methods that could be extensively used, for example, with other detectors (diode array or ultraviolet),

since expensive MS/MS detectors are not affordable for all laboratories. This implies that the seven NEOs must be baseline separated, as the coelution of analytes when using MS detectors, especially for MS/MS, is common although it is a major drawback for other types. Modification of LC methods to UHPLC entailed choosing a column of reduced physical dimensions and particle size. In addition, the commercialization of a new type of fused-core column (Kinetex® EVO) at the same time as the first study allowed us to test its potential for determining NEOs in bee products. This column provides the additional benefit of improved peak shape for bases, wide pH 1 to 12 stability, while potential signal suppression caused by the presence of polar (basic compounds) is lessened [15]; this could be especially useful in complex matrices such as bee products. We therefore decided to optimize separation of NEOs in bee pollen, which constituted the first study of the series, with the Kinetex® EVO column and similar mobile phase components to those employed in our previous studies (0.1% (v/v) formic acid in ACN and 0.1% (v/v) formic acid in water). The acidic mobile phase is recommended when conducting experiments in the positive mode of ESI, as compounds that are protonated in solution before spraying should account for most of the ions. Several experiments were conducted with diverse mobile phases and flow rates to separate the neonicotinoid insecticides in the shortest possible time. The most rapid analysis times were obtained with the chromatographic conditions described in Subheading 2.4 and Tables 3, 4 and 5. This was the fastest proposal published regarding neonicotinoid analysis in bee pollen up to the date of publication, and it was also the first application of the Kinetex® EVO for determining NEOs in any matrix. All the analytes were baseline separated (*see* Fig. 1), and subsequently the method could be applied to different detectors, not only MS-based ones.

This *modus operandi* was repeated for all the bee matrices analyzed. In relation to the publications investigating NEOs in honey, royal jelly, and beeswax, the chromatographic conditions, which were optimized on the basis of the parameters used for bee pollen, also allowed a more rapid baseline separation of all the insecticides than those reported in all the previous publications determining NEOs in these matrices. Moreover, the number of NEOs that were simultaneously determined was also greater than in most of the previous studies.

3.2.2 MS/MS (QTOF)

The optimization procedure was performed in the same manner in all the studies. However, the selection of the most adequate ionization interface was only done in the first one (bee pollen; [15]). It was performed by FIA of standard in solvent and matrix-matched solutions, with monitoring of MS/MS intensity employing atmospheric pressure chemical ionization (APCI) ESI interfaces in positive and negative ionization modes. It was decided to check both

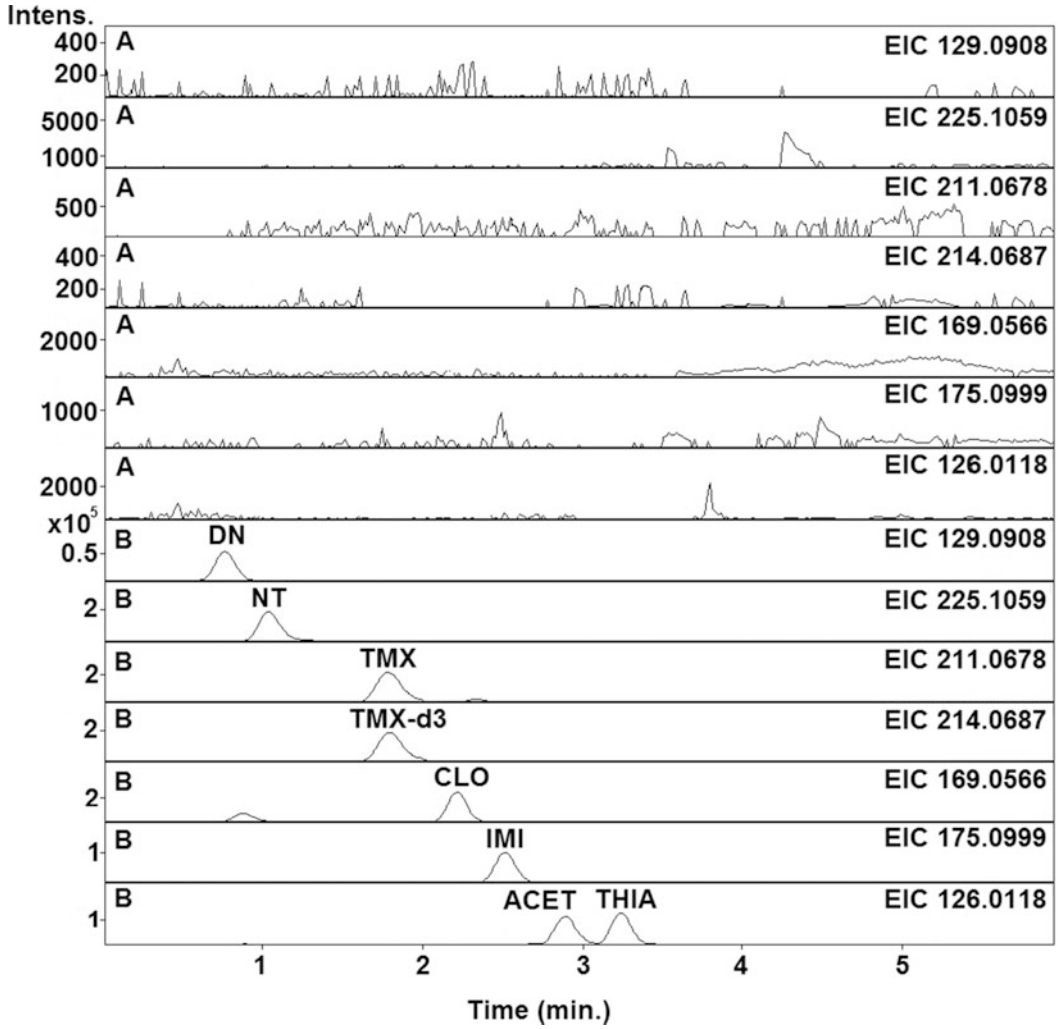

Fig. 1 Representative UHPLC-QTOF chromatograms (EIC in positive mode using the quantification ions; *see* Table 5) obtained from: (**a**) non-spiked rosemary honey sample; (**b**) spiked (50 μg/kg) rosemary honey sample. The UHPLC-QTOF conditions are summarized in Subheading 2.4 and Tables 3, 4 and 5. Reprinted from Food Chemistry, 266, Silvia Valverde, María Ibáñez, José L. Bernal, María J. Nozal, Félix Hernández, José Bernal, Development and validation of ultra high performance-liquid chromatography–tandem mass spectrometry based methods for the determination of neonicotinoid insecticides in honey, 215–22, Copyright (2018), with permission from Elsevier

interfaces as they were previously used for NEOs determination, being ESI mostly employed [4, 41–44]. Results showed that the best performance was obtained when using ESI in positive mode. Thus, this mode of operation was selected for all the publications. Then, it was evaluated with several experiments (FIA) the influence of the most relevant MS/MS parameters (capillary voltage, drying gas (N_2) temperature, drying gas (N_2) flow, nebulizer pressure, and collision energy) that could affect the signals for all the NEOs. As

can be seen in Table 4, optimal values were quite similar in all cases, being the differences mainly located in the capillary voltage, drying gas temperature, and collision energy. Finally, it must be specified that the product ions with the highest signals were used for quantification; meanwhile, the second product ions with the higher signals were used for confirmation (*see* Table 5).

3.3 Method Validation

3.3.1 Selectivity

It should be mentioned that the results were quite similar in all the studied bee products. No chromatographic interferences were observed at NEOs retention times in any of the blank samples. Meanwhile, for the identification and confirmation of NEOs presence in the samples (spiked and with endogenous neonicotinoid content), comparisons were made of the mass spectra of each of the NEOs peaks in standard solutions and beeswax samples with endogenous or spiked pesticide content; concentrations were comparable and measurements were taken under the same conditions. In all cases, both types of mass spectra were quite similar (*see* Fig. 2), although some minor differences in ion intensity were observed. In addition, the relative intensities of the ions/transitions in the matrix-matched samples concurred with the corresponding standard solutions to within ±10% for the different bee matrices. This result indicates that the selectivity of the methods is good enough in relation to the current European legislation as it is much lower than the maximum permitted rates (±30%; [25]).

3.3.2 Limits of Detection and Quantification

As can be seen in Tables 1 and 6, low LODs and LOQs were obtained for the studied NEOs in the different bee matrices assayed, being quite similar to those values in all cases. In particular, LODs ranging from 0.1 to 3.0 μg/kg or from 0.3 and 9.0 μg/kg for the LOQs. These results are better and comparable in the worst cases with to those of previous publications, and they are also much lower than the maximum residue limits (MRLs) established by the European Commission for several of these pesticides in honey and other apiculture products (50–200 μg/kg; [45]).

3.3.3 Matrix Effect

This is the validation parameter that showed more difference in relation to the bee product analyzed, as the influence of the matrix on the MS/MS signals is strongly dependent on the type of sample. This is confirmed by the data obtained for the different QC levels in each case (*see* Table 7). For example, no significant matrix effect was observed for all NEOs when analyzing bee pollen and beeswax with their corresponding methods, and in some cases when analyzing royal jelly and honey. This is justified by the fact that the comparison of the responses was in those cases always comprised between 80 and 110%, which complied with the criteria of the European Commission for pesticide residue analysis (±20% of the response from standard solutions; [25]). However, it was observed a significant signal suppression for several of the NEOs in royal jelly (DN,

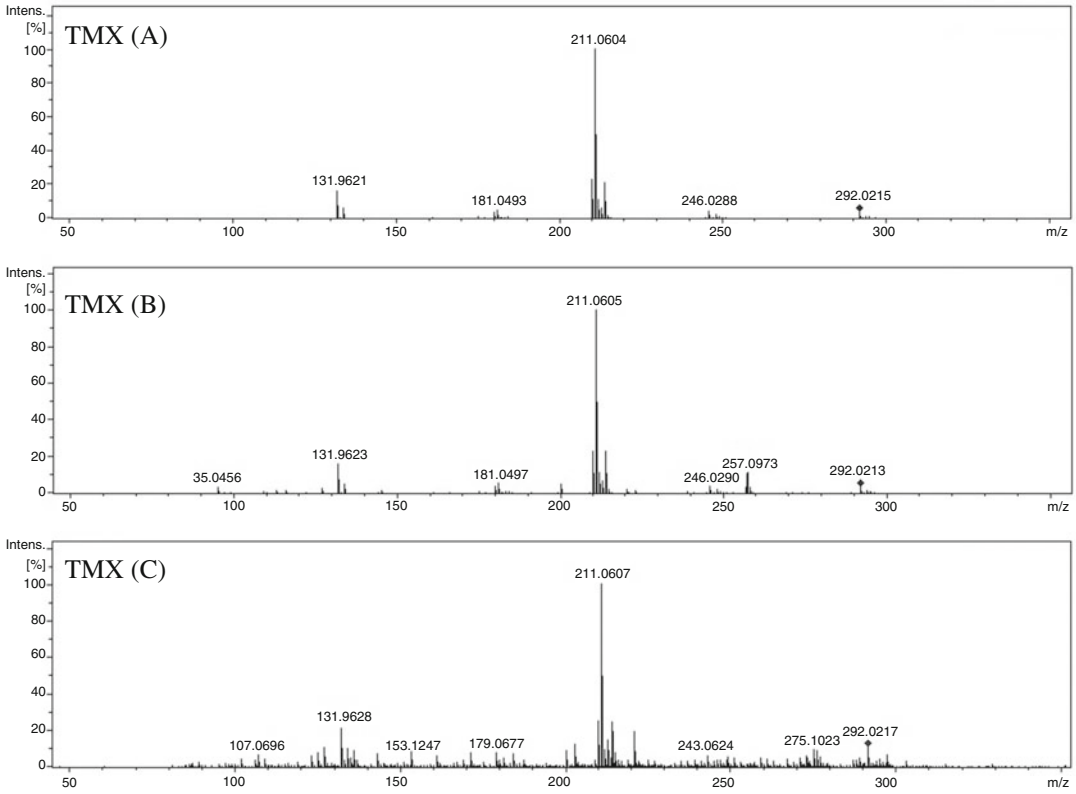

Fig. 2 Full-scan ESI-MS/MS spectra of TMX (A) standard solution (150 μg/L); (B) spiked (50 μg/kg) LDS sample; (C) spiked (150 μg/kg) FRJ sample. It must be remarked that the neonicotinoids concentrations were the same in the standard and spiked samples according to the proposed sample treatment, and the unit conversion ESI-MS/MS conditions are summarized in Subheading 2.4 and Tables 3, 4 and 5. Reprinted from Journal of Food Composition and Analysis, 70, Silvia Valverde, Ana M. Ares, Mario Arribas, José L. Bernal, María J. Nozal, José Bernal, Development and validation of UHPLC–MS/MS methods for determination of neonicotinoid insecticides in royal jelly-based products, 105–113, Copyright (2018), with permission from Elsevier

CLO, and ACET-LDS; DN, CLO, and THIA-FRJ) and honey (ACET-multifloral honey; DN, NT, CLO, and IMI-heather honey). The absence of a significant matrix effect in most cases is a relevant finding as it is not usually achieved in previous publications and has the methodological advantage of allowing the quantification of NEOs with solvent-based calibration standard curves. It could be explained by the base line separation of all NEOs and the specific optimization of the sample treatment for these compounds and matrices, which is one significant advantage of specific methodologies in relation to multi-residue approaches. In addition, the results obtained for honey are also a relevant finding as it was demonstrated the need of evaluating the matrix effect for different honey botanical origins in order to avoid potential quantification errors, which is not usually evaluated when proposing new methods in this bee product.

3.3.4 Linearity

As it was previously commented, different calibration curves were used to quantify NEOs depending on the bee product. Matrix-matched standard calibration curves should be used to quantify NEOs in the cases in which the matrix affected significantly the MS/MS signals (*see* Subheading 3.3.3); while solvent-based calibration standard curves could be employed in all other cases. The linearity ranges were quite similar for all the bee products, ranging from LOQs to 500 μg/kg. It must be also commented that the graphs obtained in all the calibration curves were straight lines, with linearity across the different concentration ranges studied; the coefficient of the determination values (R^2) was higher than 0.99 in all cases (*see* Table 6). The deviation of the back-calculated standard concentrations was equal to or less than 5% in all cases from the nominal values. Absence of bias was confirmed by a *t* test and by studying the distribution of residuals.

3.3.5 Precision

Results, expressed as the percentage of relative standard deviation (%RSD), were at all times <10% (data not shown). Moreover, these values displayed no significant differences depending on the neonicotinoid or QC level. The results indicate that the proposed methods are precise enough according to existing European norms (% RSD $\leq$ 20; [25]). This is quite significant in the case of beeswax, as with this bee product it was not necessary the employ of an internal standard.

3.3.6 Trueness

As can be seen in Table 7, mean recoveries for NEOs ranged from 80% to 110% with %RSD lower than 10% in all bee products. These are quite good results as not only they are comparable or better than those reported in the related literature but also because they fulfilled the requirements established by the European Commission [25] for pesticide residue analysis (recovery percentages between 70% and 120%; %RSD $\leq$ 20). In addition, they were observed slight differences in those values according to the bee product or NEOs, but in any case, they were not statistically significant in most cases.

3.4 Sample Analysis

The validated methods were applied to determine potential residues of NEOs in samples from the different bee products (*see* Subheading 2.3, Table 8 and Fig. 3). All of these were analyzed in triplicate, and the internal standard was added at the same concentration as in the matrix-matched samples when required (bee pollen, honey, and royal jelly). Results showed that all the beeswax and bee pollen samples analyzed were free of residues of NEOs, and this was also observed in the commercial honey samples.

What is more, residues of IMI were found in only two of the 21 samples, but in both cases the amounts were below the LOD. In addition, TMX and CLO were detected in some honeys from

Table 8
Results of the investigation of NEOs in samples of different bee products

Bee product	Nº of samples	N° of samples with NEOs	NEOs detected	Minimum concentration (μg/kg)	Maximum concentration (μg/kg)
Bee pollen	20	0	No	ND	ND
Beeswax	21	2	IMI	<LOQ	<LOQ
Royal jelly	12	0	No	ND	ND
Honey	28	6	CLO, TMX	<LOQ	141

CLO clothianidin, *IMI* imidacloprid, *LOQ* limit of quantification, *ND* not detected, *NEOs* neonicotinoids, *TMX* thiamethoxam

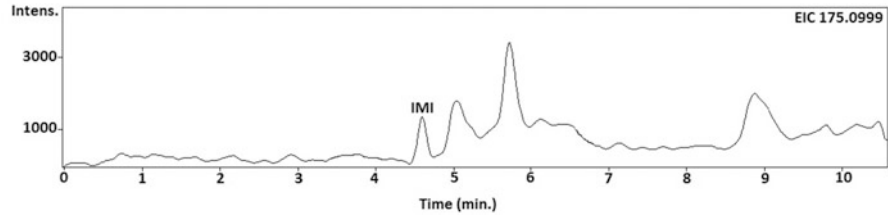

Fig. 3 Representative UHPLC-ESI-MS chromatograms (EIC in positive mode using the quantification ions, *see* Table 5) obtained from a beeswax sample with endogenous IMI content (< LOQ). The UHPLC-ESI-MS conditions are described in Subheading 2.4 and Tables 3, 4 and 5. Reprinted from Microchemical Journal, 142, Silvia Valverde, Ana M. Ares, José L. Bernal, María J. Nozal, José Bernal, Fast determination of neonicotinoid insecticides in beeswax by ultra-high performance liquid chromatography-tandem mass spectrometry using an enhanced matrix removal-lipid sorbent for clean-up, 70–77, Copyright (2018), with permission from Elsevier

experimental apiaries. TMX was detected in six samples, although it was quantified in only one (140 μg/kg); meanwhile, CLO residues were found in just one sample (40 μg/kg). TMX concentration was greater than the established MRLs for this compound (50 μg/kg). As already mentioned in several of the publications summarized in this study, the absence of NEO residues in most of the samples analyzed should not be seen as a reflection of a pointless exercise when developing specific methodologies to determine these compounds in the bee products in question. In fact, many articles have been cited in studies where residues have been detected in these same matrices but from different origins. This absence of residues can be seen as a positive result from the point of view of the consumer or beekeepers, since it signifies that some of these products are suitable for human consumption.

4 Conclusions

In this chapter, the most relevant data, findings, and conclusions of four recent studies by our research group have been summarized and discussed. Attention has been mainly focused on the most significant experimental details of these specific studies rather than on a deeper discussion of the related literature, as this has been already a feature of these publications and other chapters of this book. It has been demonstrated that there is a need for proposing specific methods to determine NEOs in each matrix. In this regard, the results of the experiments have shown that due to the different matrix components it has been necessary to choose different approaches, not only to extract the analytes but also to perform an adequate cleanup of the samples, thereby removing as many matrix components as possible so as to minimize their influence on an evaluation of NEOs. Therefore, various sample treatments have been selected for the different bee products. These include SPE (dark honey and royal jelly—LDS), QuEChERS (bee pollen and light honey), SE followed by dSPE (beeswax), and DLLME (royal jelly—FRJ). All these procedures have been shown to be efficient for NEOs extraction due to the very favorable recovery values obtained, and in several cases, they were good enough to minimize the matrix effect that usually affects the MS/MS signal in these matrices. As for chromatographic separation, we have demonstrated the practicality of a column (Kinetex® EVO) that was marketed at the same time as the studies described in this chapter began. In all cases, baseline separation of all the NEOs was achieved in a shorter time than those reported in previous publications for these same compounds and matrices. Furthermore, since the compounds were separated from each other, it allowed the methods to be used with less selective but cheaper detectors like DAD and UV-Vis. All the proposed methods were validated according to current legislation and proved to be selective, with low LODs and LOQS; these were in most cases better than existing values and much lower than existing MRLs, while also accurate, thanks largely to the use of an internal standard. Additionally, NEOs could be measured with solvent-based calibration standards in several cases, as the matrix did not significantly affect analyte ionization. This is a significant advantage compared with some of the previous proposals. Finally, the analysis of several samples from different Spanish regions revealed the absence of residues of NEOs in most cases, although it must be specified that residues of IMI, CLO, and TMX were found in some honey and beeswax samples.

To sum up, all the data, information, and arguments summarized in this chapter have an impact on the fact that we must not abandon the development of specific methods of analysis in relation to multi-residue methods. It is true that the latter allow more compounds to be analyzed in less time, but, on the other hand,

they tend to lead to a lack of separation, matrix effect, and lower recovery and precision values. Therefore, it would be interesting if both options were to be complementary and not exclusive, with the multi-residue method being used as an initial screening prior, if necessary, to evaluation with a more specific methodology.

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Chapter 3

Multiresidue Pesticide Analysis in Okra (Ladyfinger) Using Gas Chromatography Tandem Mass Spectrometry (GC-MS/MS)

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Abstract

The presence of pesticide residues in food and vegetables is a growing concern for consumers. In order to monitor these residues reliably, a selective and sensitive, multi-residue system has been developed and validated in okra (ladyfinger) by gas chromatography-tandem mass spectrometry (GC-MS/MS). The sample preparation procedure involves the extraction of the sample using the QuEChERS procedure with ethyl acetate, and the cleaning process involves the use of primary secondary amine (PSA) and the processing of two multiple reaction monitoring (MRM) transformations for each analyte by GC-MS/MS in electron impact (EI) mode. Precision and accuracy were tested by recovery studies. The process provides a cheaper and better alternative to the current multi-residue extraction techniques in the okra samples.

Key words Gas chromatography-tandem mass spectrometry, Pesticides residue analysis, Multiple reaction monitoring, QuEChERS

1 Introduction

Pesticides are used in plant growth of crops to increase plant production and eliminate plant diseases and infestations of insects [1]. Direct or indirect use of pesticides can cause pesticide accumulation inside the body, and it can cause serious diseases, even in small quantities, such as reproductive diseases, renal diseases, Alzheimer's disease, chronic cancer, and Parkinson's disease [2–7]. Since then, the possible public health consequences of pesticide residues have become a major issue [8]. Due to regional and dietary choices, fruit and vegetable use differs in various countries and even different regions, and pesticide types and residual quantities can also vary. As a consequence, pesticide residue detection is challeng-

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ing; there are several different varieties of fruit and vegetables matrix and also many pesticides. Pesticides' physical and chemical properties and low pesticide concentration require a high-performance, responsive system for examining pesticide residues in vegetables and fruits.

Techniques of gas chromatography, including flame photometric detector (FPD), nitrogen phosphorus detector (NPD) [9, 10], for liquid chromatography (LC) with ultraviolet detector (UVD) and fluorescent detector, LC [11, 12] have become popular methods for the quantitative evaluation of pesticide residues [13–15]. These techniques are, however, mostly used for a single form of pesticide samples; therefore, the amount of target analysis is restricted and the sensitivity cannot fulfil the detection of trace elements (ppb) and hence the pesticide multi-residue criterion for the high-performance and nontarget identification of the large batch samples cannot be met.

Various methods, such as solid–liquid extraction (SLE), solid-phase dispersion (SPD), and solid-phase extraction (SPE), have been employed in pesticide residue analysis [16]. The criteria for sample preparation have been simpler and much easier with the introduction of tandem mass spectrometry detectors. Developments in pesticide residue analysis today are targeted at reducing sample size, solvent use, and minimal cleaning measures.

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method developed by Annastasiades and Lehotay [17], because of its flexibility, is one of the most widely used methods for the analysis of pesticide residues. QuEChERS with slight modifications was used in herb, spice, complex pigment matrices, and secondary metabolites for the study of pesticide residues [18]. Changes to this method have also been used to evaluate chemical residues in chamomile, medicinal plants found in China and other botanicals [19]. The initial QuEChERS method has been confirmed for the *Calendula officinalis* inflorescences [20] analysis of 24 pesticides. A citrate buffered form of the QuEChERS method was used in the evaluation of pesticide residues in *Cannabis sativa* by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [21]. Addes a solid-phase extraction cleaning system for the purification of acetonitrile extracts, along with primary secondary amine (PSA) and graphitized carbon black (GCB) cartridges, and introduced this procedure to the study. In recent years, another unbuffered QuECHERS system for analyzing carbamate residues in different vegetables without using PSA in the cleanup process [22] was presented.

In this study, the development of a multi-residue system on pesticide residues in okra was investigated using QuEChERS sample preparation methods and by the sensitivity in combination with

the gas chromatography and triple quadrupole detector. The goal of the work was to find an efficient routine analysis tool. The final methodology chosen has been tested extensively.

2 Materials and Reagents

Pesticides used as certified reference standard materials (CRM) (Table 1) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Solid-phase extraction sorbent primary secondary amine cartridges (40 μm, Bondesil PSA) were obtained from Agilent Technologies (Bangalore, India).

Merck (India) supplied reagent-grade anhydrous magnesium sulfate and sodium acetate.

Ethyl acetate and glacial acetic acid have been obtained from Sigma-Aldrich (Bangalore, India) for the study of pesticide residues. Fresh okra (Lady Finger) samples (2 kg) were obtained from the farmers' field in Dharwad District (Karnataka state, India).

Table 1
Optimized GC-MS/MS parameters

Gas chromatography	Agilent 6890N with Autosampler (7683)
Mass spectrometry	RAB120 Waters, Boston, USA (Triple Quadrupole)
Software	MassLynx v4.2 version
GC column	HP-5MS (length: 30 m, 0.25 mm i.d, 0.25 μm)
Carrier gas	Helium (purity 99.999%)
Flow rate	1.3 mL/min.
Injector temp.	280 °C (splitless)
Vol. of injection	1 μL
Oven temp programming	50 °C—1 min, 25 °C/min—150 °C
	10 °C/min—280 °C (hold time 4 min)
Mode	Multiple reaction monitoring (MRM)
Interface temp.	250 °C
Source	Electron impact (EI+)
Source temp.	250 °C
Total run time	22 min
Electron energy	70 eV
Collision gas	Argon (purity 99.999%)
Collision gas pressure	$3.5 \times 10\ e^{-3}$
Source penning	$1.75 \times 10\ e^{-3}$

3 Instrumentation

(a) GC-MS/MS instrument: Auto sampler attached to Agilent 6890N (gas chromatograph) was used in the study of the pesticides with the Quatro MicroRAB120 (Waters, Manchester, UK) Triple Quadrupole Mass spectrometer. MassLynx Solutions platform was used for instrument control and data processing.

(b) Chopper and homogenizer: Vegetable chopper was used for chopping, and the homogenizer (Heidolph, Schwabach, Germany) was used for proper addition of the samples.

(c) Centrifuge: Centrifuge (Sigma 3K 10) has been used with polypropylene tubes of 2 and 50 mL.

(d) Weighing balance: Reagents for the preparation of reference standards and to weigh the chopped samples (Sartorius, Goettingen, Germany), weighing balance is used.

(e) Low volume concentrator: Inert nitrogen has been used for Turbovap (Caliper Life Sciences, Hopkinton, Massachusetts, USA) solvent evaporation.

4 Methods

4.1 Preparation of Reference Standard Solutions

Specific stock solutions (1000 μg/mL) have been produced for each CRM, weighing 10 (±0.1) mg of CRMs dissolved into 10 (±0.1) mL of ethyl acetate in a colored volumetric flask. Stock solutions have been stored at −20 °C in a cold freezer. Working standards were prepared by diluting the existing stock solutions. For the construction of the calibration curve, serial dilutions were used.

4.2 Preparation of Sample

Okra samples (2 kg) from Dharwad District (Karnataka State, India) were collected. This area is renowned for its better-quality okra. These collected samples were placed in a deep freezer at −5°C before further sample preparation. Samples of okra (0.5 kg) had been cut and homogenized, and 10 g aliquots were extracted with acetonitrile and 0.1% acetic acid (10 mL), 1.0 g sodium acetate, and 5 g magnesium sulfate were used in this study. Samples were centrifuged at 419.25 × *g* for around 3 min. For a 2 mL extraction tube consisting of 1 mL of acetonitrile extract with 150 mg magnesium sulfate and 50 mg PSA (primary secondary amine) has been added. It was then centrifuged for around 5 min at 1677 × *g*. Supernatant was moved to a 1 mL vial and filtered using a 0.2 μm filter paper. Fresh organic okra samples were used as blanks.

4.3 GC-MS/MS Analysis

Optimization was done using gas chromatography with autosampler and a mass spectrometer, and Table 1 shows the optimized GC-MS/MS parameters.

4.4 Validation Study

A single laboratory approach was used to satisfy the validation criteria, in this method.

The following validation criteria have been applied:

(a) Linearity: The calibration curve was developed using pure solvent and matrix using five calibration points between 1 and 200 ng mL and analyzed in triplicate.

(b) Selectivity: The noise reduction was determined at the compounds retention times by fixing two MS/MS transitions for each analyte considering precursor and product ions.

(c) Sensitivity: The detection limits (LOD) were calculated by a peak signal from analyte concentration to triple background chromatogram noise. The LOQ is the lowest concentration, presenting adequate accuracy and recovery. The ion ratio (Q/q) was used to assess positive samples. Q/q is the confirmation transition (q) and intensity (Q) quantification (Table 2).

5 Results and Discussion

5.1 Gas Chromatography (GC) Oven Optimization

The GC oven method was optimized to isolate pesticide molecules with a sharp peak, lower matrix interference, high resolution, and high sensitivity (S/N). At the beginning, the temperature was fixed to about 50 °C, then raised to 25 °C/min from 50 to 150 °C, which in turn shortened the compounds retention time. Subsequently, the oven temperature was raised to 280 °C at a rate of 10 °C/min. It proved to be helpful in getting a larger S/N ratio and good shape for all the compounds such as, parathion, malathion, pendimethalin, and quinalphos. The holding time of 4 min at 280 °C helped in the separation of co-eluting cypermethrin, cyfluthrin, fenvalerate, and deltamethrin. If GC-MS/MS full-scan mode (50–500 m/z) was to be used because peaks overlap, the identification of pesticides may have been challenging. In MS/MS mode, however, the confusion in identification and separation was prevented because of the unique MRM of pesticide specific transition.

5.2 Optimization of MS/MS

MS/MS optimization was applied using an EI+ ionization mode using ethyl acetate as a solvent. Three phases of optimization, i.e., precursor (parent) ion separation, ion excitation, then product dissociation, and scanning were conducted in a particular mass range [23]. The retention time (Rt) was established for each analyte to optimize MS/MS. Typically, after full scanning of each pesticide, the precursor ion was the base peak of the mass spectrum and the product ion was the next intense line of the mass spectrum. Collision energy (between 4 and 40 eV) was tuned to know the splitting sequence after the ion was formed. Analysis was specifically proposed to build an MRM with dual MS/MS transitions.

Table 2
Average percentage recoveries (% RSD) of fortified pesticides in okra (ladyfinger) from the QuEChERS extraction method with GC-MS/MS analysis

			MRM				Fortification levels (mg/kg) Okra			
Sample number	Reference standards	Rt (minutes)	Precursor ion (*Q*)	Product ion (*q*)	CE (V)	LOD (mg/kg)	0.01	0.05	0.1	0.5
1	DEET	7.06	119	65	21	0.001	95(5)	88(2)	95 (6)	91 (3)
2	Propiconazole	7.65	69	41	6	0.01	76(3)	79(10)	75 (13)	79 (10)
3	Phorate	7.85	260	75	5	0.002	90(4)	99(12)	89 (11)	84 (4)
4	Carbofuran	8.35	164	149	8	0.002	91(1)	98(9)	92 (0)	98 (3)
5	Atrazin	8.85	215	58	8	0.005	74(6)	86(8)	87 (12)	87 (4)
6	Lindane	9.04	184	145	10	0.001	86(8)	98(15)	91 (5)	96 (5)
7	Diazinon	9.74	179	137	17	0.0005	96(6)	89(1)	87 (3)	86 (6)
8	Chlorothalonil	9.95	266	133	26	0.004	82 (3)	84 (7)	98 (7)	97 (1)
9	Metalaxyl	10.37	206	59	8	0.002	86(4)	88(5)	93 (2)	92 (9)
10	Fenitrothion	10.64	125	79	11	0.002	92(7)	91(7)	84 (4)	83 (2)
11	Ethion	10.70	231	129	18	0.0001	97(6)	95(9)	90 (7)	85 (2)
12	Aldrin	11.54	263	193	22	0.003	87(13)	91(4)	98 (5)	90 (5)
13	Fenthion	11.99	278	109	12	0.005	99(5)	92 (4)	97 (4)	98 (1)
14	Chlorpyrifos	12.05	197	169	16	0.0005	88(4)	97(4)	98 (6)	96 (2)
15	Parathion	12.39	291	109	10	0.003	83(2)	90(5)	97 (14)	96 (2)
16	Triadimefon	12.77	208	181	6	0.006	99(3)	99(1)	95 (5)	99(3)
17	Pendimethalin	13.39	252	162	16	0.005	88(2)	96(1)	100 (3)	95(3)
18	Captan	13.95	79	51	20	0.002	90 (3)	84 (4)	84 (4)	83 (2)
19	Phenthoate	14.19	274	121	16	0.0005	97(5)	90 (2)	90(3)	99(0)
20	2,4-DDT	14.61	146	118	7	0.00001	84(3)	97(5)	97 (3)	88 (3)

21	Alfa-endosulfan	14.95	241	170	25	0.004	93(3)	98(9)	96 (5)	95 (3)
22	Butachlor	15.29	176	146	20	0.001	91(7)	93(6)	84 (4)	83 (2)
23	Profenofos	15.76	337	267	8	0.005	91(3)	96(10)	88 (9)	89 (2)
24	2,4-DDD	16.34	235	165	16	0.00001	94(2)	98(3)	97 (3)	100 (2)
25	Endrin	16.85	263	193	22	0.005	88 (5)	94(12)	88(2)	92(10)
26	Chlorfenapyr	17.15	247	75	17	0.02	99 (3)	94 (4)	97 (2)	82(4)
27	Beta-endosulfan	17.41	241	170	25	0.005	98(6)	84(5)	95 (4)	91 (2)
28	Quinalphos	17.87	235	165	15	0.003	93(1)	86(10)	95 (8)	95 (4)
29	Malathion	17.96	173	99	10	0.003	96 (14)	96 (1)	93 (1)	92(2)
30	Triazophos	18.72	161	77	19	0.005	91 (9)	98 (1)	96 (3)	92(4)
31	Iprodione	18.91	314	245	10	0.02	83 (10)	93 (5)	90 (4)	88(2)
32	Beta-cyfluthrin	19.63	165	127	5	0.01	90(1)	98(1)	86 (14)	85 (7)
33	Alpa Cypermethrin	20.17	163	127	6	0.005	92(2)	96(1)	90 (3)	91 (4)
34	Fenvalerate	20.72	167	125	8	0.005	89(3)	98(1)	97 (3)	88 (3)
35	Deltamethrin	21.73	181	152	18	0.008	90(3)	99(0)	97 (3)	93 (4)

(*Rt* retention time, *MRM* multiple reaction monitoring, *CE* collision energy, *LOD* limit of detection)

For each pesticide, more intensive product ions have been selected for quantitative purpose. Product ions with the next intense ion were used to confirm the pesticide compound. The product ion spectrum was held very low to determine the best S/N ratio. Table 2 indicates the product and precursor ions for conformation and/or qualitative transitions detected. For both transitions, optimized collision energy levels were kept at 5–35 eV. To obtain chromatographic peak shape and better sensitivity, dwell time for each analyte is held at 0.1 s. For measuring the Q/q ratio, matrix-matched standards were used at five different concentration levels, with RSDs usually below 16%.

5.3 QuEChERS Procedure for Extraction

In 2003, Anastassiades et al. implemented QuEChERS in order to track the levels of residue of pesticides in fruits [17]. It consists of acetonitrile as solvent for pesticide residue extraction as well as Na_2SO_4 (anhydrous) and $MgSO_4$ (anhydrous) for the good separation of acetonitrile extract and water. Initially, the extract has been added with the PSA (primary secondary amine) and is called dispersive solid-phase extraction. PSA was effectively excluded from the polar matrix, for example, organic acids and color pigments found in the matrix. This slight adjustment to the method was incorporated as the official AOAC International and the European Standard Organization (CEN) form [17, 24]. The QuEChERS method has therefore well-established and very convenient to use.

5.4 Recovery Experiments of Spiked Samples

The extraction and cleaning method typically eliminates the matrix co-extracts and removes all analytes from the matrix. During the pesticide residue analysis, the same does not apply in matrices. Practical recovery experiments have since been performed on okra samples. Table 2 sums up separate peaks with their Rt (retention times). The recovery of specific pesticides with various spiking concentrations with replicates was evaluated using the linear regression method in the okra sample matrix. Table 2 determines the average recovery rates for all spiked pesticides in okra samples for each spiked level. Total 35 pesticides analyzed had a reasonable recovery rate between 74 and 100%. To define reproducibility, RSD (relative standard deviation) was used and most RSD values were found to be below 16%.

5.5 Method Performance

The efficiency of this process was found to be very satisfactory. The MS/MS method (Fig. 1) separated pesticides that are normally problematic to resolve chromatographically due to problems of co-elution.

The linearity (straight line) of this method was tested in addition by creating solvent calibration curves and matrix standards for an $R^2 > 0.998$ for each pesticide (Fig. 2). All the pesticides have been recovered from 74 to 100% and RSD below 16%. Confirmed

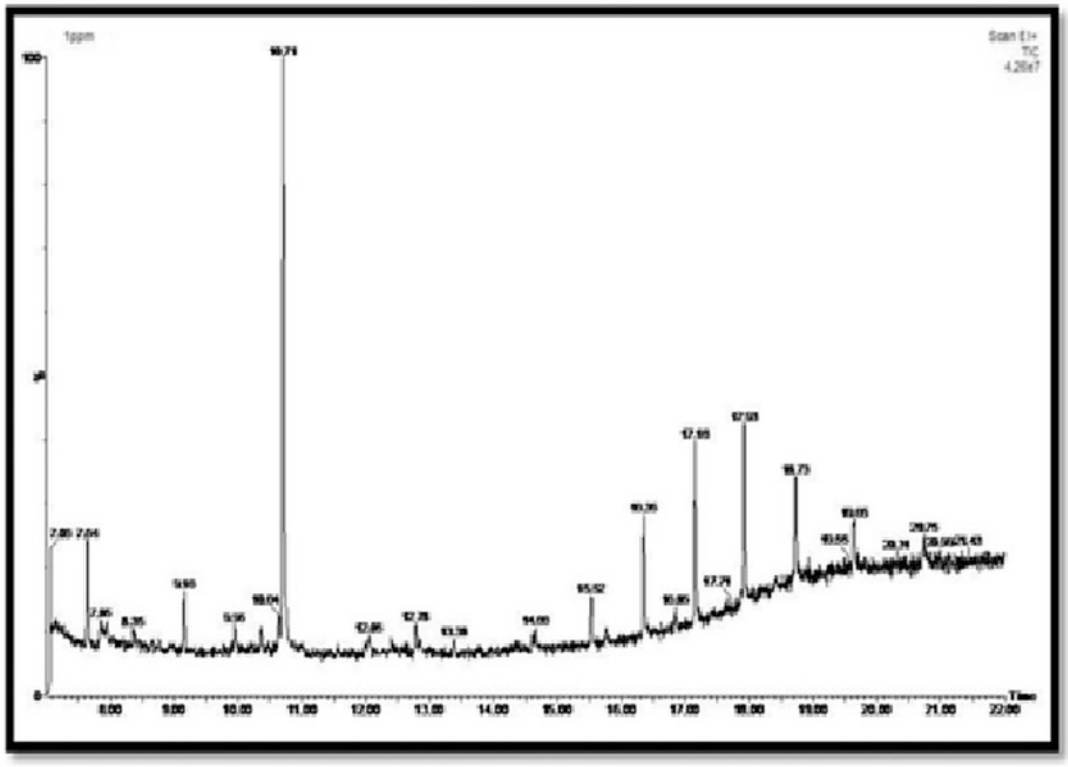

Fig. 1 Typical total ion chromatogram of certified reference standards

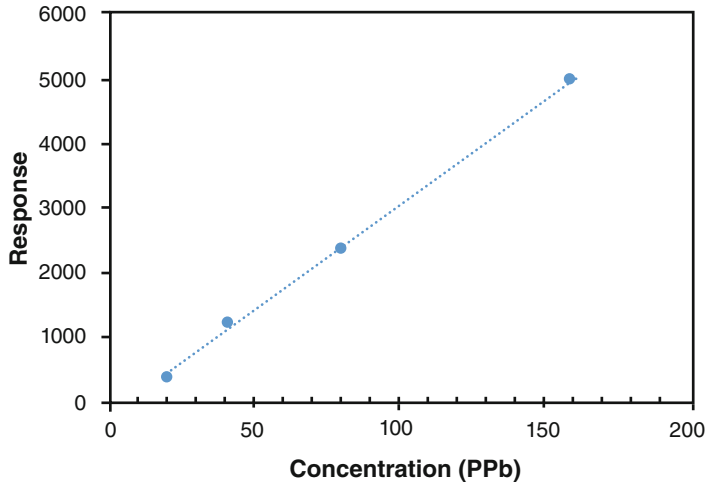

Fig. 2 Malathion linearity over the concentration range of 20–160 ppb

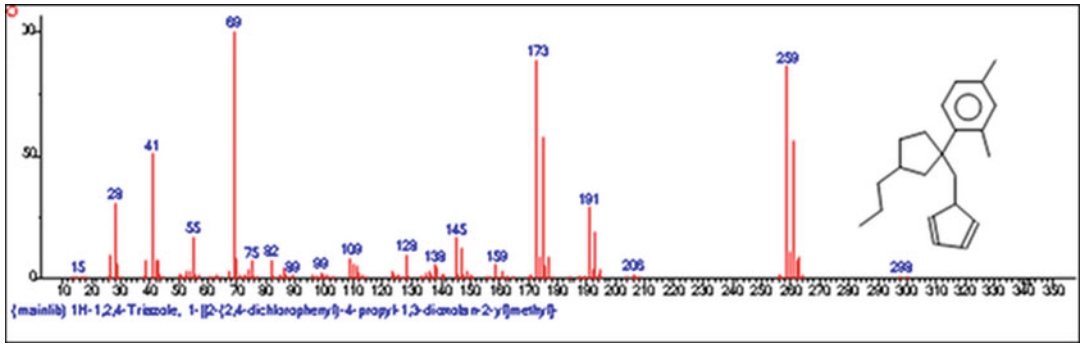

Fig. 3 Typical mass spectrum of quinalphos

Table 3
Results of okra sample analysis ($n = 20$) collected from Dharwad district

S. no.	Name of the pesticides	MRLs exceeded in samples	Residue content (ppm)	EU MRLs (ppm)
Okra (Lady Finger)				
1	Phosalone	6	0.04	0.01
2	Dimethoate	4	0.10	0.01

(*MRL* maximum residue limit, *ppm* parts per million, *EU* European Union)

residues for pesticides detected were justified on the grounds of the accuracy of the MRM target ratio (Decision 2002/657/EC of the European Commission of 12 August 2002).

When the samples were analyzed in the GC-MS/MS full scan mode, only the qualitative data were generated that fit with the NIST library (Fig. 3).

5.6 Applicability of the Developed Method

Samples of okra were collected from farms in the Dharwad District (State of Karnataka, India). These areas are known for Okra growing and excessive pesticide use. The established analytical method was used to determine pesticide residues in the okra samples and checked in triplicate. The outcomes showed that the okra samples have residues of pesticides above the required level. In the okra samples, phosalone and dimethoate (Table 3) are present above MRL level. Okra, which was examined in this report, contributed mainly to the major dietary intake of people in India.

6 Conclusion

Pesticide-contaminated okra is a major public health concern. An effective method of detecting harmful pesticides is also necessary. Thus, a multi-residue method for simultaneous detection and

quantification of 35 pesticides in the okra samples was developed and validated. The multiclass pesticide residues were detected by GC-MS/MS with a triple quadrupole analyzer. Within 22 min of running time, a greater sensitivity separated all narrowly eluted and co-eluted peaks. The two MRM transformations, one for quantification and the other for confirmation, achieve excellent sensitivity and selectivity for possible safe pesticide identification using the Q/q ratio factor. The detection limit was less than the MRL. Solid-phase extraction by solvent acetonitrile was used. Finally, two concentrations for the okra sample were successfully tested: 0.01–0.05 mg/kg and 0.1–0.5 mg/kg. The established approach tends to minimize analysis expenses and also shows low uncertainty measurements. This technique was also used to study real-world okra samples.

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Chapter 4

Analytical Methodologies for Neonicotinoid Determination in Bee Products

Silvia Valverde, Ana M. Ares, María J. Nozal, and José Bernal

Abstract

Neonicotinoids are systemic insecticides of great importance for agriculture due to their powerful activity against pests and insects. However, concerns regarding the side effects on health and the environment of this family of insecticides continue to increase, since these can then be transferred to the environment and the food chain, with potential adverse consequences for nontargeted organisms such as bees. In fact, exposure to neonicotinoids has been identified as one of the factors involved in the sudden decline in the bee population, and for this reason, numerous studies have been published relating to their extraction and determination in bee products (honey, beeswax, bee pollen, royal jelly, nectar, and bee bread). Therefore, the main goal of this chapter is to present an overview of the analytical methodologies generally employed to determine neonicotinoid insecticides and related compounds in bee products during the last 10 years (2010–2020), as this could help to facilitate their assessment. The layout of the chapter is in accordance with the different bee products, indicating and discussing the most common sample treatments and evaluation methods used to determine neonicotinoids in each of them. A list of some of the most relevant applications is provided for each bee product. The references included will provide the reader with a comprehensive overview of and insight into the analysis of neonicotinoid insecticides in bee products.

Key words Analytical methods, Bee products, Chromatography, Insecticides, Mass spectrometry, Neonicotinoids, Sample treatment

Abbreviations

ACN	Acetonitrile
CIAME	Cold-induced aggregation microextraction
DAD	Diode array detector
DCM	Dichloromethane
DI	Dilution
DLLME	Dispersive liquid–liquid microextraction
DPX	Disposable pipette extraction
dSPE	Dispersive solid-phase extraction
EMR-lipid	Enhanced matrix removal-lipid
ESI	Electrospray ionization
EV	Evaporation

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FLD	Fluorescence detector
GCB	Graphitized carbon black
HPLC	High-performance liquid chromatography
IL	Ionic liquid
IT	Ion trap
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NEOs	Neonicotinoids
PDA	Photodiode array
PSA	Primary secondary amine
QqQ	Triple quadrupole
QTOF	Quadrupole time-of-flight
QTRAP	Triple quadrupole linear ion trap mass spectrometer
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RI	Refractive index
SFC	Supercritical fluid chromatography
SQ	Single quadrupole
SULLE	Sugaring-out-assisted liquid–liquid extraction
UHPLC	Ultra-high-performance liquid chromatography

1 Introduction

Honeybees (*Apis mellifera*) play a fundamental role in the pollination of wild crops and plants [1]. In addition to improving the quality of crops, they also generate economic activity since bee products, like honey, beeswax, bee pollen, and royal jelly, are increasingly in demand. Since the year 2000, a decrease has been detected in both the population of bees and in the number of hives. This phenomenon, initially known as colony collapse disorder (CCD), is characterized by the worker bees going out to collect pollen, in order to feed the hive, but not returning to it, as a result of which the colony becomes weak. Besides the fact that the workers do not return, greater losses of bees are produced by the invasion of other insects, such as the Varroa mite or microsporidia like Nosema ceranae, which are lethal when the hive is weakened [2]. It has been recognized that CCD is a multifactorial phenomenon that may be due to either pests affecting the hive, such as those mentioned above, bad manipulation of hives by beekeepers, or the use of pesticides in crops visited by bees, such as neonicotinoids (NEOs) [3]. NEOs are systemic insecticides of great importance for agriculture due to their powerful activity against pests and insects belonging to the family *Heteroptera*, *Coleoptera*, and *Lepidoptera* [4]. They are neurotoxic substances that act selectively as the nicotinic receptor antagonist acetylcholine from the insect's central

nervous system, which activates the neurotransmitter acetylcholine. Acetylcholine is involved in several central functions, among which predominate voluntary control of movement, memory, affecting, for example in the case of bees, the routines to and from the hive, attention, and the ability to feed. In other words, it is associated with changes in the daily behavior of the bee, thereby hindering the survival of the colonies. Due to their systemic and permanent properties, NEOs are capable of contaminating plants and trees surrounding treated crops [5] and may permeate from groundwater and contaminate other plants and crops. Pollinating insects, then, are likely to be exposed to multiple sources of NEOs. In this regard, the European Commission has severely restricted the use of plant protection products and treated seeds containing three of these NEOs (clothianidin, imidacloprid, and thiamethoxam) to protect honeybees [6]. Since the identification of exposure to NEOs as one of the factors involved in the sudden decline in the bee population, numerous studies have been published in the last few years relating to the extraction and determination of these insecticides in different bee products such as honey, beeswax, bee pollen, royal jelly, nectar, or bee bread (*see* Fig. 1). As this issue is a global concern, it is not surprising to notice the large list of countries from which these studies originate (*see* Fig. 2).

As can be concluded from Fig. 3, NEOs have been mainly studied in honey, followed by beeswax and bee pollen; they have, however, received less attention in other bee matrices like, royal jelly, nectar, or bee bread.

NEOs have generally been measured in bee products by high-performance liquid chromatography (HPLC) techniques, such as high-performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC), or nano-high performance liquid chromatography (*see* Fig. 4). This is due to the thermolability, low volatility, and high polarity of those compounds. Also, HPLC is gaining attention because of the greater degree of resolution and sensitivity attained as well as the shorter running times in comparison with HPLC. Other techniques, such as supercritical fluid chromatography (SFC) or capillary electrophoresis (CE), have rarely been employed.

HPLC analyses have usually been performed in reverse-phase mode with C_{18} columns, coupled with mass (MS) or tandem mass spectrometry (MS/MS) detectors. MS/MS has been predominant in the last few years due to its excellent performance in terms of sensitivity, selectivity, and robustness, as well as reliable identification and quantification of the analytes. In order to achieve accurate and reliable analytical data, an efficient sample treatment is required prior to determining residues of NEOs in bee products, even when sensitive and selective detection systems like MS/MS are used. As can be observed in Fig. 5, solvent extraction (SE) and solid-phase

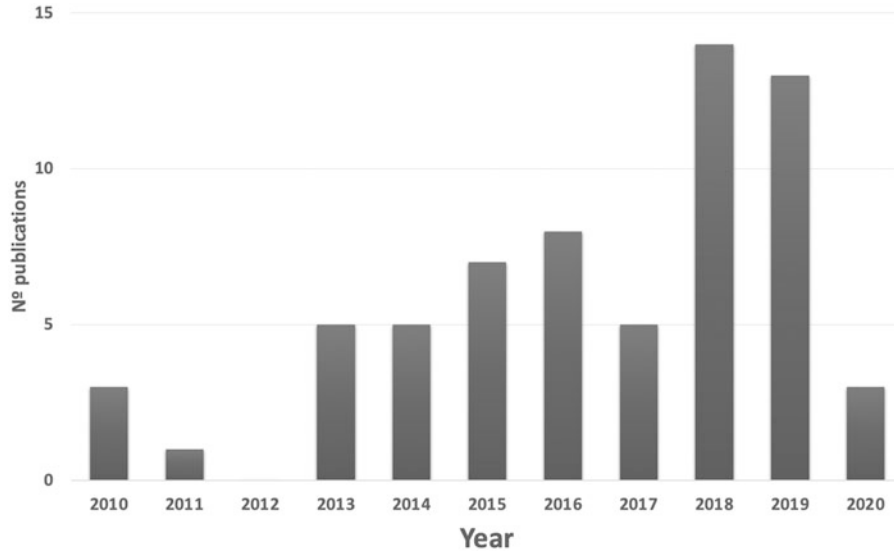

Fig. 1 Evolution of the published works in the last years (2010–2020) in the analysis of neonicotinoids in bee products. The sources of information were the databases ISI-Web of Knowledge and Scopus. The search has been done using as keywords. [(Bee products) or (honey) or (beeswax) or (bee pollen) or (nectar) or (royal jelly)] and [(neonicotinoids) or (insecticides) or (pesticides) or (determination) or (analysis)] among several others

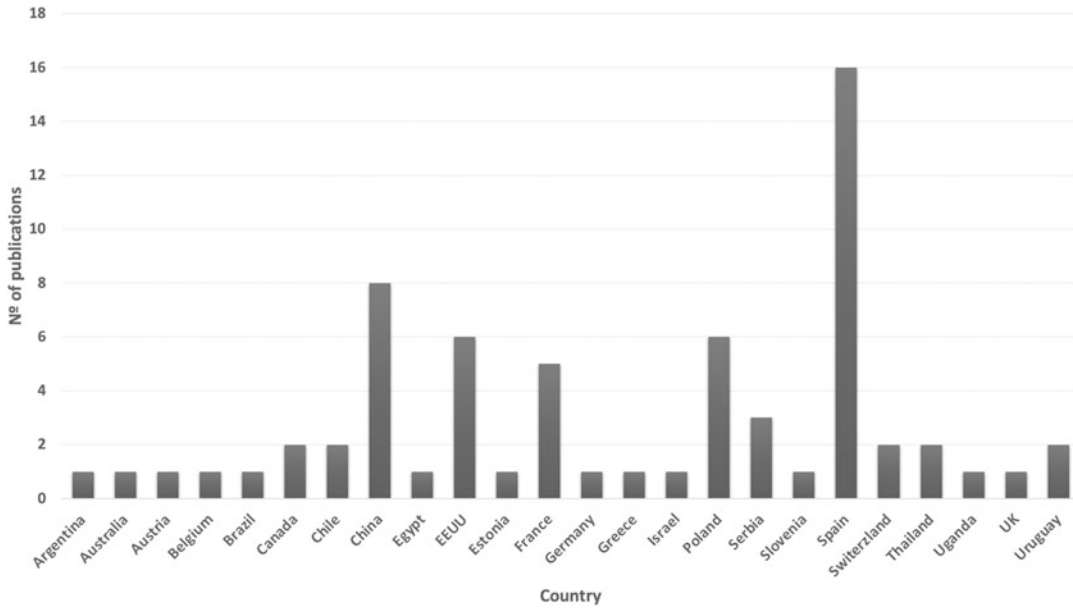

Fig. 2 Summary of the number of publications per country related to the determination of neonicotinoids in bee products in the last years (2010–2020)

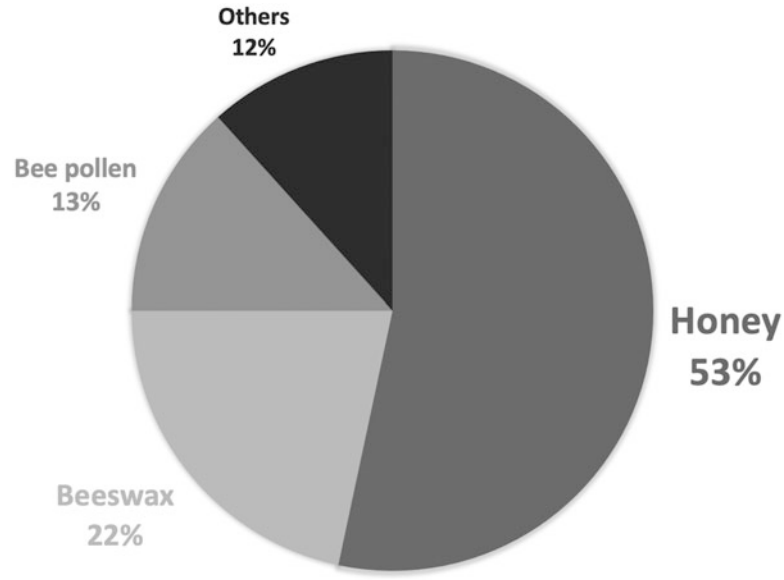

Fig. 3 Summary of the bee products in which neonicotinoids were predominantly determined in the last years (2010–2020)

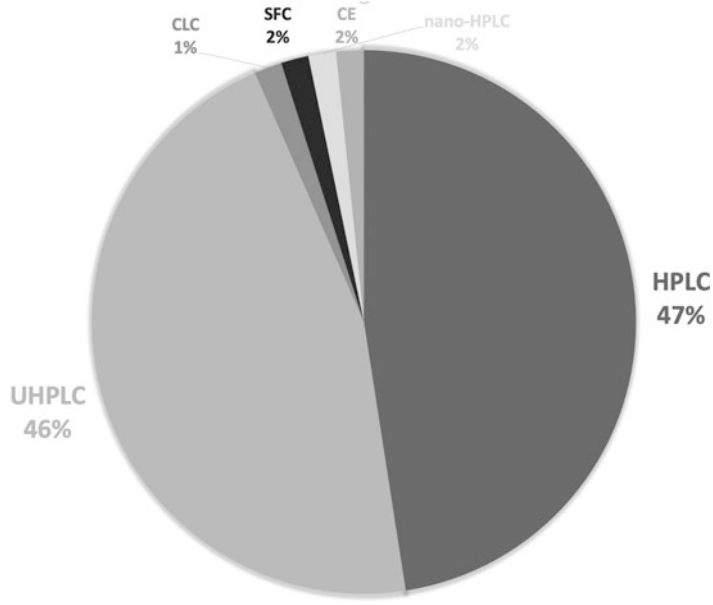

Fig. 4 Summary of the determination methods/techniques used to determine neonicotinoids in bee products in the last years (2010–2020). *CE* capillary electrophoresis, *CLC* capillary liquid chromatography, *HPLC* high-performance liquid chromatography, *nano-HPLC* nano-high performance liquid chromatography, *SFC* supercritical fluid chromatography, *UHPLC* ultra-high-performance liquid chromatography

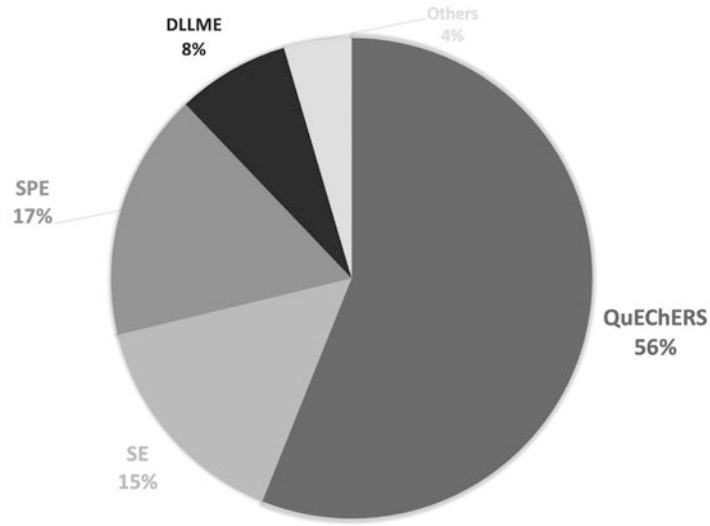

Fig. 5 Summary of the sample treatments employed to determine neonicotinoids in bee products in the last years (2010–2020). *DLLME* dispersive liquid–liquid microextraction, *QuEChERS* Quick, Easy, Cheap, Effective, Rugged, and Safe, *SE* solvent extraction, *SPE* solid-phase extraction

extraction (SPE) have been used in several studies, although these usually require huge amounts of solvents or they are slower.

It is not, therefore, surprising that the sample treatment known as quick, easy, cheap, effective, rugged, and safe (QuEChERS) has recently attracted the interest of many researchers. Dispersive liquid–liquid microextraction (DLLME) and other sample treatments have been also employed in some cases. Several interesting reviews focusing on an analysis of pesticides/insecticides in foods have been published in the last years [7–12], but only a few of them specifically focused their attention on NEOs and/or bee products [11, 12]. In the former study [11], a discussion took place of the methods of analysis developed/published up to 2018 for measuring NEOs in the agri-food sector. A large number of texts were reviewed and discussed, and the main method parameters of some of them were summarized in tables. However, the chapter does not undertake a specific discussion of bee matrices, which is a relevant issue when developing sample treatments. Meanwhile, the most recent publication [12] is specifically devoted to discussing the most commonly employed analytical methodologies for determining NEOs in bees and bee products. In this text, the authors mainly focused their attention on sample treatments. They gave a detailed explanation of the main characteristics of the most relevant ones, summarizing some of these in tables, but without considering/discussing the differences in such procedures in terms of the bee matrix; this is of great importance when investigating honeys from different botanical origins. In addition, little attention was paid to

separation and identification techniques. The current chapter presents the different analytical methodologies that have been reported for determining NEOs in bee products, paying special attention to assessing the techniques described in the publications of the last 10 years (2010–2020). The chapter is laid out according to the different bee products (honey, beeswax, bee pollen, and other products). Those readers who are interested in more specific aspects relating to toxicity of NEOs, extraction/separation methods in other matrices or from previous years, can consult some of the previously mentioned texts.

2 Honey

Honey, one of the most commonly used products of the hive, is a natural, unprocessed, and easily digested food. It is a natural product of great value due to its characteristic taste, nutritional value, and therapeutic applications. This has led to a significant increase in its consumption in the last years [13]. However, food alerts caused by detected pollutants such as pesticides have recently affected its healthy image, as these could represent a potential risk to consumers [14]. The presence of these contaminants may be due to direct contamination from beekeeping practices as well as to indirect contamination from environmental sources. In order to evaluate accurately and reliably NEOs in honey, it is usually necessary to carry out a preconcentration and/or separation step prior to identification and quantification. Nowadays, the current trend in sample preparation techniques is focused on simplifying these procedures to reduce costs, the number of reagents, and the time spent on these steps [15]. Thus, in recent years, the sample preparation known as quick, easy, cheap, efficient, resistant, and safe (QuEChERS) has commonly been employed for extracting NEOs from honey (*see* Table 1). This method is generally based on liquid–liquid partition with acetonitrile followed by a cleaning step by dispersive SPE (d-SPE), with different compounds such as primary secondary amine (PSA), C_{18}, or $MgSO_4$ [15]. As can be seen in Table 1, more than half of the summarized sample treatments were based on QuEChERS methodology [13, 15, 17–34] and employed the abovementioned most conventional solvents and reagents. However, it was usually necessary to dilute with water prior to conducting the QuEChERS protocols or to use mixtures of ACN with water or aqueous solutions in the partition step in order to facilitate sample treatment; this was because of the viscosity of the honey, which could be related to the fact that it is considered a highly concentrated sugar solution (mainly fructose). However, some authors have proposed alternative QuEChERS protocols with certain modifications, for example, removing the dSPE stage [15, 16] or including a freezing step [26], which could be helpful for

Table 1
Applications in the analysis of neonicotinoids in honey

Analytes (Multiresidue method)	Botanical origin (Specific method)	Sample treatment	Characterization method (SP, ISO, MSA)	Recoveries (Matrix effect, specific validation)	References
5, 6 (Yes)	MF	DI (water) + QuEChERS (ACN; PSA)	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	91–113% (No, No)	[13]
1–7 (No)	MF, RO, HT (Yes)	[1]DI (water) + QuEChERS (ACN:EA; NO dSPE) + EV [2]DI (AFO) + SPE + EV (Strata® X)	[1]UHPLC-MS/MS (C_{18}, ESI+, QTOF) [2]UHPLC-MS/MS (C_{18}, ESI+, QqQ)	[1]80–109% (Yes, Yes) [2]80–108% (No, Yes)	[15]
1, 3–5 and MET (No)	NS (NS)	DI (water) + QuEChERS (TEA:ACN; NO dSPE) + SPE (C_{18}) + EV	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	35–124% (NS, NS)	[16]
1–7, 9 and MET (No)	NS (NS)	[1]SE (MeOH: water) + SPE (DE) + EV [2]DI (water) + QuEChERS (ACN; $MgSO_4$ and PSA) + EV	HPLC-MS/MS (C_{18}, ESI+, QqQ)	[2]60–114% (Yes, No)	[17]
3–7 (Yes)	AC, CH, MF, RS (No)	DI (water) + QuEChERS (ACN; $MgSO_4$ and PSA) + EV	HPLC-MS/MS (C_{18}, ESI+, QTRAP)	65–127% (NS, No)	[18]
3–7 (No)	NS (NS)	DI (water) + QuEChERS (hexane and ACN; $MgSO_4$ and PSA) + EV	HPLC-MS/MS (C_{18}, ESI+, QqQ)	79–90% (Yes, NS)	[19]
1–7 (No)	AC, LD, SF, WF (No)	[1]DLLME (ACN, DCM) + EV [2]QuEChERS (ACN; $MgSO_4$ and PSA) + EV	HPLC-DAD (C_{18}, NR, NR)	[1]78–116% (Yes, No) [2]78–97% (Yes, No)	[20]
1 and MET (No)	NS (No)	DI (water) + QuEChERS (ACN:AA; PSA, GCB and C_{18})	SFC-MS/MS (Trefoil AMY1 3.0, ESI+, QqQ)	78–100% (Yes, NS)	[21]

(continued)

Table 1
(continued)

Analytes (Multiresidue method)	Botanical origin (Specific method)	Sample treatment	Characterization method (SP, ISO, MSA)	Recoveries (Matrix effect, specific validation)	References
7 (Yes)	EU, WF, NS (No)	DI (water) + QuEChERS (ACN:EA; $MgSO_4$, PSA, and florisil)	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	96–102 (Yes, No)	[22]
2–7, 9 (No)	AF, CA, WF (No)	DI (water) + QuEChERS (ACN; $MgSO_4$ and PSA) + EV	HPLC-MS/MS (C_{18}, ESI−, QqQ)	68% (NS, NS)	[23]
3, 5–7 (No)	NS (No)	DI (water) + QuEChERS (ACN; $MgSO_4$ and PSA)	HPLC-MS/MS (C_{18}, ESI+, QIT)	58–97% (Yes, No)	[24]
1, 3–7 and MET (Yes)	NS (No)	DI (water) + QuEChERS (ACN; $MgSO_4$ and PSA)	HPLC-MS/MS (C_{18}, ESI−, QqQ)	68% (NS, NS)	[25]
3, 5, 6 (Yes)	NS (NS)	DI (water) + QuEChERS (ACN, freezing) + DI	UHPLC-MS/MS (NS, ESI+, QqQ)	NS (NS, No)	[26]
5 (No)	NS (No)	DI (water: FA) + QuEChERS (ACN; $MgSO_4$, PSA and C_{18}) + EV	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	85–88% (No, No)	[27]
1–7 (Yes)	MF (No)	DI (water) + QuEChERS (ACN:FA; chitosan/Aluminum oxide/C8)	UHPLC-MS/MS (C_{18}, ESI+, QTRAP)	44–156% (Yes, Yes)	[28]
3, 5–7 (No)	MF, UF (No)	DI (water) + QuEChERS (ACN; $MgSO_4$, PSA and C_{18}) + EV	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	79–101% (NS, No)	[29]
5–7 (Yes)	NS (NS)	DI (water) + QuEChERS (ACN; $MgSO_4$, and PSA) + EV + DI	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	NS (NS, No)	[30]
2–7 and MET (Yes)	NS (No)	DI (water) + QuEChERS (ACN:FA; $MgSO_4$, PSA and ZSep+)	HPLC-MS/MS (PH, ESI+, QTRAP)	70–118 (Yes, No)	[31]
3–7 (No)	AC, CH, FO, LI, MF (No)	[1]DI (water) + SPE + EV (OASIS® HLB) [2]DI (water) + QuEChERS (ACN; $MgSO_4$ and PSA) + EV	HPLC-MS/MS (C_8, ESI+, QqQ)	[1]70–114% (Yes, No) [2]68–107% (Yes, No)	[32]

(continued)

Table 1
(continued)

Analytes (Multiresidue method)	Botanical origin (Specific method)	Sample treatment	Characterization method (SP, ISO, MSA)	Recoveries (Matrix effect, specific validation)	References
3, 5–7 (No)	MF, UF (No)	QuEChERS (ACN:AA; $MgSO_4$, PSA and C_{18})	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	NS (NS, No)	[33]
1–8, 12 (No)	NS (No)	QuEChERS (water: ACN; $MgSO_4$, PSA and C_{18})	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	73–95% (Yes, No)	[34]
3–7 (No)	MF, EU, OR, HT, RO (No)	DI (water) + SPE (DSC-18Lt) + DLLME (ACN, DCM) + EV	[1]HPLC-DAD [2]HPLC-MS/MS (C_{18}, APCI+, IT)	[1]NS (NS, Yes) [2]90–104% (Yes, Yes)	[35]
1–7 and MET (No)	NS (No)	SE (ACN, TEA and ethyl acetate) + SPE (strata® X-CW) + EV	HPLC-MS/MS (C_{18}, ESI+, QqQ)	89–112% (No, No)	[36]
3, 5–7 (No)	FO, LA, MF, RO (No)	DI (water) + SPE (Strata® X)	UHPLC-MS/MS (C_{18}, ESI+, QTOF)	79–105% (Yes, Yes)	[37]
1–7, 9, 11, 13 (No)	NS (No)	DI (water) + SPE (Oasis® HLB) + EV	HPLC-MS/MS (C_{18}, ESI+, QqQ)	72–107% (Yes, No)	[38]
1–7 (No)	NS (No)	DI (water) + DLLME (ACN, DCM) + EV	HPLC-MS/MS (C_{18}, ESI+, QqQ)	76–114 (Yes, No)	[39]
3–7 (No)	NS (No)	IS-DLLME (octanol and water)	HPLC-DAD (C_8, NR, NR)	97–108% (Yes, No)	[40]
1–7 (No)	MF, EU, OR, RO (No)	DI (water) + DLLME (ACN and DCM) + EV	CLC-DAD (C_{18}, NR, NR)	80–100% (NS, No)	[41]
5–7, 11 (No)	NS (No)	DI (water) + IL-DLLME ([C4MIM][Br], K[PF6])	UHPLC-UV (C_{18}, NR, NR)	81–111% (NS, No)	[42]
4–7 (No)	NS (No)	IL-CIAME ([C4MIM][PF6])	HPLC-PDA (C_{18}, NR, NR)	84 (Yes, No)	[43]
1–7, 11 (Yes)	NS (No)	DI (water) + DPX (Anion exchanger, QAE Sephadex A-25)	HPLC-MS/MS (C_{18}, ESI+, QqQ)	72–111% (No, No)	[44]

(continued)

Table 1
(continued)

Analytes (Multiresidue method)	Botanical origin (Specific method)	Sample treatment	Characterization method (SP, ISO, MSA)	Recoveries (Matrix effect, specific validation)	References
5–7 (No)	NS (No)	SULLE (water:ACN)	HPLC-PDA (C_{18}, NR, NR)	91–98% (No, No)	[45]

1 Dinotefuran, *2* Nitenpyram, *3* Thiamethoxam, *4* Clothianidin, *5* Imidacloprid, *6* Acetamiprid, *7* Thiacloprid, *8* Sulfoxaflor, *9* Flonicamid, *10* Cicloxaprid, *11* Imidaclothiz, *12* Flupyradifurone, *13* Pymetrozine, *AA* acetic acid, *AC* acacia, *ACN* acetonitrile, *AF* alfalfa, *AFO* ammonium formate, *APCI* atmospheric pressure chemical ionization, *BW* buckwheat, *CA* canola, *CD* common dandelion, *CF* cornflower, *CH* chestnut, *CIAME* cold-induced aggregation microextraction, *CL* clover, *CLC* capillary liquid chromatography, *DAD* diode array detector, *DCM* dichloromethane, *DE* diatomaceous earth, *DI* dilution, *DLLME* dispersive liquid–liquid microextraction, *DPX* disposable pipette extraction, *dSPE* dispersive SPE, *EA* ethyl acetate, *ESI* electrospray ionization, *EU* eucalyptus, *EV* evaporation, *FA* formic acid, *FO* forest, *GCB* graphite carbon black, *HPLC* high-performance liquid chromatography, *HT* heather, *IL* ionic liquid, *IS* in-syringe, *ISO* ionization source, *IT* ion trap, *LA* lavender, *LD* linden, *MeOH* methanol, *MET* metabolites, *MF* multiflower/multifloral, *MSA* mass spectrometry analyzer, *MS/MS* tandem mass spectrometry, *NR* not required, *NS* not specified, *OR* orange blossom, *PDA* photo diode array, *PH* phenyl hexyl, ***PHA*** phacelia, *PSA* primary secondary amine, *QqQ* triple quadrupole, *QTOF* quadrupole-time-of-flight, *QTRAP* triple quadrupole and linear ion trap, *QuEChERS* quick, easy, cheap, effective, rugged and safe, *RO* rosemary, *RP* rape, *SE* solvent extraction, *SF* sunflower, *SP* stationary phase, *SPE* solid phase extraction, *SULLE* sugaring-out liquid–liquid extraction, *TFA* trifluoroacetic, *UF* uniflower/unifloral, *UHPLC* ultra-high-performance liquid chromatography, *WF* wildflower

precipitating proteins and lipids and facilitating their removal, but in most cases the novel protocol involved selecting new compounds for the dSPE [21, 22, 28, 31]. For example, graphitized carbon black (GCB) was employed in combination with PSA and C_{18} for dSPE when determining dinotefuran and its metabolites [21]**.** The strong affinities of PSA for carbohydrates, C_{18} for nonpolar compounds, and GCB for pigments facilitated the cleanup process. Other authors selected florisil due to the interaction of the honey's sugars with the polar surface of this sorbent [22], with the aim of removing as many matrix components as possible; meanwhile, in a different study, six different dSPE sorbents (chitosan, C_8, C_{18}, silica gel, aluminum oxide, and florisil) were evaluated to test their ability to remove interfering matrix components from honey samples [28]**.** The results obtained showed that the combination of chitosan, aluminum oxide, and C_8 not only provided the best recoveries but also minimized the matrix effect for many compounds, compared with the other possible combinations of sorbents, some of them NEOs. This is a quite relevant issue, as the presence of a matrix effect implies the need to use matrix-matched calibration curves instead of solvent-based ones, which makes quantification more complex. It must be pointed out that the matrix effect was observed in most of the QuEChERS-based publications (*see* Table 1).

Finally, a different sorbent (Z-Sep+), which is a silica gel modified with C_{18} and zirconium dioxide group, was used in the dSPE together with PSA and $MgSO_4$, when investigating the presence of NEOs and 199 other pesticides in honey [31]. The cleanup mechanism of Z-Sep+ sorbent involves pH-independent Lewis acid and hydrophobic interactions. After dilution with water or an aqueous solution, honey can be extracted using protocols similar to those applied to water, such as SPE, as seen in several publications [15, 16, 32, 35–38]. The SPE procedure usually provides good results in terms of sensitivity, recovery, and matrix effect, although it also requires a significant cost regarding reagents and equipment, especially due to the SPE sorbents, when compared with QuEChERS protocols. Polymeric-based SPE sorbents like Oasis® HLB [32, 38], Strata® X [15, 37], and Strata® X-CW [36] have generally been employed in honey samples. Oasis® HLB is a copolymer macroporous sorbent based on poly(*N*-vinylpyrrolidone divinylbenzene) that has been selected in the abovementioned publications due to its superior overall performance (recovery, cleanup, matrix-effect) in comparison with two other SPE sorbents (C_{18} and Oasis MCX). Strata® X is an SPE sorbent that is composed of macroporous poly(styrene-divinylbenzene) with pyrrolidone groups; meanwhile, Strata® X-CW is a modification of the conventional Strata® X with carboxylic groups and which acts as a weak cation exchanger. These latter sorbents together with two others (C_{18} and Oasis® HLB) were checked for cleanup of honey samples [36], and in this case, the Strata® X-CW sorbent was selected due to the higher rate of recoveries and the absence of a matrix effect. C_{18}-based SPE sorbents were selected in two studies [16, 35], although in one of these works [16], the SPE step was used as a modification of a QuEChERS protocol in replacement of PSA. Dispersive liquid–liquid microextraction (DLLME) is a sample-preparation technique offering high enrichment factors from low amounts of water samples. It has attracted the attention of many researchers in the last few years, including those focusing on the analysis of honey samples [20, 35, 39–42], due its advantages in more conventional procedures such as simplicity, low cost, and simple method development, which has made it available to virtually all analytical laboratories [39]. In one of these studies, the authors compared the performance of a previously developed QuEChERS method with a DLLME proposal [20]. The results showed that although the matrix effect was observed in both protocols, in the case of most of the NEOs, samples cleaned up by the QuEChERS procedure exhibited a greater matrix effect than with the DLLME procedure. Moreover, lower limits of detection (LODs) and quantification (LOQs) were obtained with DLLME, although recovery and precision results were quite similar in both cases. Occasionally, DLLME was employed in combination with other techniques such as SPE, since due to the complexity of honey, it is sometimes

necessary to include an extra cleanup step so as to avoid low LODs and LOQs [35]. A modification of this sample treatment, which is known as in-syringe DLLME (IS-DLLME), was successfully employed for determining NEOs in honey [40]. Simplicity and rapidness are the most relevant benefits of this technique. A glass syringe is used as an extraction, separation, and preconcentration container. The significant increase in the interaction surface with the sample makes possible an efficient mass transfer of the analyte into the extraction solvent droplets. Another alternative to conventional DLLME is the in situ ionic liquid DLLME (IL-DLLME) [42]. It has the main advantage of eliminating the use of an organic disperser, and, in addition, the in situ reaction can be completed in a shorter time with excellent extraction efficiency. Different hydrophilic ILs with different cations and anions were tested, and IL [C4MIM][PF6] was chosen due to its greater extraction efficiency and because it was more suitable for HPLC systems than other solvents. The same IL was chosen for developing an IL-based cold-induced aggregation microextraction (IL-CIAME) procedure [43]. This is a simple, fast, and effective preconcentration method that can be applied to an analysis of sample solutions containing high concentration of salt and water miscible organic solvents. The suitability of this proposal was corroborated by high preconcentration factors, good rates of recovery, and high reproducibility. In relation to liquid–liquid extraction-based methods, it is worth mentioning one study in which a sugaring-out-assisted liquid–liquid extraction (SULLE) method was proposed for determining three NEOs in honey [44]. Sugaring-out is a phase separation method introducing monomeric sugars or disaccharides into an acetonitrile aqueous solution. Thus, acetonitrile can be separated from water to form a new phase. The authors compared the performance of SULLE with previously reported methods such as DLLME or QuEChERS; the results (recoveries and sensitivity) were quite similar or even better but with the advantage of the new proposal's simplicity. Finally, disposable pipette extraction (DPX) was also evaluated as an alternative sample treatment in one recent study [45]. In this procedure, solutions were mixed with the sorbent (anion exchanger) in a both dynamic and dispersive manner to obtain fast equilibration partitioning and enhance contact between NEOs and the sorbent.

As mentioned in the introduction, when taking into account the physicochemical properties of this family of insecticides, reverse-phase HPLC and UHPLC are the most frequently selected techniques, with analytical columns and C_{18}-based stationary phases (*see* Table 1). All the same, phenyl hexyl [31] and C_8 [32, 40] stationary phases were chosen in some studies. It should also be mentioned that a miniaturized technique, capillary liquid chromatography (CLC), has been studied as an alternative to conventional HPLC [41]. This technique is characterized by the use of

capillary columns of a narrow internal diameter (<500 μm) providing short analysis times with lower consumption of mobile phases and/or amounts of sample Meanwhile, a different technique, supercritical fluid chromatography (SFC), was selected with the aim of achieving enantioseparation of dinotefuran and its chiral metabolite [21]. In this study, 16 chiral columns and different CO_2-based mobile phases were evaluated in order to obtain optimal separation; the best performance was obtained with a Trefoil AMY1 3.0 chiral column and a formic acid–methanol cosolvent. In relation to detection systems, MS/MS with a triple quadrupole analyzer (QqQ) has mostly been employed, although other analyzers, such as ion trap (IT), quadrupole-time of flight (QTOF), or triple quadrupole linear ion trap (QTRAP), were chosen in other studies (*see* Table 1). It should be specified that a QTRAP system can be operated as a conventional QqQ. However, in a QTRAP device, the third quadrupole can be configured as a linear ion trap to enhance performance and flexibility. In almost all cases, electrospray (ESI) operating in positive mode was the ionization source chosen, while atmospheric pressure chemical ionization (APCI) in positive mode was selected only once [35]. UV-visible-based detectors like diode array (DAD) or photodiode array (PDA) ones have also been employed in several studies [35, 40–44]. To sum up, a methodology composed of a QuEChERS-based protocol followed by UHPLC-MS/MS (ESI+, QqQ) determination is the most suitable for determining NEOs, according to current trends in analytical chemistry and the literature reviewed. Nevertheless, it is surprising how in few studies sample treatment and/or validation was specifically evaluated/optimized in order to measure NEOs in different botanical sources of honey. Several of the studies summarized in Table 1 were multiresidue methods with attention focused more on providing a fast-screening protocol rather than on obtaining optimal conditions for each family of pesticides in the different types of honey. This is a relevant issue because, due to the different physicochemical characteristics of the honey depending on its botanical origin, the application of different sample treatments would be necessary in order to obtain optimal conditions. In fact, this has been demonstrated in a recent study in which different sample treatments were selected when analyzing light (QuEChERS; multifloral and rosemary) or dark (SPE; heather) colored honeys, in order to obtain the best recoveries and sample cleanup, while preventing the potential matrix effect that could potentially affect the MS/MS signals [15].

3 Beeswax

Beeswax is an extremely complex matrix composed of mixtures of many lipophilic compounds, among which are to be found the esters of long chain fatty acids with aliphatic alcohols. It also

contains free fatty acids, alcohols, and diesters. Pesticides can come into contact with it either directly, if these are added to the hive to exterminate a plague affecting it, or indirectly, when the compounds are added to plants to control other pests that might be affecting them. Interest in beeswax is related to the fact that it is in permanent contact with bees and can act as a reservoir of pesticides, including NEOs, releasing them little by little. A few studies have been published in which a comparison was made with honey, and in several cases, these were multiresidue methodologies where the pesticides analyzed belonged to different families. As can be deduced from the analysis in Table 2, the proposed sample treatments were based on two different strategies, QuEChERS and solvent extraction, but in both cases the usual necessary step was included in which beeswax was melted to obtain more homogenous samples and optimize sample treatment. As to be expected, it was followed by cooling at room temperature. In addition, there was a freezing step in several of the selected treatments [26, 27, 49, 50, 53, 54], quite similar to the one mentioned in the section devoted to honey samples; the aim was to remove by precipitation certain beeswax components, mainly lipids and proteins. Regarding QuEChERS-based protocols [26, 27, 30, 52–55], it could be said that all of them employed conventional reagents, such as ACN in the extraction stage and PSA, C_{18}, and $MgSO_4$ for the dSPE. Several of these studies were multiresidue methods, and in only two of them a matrix effect was not present.

However, in one study [52], a modification was introduced which entailed buffering during extraction, thereby further improving the results in a simple, fast, and inexpensive way. The authors employed a buffer composed of acetic acid and sodium acetate, instead of the more conventional sodium chloride that implies less acidic conditions, as it had shown good results in terms of recoveries in previous studies with other pesticides [56]. Meanwhile, three different protocols were adopted in the studies in which solvent extraction was performed. The first used a hexane and isopropanol mixture, followed by a heating step to dissolve the beeswax and the addition of water. Then an SPE was carried out with diatomaceous earth cartridges and an evaporation stage. Good recoveries were obtained in all cases, although a significant matrix effect was also observed [46–48]. A diatomaceous earth sorbent was also employed in other studies [49, 50], but without the need for a specific SPE step. This procedure displayed certain additional differences, such as the use of two solvents: (1) pentane, which was added before dissolving the beeswax in an ultrasonic bath and which was evaporated following centrifugation with the diatomaceous earth sorbent and (2) acetonitrile with acetic acid, which was added twice before the freezing stage. Further differences include the use of an ultrasonic bath, instead of heating, to dissolve the wax, and the inclusion of a freezing step (15 h). Low rates of recovery were reported in some cases, and it was not

Table 2
Applications in the analysis of neonicotinoids in beeswax

Analytes (Multiresidue method)	Sample treatment	Characterization method (SP, ISO, MSA)	Recoveries (Matrix effect)	References
3, 5, 7 (Yes)	QuEChERS (ACN; freezing; PSA and C_{18})	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	NS (NS)	[26]
5 (No)	QuEChERS (ACN; freezing; PSA and C_{18}) + EV	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	97–101% (No)	[27]
5–7 (Yes)	QuEChERS (water:ACN; PSA and C_{18})	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	NS (NS)	[30]
5 (No)	SE (hexane:IPA, water) + SPE (DE) + EV	HPLC-MS (C_{18}, ESI+, SQ)	95–103% (Yes)	[46]
1–7 (No)	SE (hexane:IPA, water) + SPE (DE) + EV	[47]HPLC-MS (C_{18}, ESI+, SQ) [48]CE-MS/MS (QTOF)	[48]85–105% (Yes)	[47, 48]
3–7 and MET (Yes)	SE (pentane; DE; ACN) + Freezing + EV	UHPLC-MS/MS (PH, ESI+, QqQ)	72–97% (NS)	[49, 50]
1–7 (No)	SE (MeOH:EA, water) + freezing + dSPE (EMR-lipid) + EV	UHPLC-MS/MS (C_{18}, ESI+, QTOF)	93–106% (No)	[51]
3–7 (Yes)	QuEChERS (water:ACN:AA; $MgSO_4$, PSA and C_{18})	HPLC-MS/MS (C_{18}, NS, QqQ)	NS (NS, NS)	[52]
3, 5, 7 (Yes)	QuEChERS (ACN; freezing; PSA and C_{18})	UHPLC-MS/MS (C_{18}, ESI+, QTRAP)	100–120% (No)	[53]
3–7 (Yes)	QuEChERS (ACN; freezing; PSA and C_{18})	UHPLC-MS/MS (C_{18}, ESI+, QTRAP)	NS (NS)	[54]
2–7 (Yes)	QuEChERS (ACN; $MgSO_4$ and PSA)	HPLC-MS/MS (C_{18}, ESI+, QTRAP)	73–120 (NS)	[55]

1 Dinotefuran, *2* Nitenpyram, *3* Thiamethoxam, *4* Clothianidin, *5* Imidacloprid, *6* Acetamiprid, *7* Thiacloprid, *8* Sulfoxaflor, *9* Flonicamid, *10* Cicloxaprid, *11* Imidaclothiz, *12* Flupyradifurone, *13* Pymetrozine, *AA* acetic acid, *ACN* acetonitrile, dichloromethane, *CE* capillary electrophoresis, *DE* diatomaceous earth, *DLLME* dispersive liquid–liquid microextraction, *dSPE* dispersive SPE, *EA* ethyl acetate, *ESI* electrospray ionization, *EV* evaporation, *FA* formic acid,

specified whether a matrix effect was observed. Finally, a different sorbent, known as an enhanced matrix removal lipid (EMR-lipid), was employed in the last approach [51]. This recently commercialized sorbent contains C_{18} and certain special polymers that are not specified by the manufacturer, and it had the advantage of removing lipids without losing the analytes. In this case, extraction was performed with a methanol and ethyl acetate mixture. A freezing step was also required followed by a cleanup with the dSPE EMR-lipid sorbent and an evaporation step. This procedure had additional advantages in terms of simplicity, length, and absence of a significant matrix effect. As previously mentioned in the Introduction and in the section devoted to honey samples, HPLC and UHPLC in reverse-phase mode with C_{18}-based stationary phases have generally been selected (*see* Table 2). A similar comment could be made in relation to the detection systems, as MS/MS was the preferred choice, with QqQ being the most commonly employed analyzer, despite the use of QTRAP and QTOF in some studies. An MS detector with a single quadrupole was chosen in three of the studies [46–48], and results in terms of sensitivity were sufficiently favorable when compared with the most powerful MS/MS detectors. ESI in positive mode was selected in all the MS and MS/MS experiments. However, in one study, capillary electrophoresis coupled to MS/MS (CE-MS/MS) was evaluated as an alternative to an HPLC-based method for determining NEOs in beeswax. This, in fact, was the first report of a CE-MS/MS method for a simultaneous evaluation of this family of pesticides. The good results which were obtained (recovery, precision, overall run time) demonstrate that this technique may be considered a promising alternative to HPLC-based strategies, due mainly to the low consumption of solvents, reagents, and samples.

Therefore, after analyzing the data summarized in Table 2, it can be concluded that HPLC/UHPLC-MS/MS (ESI; QqQ) is the best option for determining NEOs in beeswax. Meanwhile, a QuEChERS methodology is the most recommendable sample treatment in terms of reagent consumption and simplicity, although in this case the similarity here with some of the proposed solvent extraction methods [49–51] is considerable, making this choice a more difficult one and dependent on the researcher's personal decision.

HPLC high-performance liquid chromatography, *ISO* ionization source, *MeOH* methanol, *MET* metabolites, *MSA* mass spectrometry analyzer, *MS/MS* tandem mass spectrometry, *NS* not specified, *PH* phenyl hexyl, *PSA* primary secondary amine, *QqQ* triple quadrupole, *QTOF* quadrupole-time-of-flight, *QTRAP* triple quadrupole and linear ion trap, *QuEChERS* quick, easy, cheap, effective, rugged, and safe, *SE* solvent extraction, *SP* stationary phase, *SPE* solid-phase extraction, *SQ* single quadrupole, *UHPLC* ultra-high-performance liquid chromatography

4 Bee Pollen

Due to the systemic characteristics of these compounds, they can be distributed to the rest of the plant by translocation; as a consequence, neonicotinoid residues can be found in pollen, the main food source for honey bees, from flowers or hives. In addition, bee pollen in particular is gaining attention as a functional food for human consumption owing to its high content of compounds with health-promoting effects, such as amino acids, antioxidants, vitamins, and lipids [57]. The most common sample treatment for determining NEOs in bee pollen is again the QuEChERS protocol ([16, 58–62]; *see* Table 3), while solvent extraction-based procedures have been employed in only two cases [63, 64]. As with other bee products, conventional QuEChERS conditions are the norm in most cases. This entails the use of water and ACN in the first stage of the protocol, followed by a dSPE procedure with $MgSO_4$, PSA, and C_{18} [60–62]. However, certain authors have decided to employ different reagents and/or approaches. For example, Kamel [16] decided to use SPE cartridges (C_{18}) rather than a dSPE with C_{18} and PSA, because loss of some of the analytes was noted if vacuum or positive pressure was absent during elution, with several metabolites tightly bound to PSA and low recoveries obtained. Other authors include hexane in combination with ACN as the reagents for the first step of the QuEChERS protocol [58]. The aim was to remove lipids from the ACN extract, as these are likely to be dissolved in this solvent and can be easily eliminated after centrifugation. The results showed that cleaner extracts, chromatograms with a lower background, and enhanced method sensitivity were obtained. GCB was added to the dSPE sorbent mixture in another study, due to the improved sensitivity obtained in previous publications in which NEOs were investigated in pollen [59]. However, the amount of the dSPE sorbents should be carefully selected as the combined use of C_{18} and GCB could cause some of the insecticides to be absorbed. Regarding solvent extraction-based procedures, dichloromethane (DCM; [63]) and a combination of water, hexane, and ACN were employed [64]. In the first study, a further cleaning step was not included [63], while in the other different resins for sample purification were tested, namely, silica, C_{18}, and Envi-Carb II/PSA [64].

The best results in terms of recovery percentages were obtained with Envi-Carb II/PSA sorbents, as recoveries ranged between 81% and 99% for all the compounds.

As can be observed in Table 3, HPLC-MS based methods with C_{18} stationary phases were employed in all cases for assessing neonicotinoid in bee pollen. However, in one study [61], nanoflow LC coupled to ESI was proposed as an alternative to conventional

Table 3
Applications in the analysis of neonicotinoids in bee pollen

Analytes (Multiresidue method)	Sample treatment	Characterization method (SP, ISO, MSA)	Recoveries (Matrix effect)	References
1, 3–5 and MET (No)	QuEChERS (TEA:ACN; NO dSPE) + SPE (C_{18}) + EV	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	41–145% (NS)	[16]
1–7, 9 (No)	QuEChERS (water, ACN, hexane; $MgSO_4$, PSA and C_{18}) + EV	HPLC-MS/MS (C_{18}, ESI+, QqQ)	37–113% (Yes)	[58]
3–7 (Yes)	QuEChERS (water, ACN; GCB, PSA and C_{18}) + EV	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	79–90% (No)	[59]
1–7 (No)	QuEChERS (water, ACN; $MgSO_4$, PSA and C_{18}) + EV	UHPLC-MS/MS (C_{18}, ESI+, QTOF)	91–105% (No)	[60]
2–7 (Yes)	QuEChERS (water, ACN; $MgSO_4$, PSA and C_{18}) + DI	Nano-HPLC-MS/MS (C_{18}, ESI+, Orbitrap)	94–97% (No)	[61]
3–5 and MET (No)	QuEChERS (water, ACN; $MgSO_4$, PSA and C_{18})	UHPLC-MS/MS (C_{18}, ESI+, QqQ)	89–101% (NS)	[62]
1–7 (No)	SE (DCM) + EV	HPLC-MS (C_{18}, ESI+, SQ)	86–106% (Yes)	[63]
1–7 (No)	SE (hexane:ACN, water) + SPE (ENVI-carb II/PSA) + EV	HPLC-MS/MS (C_{18}, ESI+, QqQ)	81–99% (Yes)	[64]

1 Dinotefuran, *2* Nitenpyram, *3* Thiamethoxam, *4* Clothianidin, *5* Imidacloprid, *6* Acetamiprid, *7* Thiacloprid, *8* Sulfoxaflor, *9* Flonicamid, *10* Cicloxaprid, *11* Imidaclothiz, *12* Flupyradifurone, *13* Pymetrozine, *AA* acetic acid, *ACN* acetonitrile, *DCM* dichloromethane, *DI* dilution, *DLLME* dispersive liquid-liquid microextraction, *dSPE* dispersive SPE, *ESI* electrospray ionization, *EV* evaporation, *HPLC* high-performance liquid chromatography, *ISO* ionization source, *MeOH* methanol, *MET* metabolites, *MSA* mass spectrometry analyzer, *MS/MS* tandem mass spectrometry, *Nano-LC* nano-liquid chromatography, *NS* not specified, *PSA* primary secondary amine, *QqQ* triple quadrupole, *QTOF* quadrupole-time-of-flight, *QuEChERS* quick, easy, cheap, effective, rugged, and safe, *SE* solvent extraction, *SP* stationary phase, *SPE* solid-phase extraction, *SQ* single quadrupole, *TEA* triethylamine, *UHPLC* ultra-high-performance liquid chromatography

HPLC methodologies because this involves a significant advantage in terms of sensitivity (nanospray is a more effective process than pneumatically assisted electrospray) and matrix effect, as it is less intense in nanospray. QqQ was preferred as the MS/MS analyzer [16, 58, 59, 62, 64], while QTOF [60] and Orbitrap [61] were scarcely chosen; an MS analyzer was employed in only one publication [63].

To conclude this section on an evaluation of neonicotinoid analysis in bee pollen, it can be affirmed that a methodology comprising a QuEChERS protocol followed by a UHPLC-MS/MS (C_{18}, ESI+, QqQ) is the best option not only in terms of overall method time, solvent consumption, or complexity, but also in

relation to matrix effect. In most of the QuEChERS-based studies, we have cited, this was almost negligible, despite having a strong influence on neonicotinoid signals in both solvent-based extraction protocols.

5 Other Bee Products

5.1 Royal Jelly

In order to evaluate the presence of neonicotinoid residues and provide data regarding possible explanations of the disappearance of pollinators, it may be appropriate to consider the hypotheses that bees are born weakened and with a shorter half-life, which directs attention to larval nutrition mainly by royal jelly. This is because bees may come into contact with these compounds and transport them to the hive, distributing them around the different compartments. One possibility is that royal jelly may become contaminated and affect the survival of the hive. Royal jelly is a product secreted by the hypopharyngeal glands and by the mandibular glands of nurse bees (workers between 5 and 15 days old). It is viscous and yellowish in color and is intended to feed bee larvae during the first 3 days after birth, after which they go on to feed on a mixture of water, honey, and pollen. The queen feeds on pure royal jelly throughout her life, which is also the case of royal larvae, that is, larvae that are destined to become new queens. Different proposals have been published in the last years devoted to investigating the presence of NEOs in this bee product (*see* Table 4). For example, Hou et al. [38] proposed sample treatment comprising three steps, namely, dilution with water and a further protein precipitation with methanol, a cleanup step based on an SPE with polymeric cartridges, followed by evaporation. Different SPE sorbents were evaluated (C_{18}, Oasis® HLB and Oasis® MCX), and the best results in terms of recovery and reproducibility were obtained with the Oasis® HLB sorbent. The same research group published a different method grounded in a modified QuEChERS methodology [65]. In this study, the first steps were identical to those in the previous study (dilution with water and protein precipitation with methanol), and the main difference concerned the cleanup stage, which involved QuEChERS reagents such as PSA, C_{18}, and $MgSO_4$. This procedure had the advantage of low solvent consumption and the absence of a significant matrix effect. The limitation of the matrix effect was also achieved by means of a salting-out liquid–liquid extraction, which is quite similar to the first step of a QuEChERS method [66], followed by a time-consuming cleaning step based on freezing (15 h). The solvents employed in this study were water, ACN, and acetate buffer.

Finally, two new methodologies have been developed for analyzing NEOs in products based on royal jelly, pure royal jelly, and royal jelly lyophilized in the form of ampoules [67]. For extraction of these in pure royal jelly, a dispersive liquid–liquid microextraction was proposed (ACN and chloroform), while for lyophilized

royal jelly, an SPE methodology (Strata® X) was performed following dilution with ammonium formate. It should be pointed out that this is the first time a specific methodology was developed to examine NEOs in different products containing royal jelly. However, a significant matrix effect was observed on certain compounds in both cases. HPLC-MS/MS (C_{18}, ESI+, QqQ) was employed in most of the above studies, with the exception of two in which a phenyl hexyl-based stationary phase [66] or a UHPLC with a QTOF analyzer [67] was chosen. According, then, to the related literature, it can be concluded that the recommended sample treatment should consist of a dilution stage, followed by precipitation with methanol, a cleanup step with QuEChERS reagents, and an evaporation. As a result, the overall procedure time is lower, recoveries are sufficiently acceptable, and the matrix does not have a significant influence on neonicotinoid signals. Regarding the determination technique, UHPLC-MS/MS with a C_{18} column and a QqQ (ESI+) seems to be the best choice in terms of sensitivity and rapidness.

5.2 Bee Bread

Bee bread is the main source of protein for adult bees and their larvae and is made up of pollen, honey, and various enzymes added by bees, transforming the product through lactic fermentation. It is not the pollen that is marketed as a dietary supplement, nor does it have the same taste, since what is simply called pollen (without being processed by bees) is collected at the entrance of the hive by means of special traps. Instead, bee bread is accumulated and stored by the bees in the wax combs within the hive [50]. Two studies have been published dealing with an evaluation of NEOs in this substance [50, 52]. Modified QuEChERS methods were used in both cased, but with several differences. For instance, the solvents employed in the first stage of the procedure were not the same, as in one case heptane was selected in combination with water and ACN containing triethylamine [50], while in the other study, water and ACN acidified with acetic acid were chosen [52]. Freezing and evaporation steps were employed in only one of the publications [50], drastically increasing the length of the procedure (15 h), while the reagents selected for the dSPE also differed (*see* Table 4). No mention of a potential matrix effect was reported in either publication. As with other bee products, HPLC and UHPLC coupled to MS/MS were selected to determine NEOs in this matrix, with two different stationary phases, namely, C_{18} and phenyl hexyl and QqQ analyzer. Therefore, a recommended protocol is one involving a modified QuEChERS method, but without a freezing step, and a UHPLC-MS/MS (C_{18}, ESI+, QqQ), as this would permit faster analysis without compromising the sensitivity and selectivity of the proposal.

5.3 Nectar

Nectar is an aqueous solution of sugars, amino acids, mineral ions, and aromatic substances. It is produced by flowers as an attractant and reward for animals that perform the pollination service. The nectar is produced by differentiated glands, called nectaries, at the base of the stamens or petals, and is deposited in many cases in bags or spurs at the base of the corolla. Floral nectar is the energy food of various groups of animals, such as hummingbirds, day and night butterflies, bees or flies, while it is the most important raw material for the production of honey by the honey bee. As can be seen in Table 4, only one study has been published on the evaluation of NEOs in nectar [68]. A conventional QuEChERS protocol was used in which prior dilution of the sample was required. The

Table 4
Applications in the analysis of neonicotinoids in other bee products

Bee product	Analytes (Multiresidue method)	Sample treatment	Characterization method (SP, ISO, MSA)	Recoveries (Matrix effect)	References
Royal jelly	1–7, 9, 11, 13 (No)	DI (water, MeOH) + SPE (Oasis® HLB) + EV	HPLC-MS/MS (C_{18}, ESI+, QqQ)	72–107% (Yes)	[38]
Bee bread	3–7 and MET (Yes)	SE (water, heptane, ACN: TEA) + freezing + dSPE (PSA) + EV	UHPLC-MS/MS (PH, ESI+, QqQ)	53–119% (NS)	[50]
Bee bread	3–7 (Yes)	QuEChERS (water:ACN:AA; $MgSO_4$, PSA, and C_{18})	HPLC-MS/MS (C_{18}, NS, QqQ)	NS (NS)	[52]
Royal jelly	1–7, 9, 11, 13 (No)	DI (water, MeOH) + dSPE ($MgSO_4$, PSA and C_{18}) + EV	HPLC-MS/MS (C_{18}, ESI+, QqQ)	81–119% (No)	[65]
Royal jelly	3–7 (No)	SLLME (water, ACN, AB) + US + freezing + EV	UHPLC-MS/MS (PH, ESI+, QqQ)	94–105 (No)	[66]
Royal jelly	1–7 (No)	[1]DLLME (ACN, chloroform) + EV [2]DI (AFO) + SPE (Strata® X) + EV	UHPLC-MS/MS (C_{18}, ESI+, QTOF)	[1]90–109% (Yes) [2]85–107% (Yes)	[67]
Nectar	3–5 and MET (No)	DI (water) + QuEChERS (water, ACN; $MgSO_4$, PSA, and ENVI-Carb)	HPLC-MS/MS (NS, ESI+, NS)	95–115% (NS)	[68]

1 Dinotefuran, *2* Nitenpyram, *3* Thiamethoxam, *4* Clothianidin, *5* Imidacloprid, *6* Acetamiprid, *7* Thiacloprid, *8* Sulfoxaflor, *9* Flonicamid, *10* Cicloxaprid, *11* Imidaclothiz, *12* Flupyradifurone, *13* Pymetrozine, *AA* acetic acid, *AB* acetate buffer, *ACN* acetonitrile, *AFO* ammonium formate, *DI* dilution, *DLLME* dispersive liquid–liquid microextraction, *dSPE* dispersive SPE, *ESI* electrospray ionization, *EV* evaporation, *HPLC* high performance liquid chromatography, *ISO* ionization source, *MeOH* methanol, *MET* metabolites, *MSA* mass spectrometry analyzer, *MS/MS* tandem mass spectrometry, *NS* not specified, *PH* phenyl hexyl, *PSA* primary secondary amine, *QqQ* triple quadrupole, *QTOF* quadrupole-time-of-flight, *QuEChERS* quick, easy, cheap, effective, rugged and safe, *SE* solvent extraction, *SLLME* salting-out liquid–liquid extraction, *SP* stationary phase, *SPE* solid phase extraction, *TEA* triethylamine, *US* ultrasound, *UHPLC* ultra-high performance liquid chromatography

reagents were those typical for this procedure, that is, water and ACN for the first stage, together with PSA, C_{18}, and $MgSO_4$ for the dSPE. NEOs were determined by HPLC-MS/MS, but the authors did not specify either the stationary phase or the MS/MS analyzer.

6 Conclusions

In this chapter, an overview has been presented of the publications relating to determining NEOs in bee products. Monitoring neonicotinoid residues in bee products is necessary not only to ensure the safety of these products for potential consumers, including honey bees and humans, but also to protect the environment. It may be concluded that honey is the bee product in which NEOs have been most frequently investigated, which could be expected due to the high demand for this food compared with other bee products. In terms of sample preparation techniques, QuEChERS have generally been employed in all such products, probably due to its main characteristics, especially simplicity and rapidness. However, other procedures such as SPE and solvent extraction have also been extensively used. HPLC/UHPLC in reverse-phase mode (C_{18} columns) coupled to MS/MS detectors, and especially QqQ, has been the technique of choice when performing individual analysis of the NEOs. This could be attributed to the physicochemical characteristics of this family of insecticides, discarding the use of GC, and the high degree of selectivity and sensitivity provided by the QqQ analyzer. Finally, all the data and information summarized in this chapter should facilitate extraction and determination of NEOs in bee products, thereby providing useful information for researchers involved in assessing these insecticides in the latter.

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Chapter 5

Determination of Pyrethroid Insecticides Metabolites in Wastewater

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Abstract

Pyrethroids are one of the most frequently used insecticides, particularly due to their low toxicity and high efficiency in small doses. Human exposure to pyrethroids usually occurs through the ingestion of residues present in food. The measurement of pyrethroid metabolites in wastewater can be used for the evaluation of the exposure of a given population to these compounds. In this chapter, a sensitive and specific assay for the determination of the three major biomarkers of pyrethroids exposure in wastewater, namely 3-PBA, *cis*, and *trans*-DCCA, is presented. The procedure includes a simple solid-phase extraction step, followed by analyte measurement using a liquid chromatography-tandem mass spectrometry system.

Key words Pyrethroid insecticides, Pyrethroid metabolites, Wastewater, Mass spectrometry, Solid-phase extraction

1 Introduction

The expansion of the world population demands a constant increase in food production. Along with the development of industrial agriculture, the use of pesticides is constantly escalating [1]. After the prohibition of organochlorines, due to their long-term stability in the environment and high toxicity to mammals [2], pyrethroids became one of the most frequently used pesticides in homes, agriculture, and public health [3, 4]. The increasing interest in the use of these insecticides is associated to their low human toxicity and adequate effectiveness [2]. Synthetic pyrethroid insecticides are human-made derivatives of pyrethrins, which are natural compounds found in plants such as chrysanthemums. They are designed to be more potent and environmentally stable than natural pyrethroids [5].

Human exposure to pyrethroid insecticides occurs in the occupational setting, in farming, chemical industry, and pest control activities, and also by the consumption of food containing these

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compounds [3, 4, 6, 7]. In humans, pyrethroids are metabolized by liver enzymes and eliminated in urine. The main human metabolites of pyrethroid insecticides are 3-phenoxybenzoic acid (3-PBA, a common metabolite of 20 synthetic pyrethroids) and both *cis* and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethyl-1-cyclopropane) carboxylic acid (*cis* and *trans*-DCCA, a common metabolite of permethrin, cypermethrin, and cyfluthrin) [7–9].

Human biomonitoring of pyrethroid exposure is usually performed using urine specimens [7]. A useful alternative for evaluating the exposure of an urban population to these compounds is to measure the concentration of metabolites in wastewater [8, 10]. Using appropriate mathematical extrapolations, the mass discharge of the ingested compound in the studied population can be estimated. This approach, named wastewater-based epidemiology (WBE), is a strategy that provides information on population exposure to contaminants in a short time, with lower cost when compared to the collection of multiple biological specimens. WBE allows back-calculation, using human biotransformation metabolism data, to estimate the population exposure to the compound of interest [7, 8, 11, 12].

As the concentrations of 3-PBA, *cis,* and *trans*-DCCA in wastewater are expected to be very low, very sensitive assays are needed for their measurement [13]. Moreover, the complexity of the wastewater matrix requires efficient analyte concentration and cleanup. The combination of selective solid-phase extraction (SPE) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the more straightforward approach for the measurement of these compounds in wastewater [7, 8]. In this context, the presented method was developed to identify the three major human pyrethroids biomarkers (3-PBA, *cis, and trans*-DCCA) in wastewater, using SPE and LC-MS/MS. Previous to the SPE procedure, specimens are alkalinized with ammonium hydroxide, and internal standards (3-PBA-13C6, *cis*-DCCA-d3, and *trans*-DCCA-d3) are added. This mixture is applied to a conditioned anion-exchange SPE cartridge, which is washed with an ammonium hydroxide solution and methanol. The analytes are eluted from the SPE cartridges with a methanolic solution of formic acid, evaporated to dryness, and reconstituted with a water and methanol mixture. The extract is separated in a CSH phenyl-hexyl chromatographic column, and analytes are measured by mass spectrometry, using electrospray ionization in negative ionization mode, in multiple reaction monitoring mode (MRM). The quantification of the biomarkers is made using a seven-point calibration curve, with concentrations ranging from 10 to 2000 ng mL^{-1}.

2 Materials

All reagents are HPLC grade, and all standards are Certified ACS grade.

2.1 Extraction

1. SPE wash solvent (5% ammonium hydroxide in water): To prepare 9 mL, add 450 μL of concentrated ammonium hydroxide to 8550 μL of ultrapure water (*see* **Note 1**). Prepare daily.
2. SPE elution solvent (2% formic acid in methanol): To prepare 9 mL, add 180 μL of formic acid to 8820 μL of methanol. Prepare daily.
3. 10,000 ng L^{-1} working calibration standard (WCS): 3-PBA and *trans*-DCCA are purchased as 1 mg mL^{-1} in methanol, and cis-DCCA are purchased as 100 μg mL^{-1} in methanol, and they are stored at room temperature until consumed. To prepare the WCS, dilute stock solutions to 1 μg/mL, then prepare the WCS by adding 10 μL of 3-PBA, *cis/trans* DCCA 1 μg/mL and complete the volume with 970 μL of methanol:water (60:40, v/v). Store in the freezer.
4. 500 ng mL^{-1} internal standard (IS) solution. 3-PBA^{13}C6 is purchased at 1 mg mL^{-1}, and *cis*-DCCA d3 e *trans*-DCCA-d3 are purchased as 0.1 mg mL^{-1}. 3-PBA-^{13}C6 and *trans*-DCCA-d3 are stored in the freezer and *cis*-DCCA-d3 is stored at room temperature. Using the stock solution, prepare an intermediate solution at 10,000 ng mL^{-1} by adding 10 μL of 3-PBA-^{13}C6 and 50 μL of both *cis* and *trans*-DCCA-d3 in 450 μL of methanol:water (60:40, v/v). To prepare the IS solution, 200 μL of each intermediate solution was added with 3400 μL of methanol:water (60:40, v/v). Store in the freezer.
5. Pyrethroid Quality Control for SPE (Quality control, *see* **Note 2**): Add 10 μL of pyrethroid WCS 10,000 ng L^{-1}, 50 μL of IS 500 ng mL^{-1}, and complete to 50 mL with water.
6. SPE cartridges: The employed cartridges are Oasis® MAX 6 cm^3/500 mg, from Waters. Store in a cool dry area.

2.2 Instrument

1. Mobile phase A (MP-A): Formic acid 0.1% in water. In a 500 mL volumetric flask, add 450 mL of ultrapure water and 50 μL of formic acid. The volume is completed until 500 mL with ultrapure water. Store at room temperature, valid for 7 days.
2. Mobile phase B (MP-B): Formic acid 0.1% in acetonitrile. In a 500 mL volumetric flask, add 450 mL of acetonitrile HPLC grade and 50 μL of formic acid. The volume is completed until 500 mL with acetonitrile. Store at room temperature, valid for 7 days.

Table 1
Employed mobile-phase gradient for the separation of 3-PBA and *trans/cis*-DCCA from wastewater

Time (min)	MP-A (%)	MP-B (%)
0	60	40
3.5	49	51
4.5	30	70
5	30	70
5.1	1	99
6.5	1	99
6.51	60	40
8	60	40

3. Mobile phase A2 (MP-A2): Water:acetonitrile (90/10). In a 500 mL volumetric flask, add 450 mL of ultrapure water, then complete the volume with acetonitrile HPLC grade.
4. Mobile phase B2 (MP-B2): Acetonitrile. In a clean bottle, add 300 mL of acetonitrile HPLC grade.
5. Working mobile phase: Chromatographic separation was performed using a mobile phase gradient, at 0.25 mL min^{-1} flow rate, as showed in Table 1.
6. Wash solvent: Acetonitrile/methanol/isopropanol/water (55:20:15:10, v/v/v/v). In a 500 mL graduate cylinder, add 275 mL of acetonitrile HPLC grade, 100 mL of methanol HPLC grade, 75 mL of isopropanol HPLC grade, and 50 mL of ultrapure water. Store at room temperature.
7. LC Column: Waters CSH phenyl-hexyl 1.7 μm (2.1 × 100 mm), maintain at 40 °C during analysis (*see* **Note 3**).
8. LC-MS/MS system: Analysis was performed using an Acquity XEVO TQS-Micro ultra-performance liquid chromatograph coupled to a triple quadrupole mass spectrometer in tandem, from Waters Technologies.

3 Methods

The total run time of the LC separation is 8 min, with retention times and mass transitions presented in Table 2 (*see* **Notes 4** and **5**).

3.1 Sample Preparation

1. Wastewater sample is collected and transferred to amber glass bottles. Samples are filtered using a glass microfiber filter GF/A (1.6 μm), followed by a second filtration step through a mixed

Table 2
Compound-specific optimized mass spectrometry acquisition parameters for the analysis of 3-PBA and *trans/cis*-DCCA

Analyte	MRM transition (*m/z*)[a]	Cone energy (V)	Collision energy (V)	Retention time (min)
Trans-DCCA	207 > 35 209 > 37	−10	−8 −8	3.67
Trans-DCCA-d_3	210 > 35	−10	−8	3.67
Cis-DCCA	207 > 35 209 > 37	−10	−8 −8	4.02
Cis-DCCA-d_3	210 > 35	−10	−8	4.02
3-PBA	213 > 169.1 213 > 93.1	−55	−22 −10	3.98
3-PBA-^{13}C6	219.05 > 175.1	−30	−12	3.98

[a]Quantification ions are underlined

Table 3
Preparation of calibrators for the quantification of 3-PBA and *trans/cis*-DCCA

Calibrator (ng L^{-1})	Original solution (ng L^{-1})	Spiked volume (μL)	Spiked IS volume (μL)	Diluent (methanol/water, 60:40, v/v) (μL)
2000	WCS 10000	100	50	350
1000	WCS 10000	50	50	400
500	WCS 10000	25	50	425
200	WCS 10000	10	50	440
100	1000	50	50	400
50	1000	25	50	425
10	100	50	50	400

cellulose membrane filter (0.45 μm). Aliquots of 50 mL of filtered specimen are alkalinized with 250 μL of concentrated ammonium hydroxide, to improve the retention of the analytes on the SPE cartridge.

2. Spiked calibrators are prepared according to Table 3 (*see* **Note 6**). Calibrators are spiked with the analytes of interest and with the IS. A full calibration curve is analyzed within each analytical run (*see* **Note 7**).

3.2 Extraction

Liquid flows through the SPE cartridge are drawn only by gravity, unless otherwise stated.

1. Add 50 μL of IS to the sample and vortex thoroughly.
2. Add 250 μL of concentrated ammonium hydroxide solution to the SPE calibrator and testing samples, vortex-mix.
3. Condition the SPE cartridge with 2 mL of methanol.
4. Load samples or SPE calibrator on the cartridges after conditioning is completed. Carefully check for the presence of suspended solids on the sample, once they can disrupt flow through the SPE cartridge. If suspended material is present, repeat the sample filtration step.
5. After samples are completely loaded, wash cartridges with 2 mL of 2% ammonium hydroxide in water, followed by 2 mL of methanol. When all liquid passed through the cartridge, dry under vacuum (>15 mm Hg) for 20 min.
6. Elute the analytes to a 5 mL polypropylene tube with 2 mL of 2% formic acid in methanol; apply 1 mL of elution solvent, and when almost all volume had passed through the cartridge, apply another 1 mL.
7. Evaporate the collected extract to dryness under a gentle stream of air, at 60 °C.
8. Reconstitute the dried extracts with 100 μL of methanol, vortex-mix, add 100 μL of ultrapure water, and vortex-mix again (*see* **Note 8**). Transfer the recovered extract to a vial and inject 2 μL in LC-MS/MS system.

3.3 Instrument Preparation

1. Place adequate volumes of MP-A and MP-B in correct bottles, and startup the LC system, by flushing lines, washing the syringe (set three cycles), and prime the instrument for 5 min at flow 4 mL min^{-1} (*see* **Note 9**).
2. While the system is starting up, install the column in the oven, and then, check for the absence of leak. Take corrective measures if needed.
3. Download the method to the instrument and allow the system to equilibrate at initial conditions, for at least 10 min. Table 4 shows the instrument parameters. A typical ion chromatogram obtained with the method is presented in Fig. 1.

Table 4
Instrumental mass spectrometer parameters for 3-PBA and *trans/cis*-DCCA analysis

Source voltage	
Capillary (kV)	−1
Source temperature	
Desolvation temperature (°C)	550
Source gas flow	
Desolvation (L/h)	1100
Cone (L/h)	50
Analyzer	
LM resolution 1	11.7
HM resolution 1	14.9
Ion energy 1	0.5
LM resolution 1	9.8
HM resolution 1	14.9
Ion energy 1	1.5

4 Notes

1. As ammonium hydroxide is rapidly volatilized, prepare the solution daily and keep at tightly closed vials.
2. To evaluate SPE procedure, a quality control sample is prepared on analysis day. It goes through SPE process as well as the samples, thus it acts as a quality control, to evaluate possible analyte losses during the SPE process.
3. After running each batch of samples, the column is washed with water and acetonitrile (MP-A2 and B2) for 30 min at flow rate of 0.3 mL min^{-1}. The mobile phase gradient for column wash is presented in Table 5.
4. As *cis* and *trans*-DCCA are isomers, the mass transitions and fragmentation ions are the same. However, these compounds are separated by the employed chromatographic conditions, with the *trans* isomer eluting few seconds before the *cis*.
5. The selection of collision energies and fragmentation voltages were made using the MassLynx software (Waters). Initially, the molecular mass is informed on the software and masses are scanned. Once the signal of the parent ion is found, the best capillary voltage (higher signal) is selected. Then, collision gas is released, allowing the formation of product ions. Specific

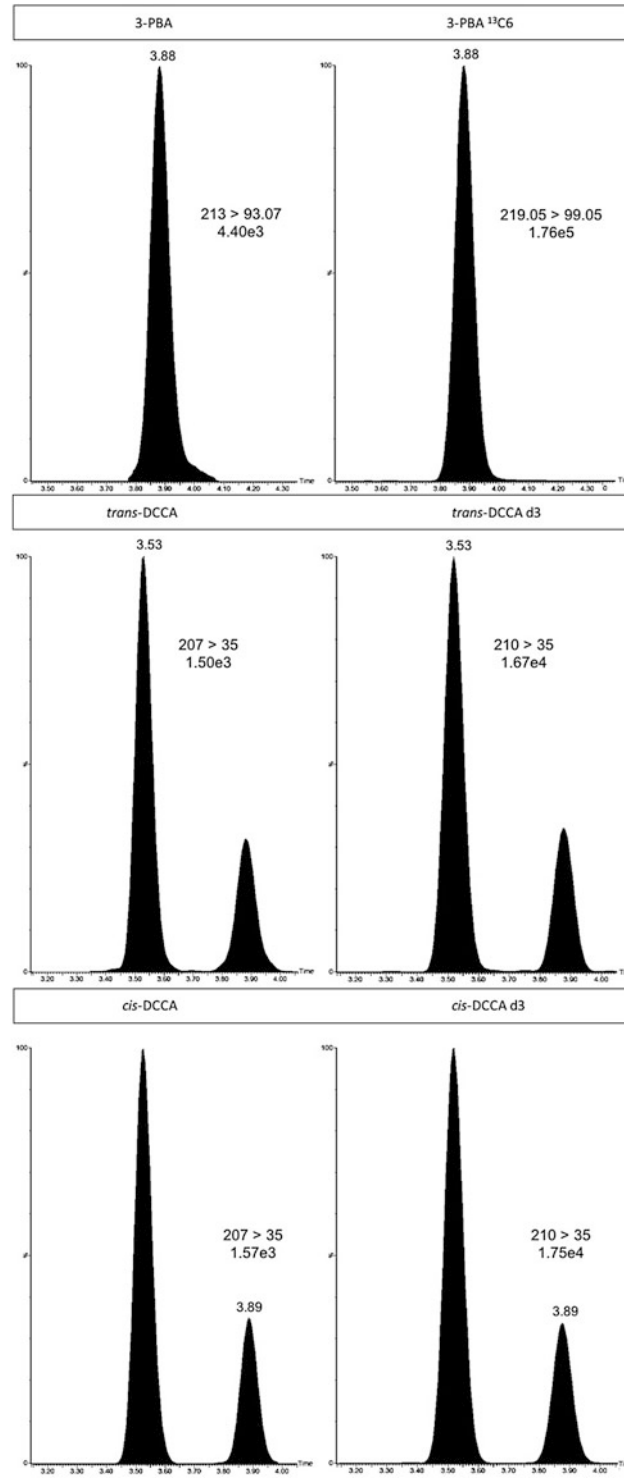

Fig. 1 Chromatogram obtained with the method, presenting 3-PBA and *trans/cis*-DCCA at the concentration levels of calibrator 1

Table 5
Employed mobile phase gradient for washing the chromatographic column

Time (min)	MP-A2 (%)	MP-B2 (%)
0	99	1
10	99	1
10.1	50	50
15	50	50
15.1	0	100
30	0	100

ions showing the higher signals are selected, and the collisions energies that provide the higher signal for each daughter ion are selected.

6. Calibrators are prepared once in diluent (methanol/water, 60:40, v/v), added with IS, and stored in the freezer. When performing analysis, remove the calibrator from the freezer, vortex-mix, and inject into the LC-MS/MS. After analysis, store in the freezer again.
7. The lowest cumulative percentage relative error of the calibration model was obtained using $1/x$ weighting. Weighted regression allows better accuracy along the linear range of the assay.
8. The reconstitution of the dried extract must be performed using first only methanol, vortex-mixed, then followed by addition of water. In our experience, this procedure increased the recovery of the analytes from the tube.
9. The procedure of priming, washing, and equilibration of the chromatographic system is essential for an adequate analytical performance. Residuals of ion-pairing agents, like HFBA, must be completely removed before analysis due to their significant ionization suppression effects.

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Chapter 6

Agricultural Pesticide Exposure and Risk Assessment to Human Health in Mexico

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Abstract

In Mexico, pesticides are widely used in agricultural production to control pests, diseases, weeds, and other plant pathogens that allow maintaining good product quality. Occupational exposure to pesticides occurs in the case of agricultural workers in open fields and greenhouses, workers in the pesticide industry, and in domestic use for pest control. Exposure of the population happens mainly through the consumption of food and water contaminated with pesticide residues. Regarding the adverse impacts on the environment and on wildlife, fish, and plants, many of these effects depend on the toxicity of the pesticide, the way it is applied, the weather conditions prevailing during and after application, and its persistence in the environment. All these reasons imply a high risk for exposed populations. For the evaluation of the real levels of pesticide residues in environmental and biological samples, sophisticated analytical methodologies are required, as well as equipment with a high degree of precision and resolution, which allows to detect the compounds of interest unequivocally, which represents a great challenge, given the high costs that this technology represents. A great concern has been manifesting on some part of the Mexican population, who knows about the negative impacts on human health, represented by exposure to certain types of pesticides. For these reasons, this chapter aims to give an overview of the current situation of the type of pesticides that are used in greater proportion in agricultural activity, as well as their levels of concentration.

Key words Pesticide residues, Exposure, Risk, Challenges

1 Introduction

The intensive use of pesticides in agricultural activity has allowed increasing productivity due to its capacity in the control of vector-borne diseases [1]. The vast majority of these compounds are persistent in the environment, even at very low concentration levels (ppb). Consequently, the exposure of the general population to different pesticide residues is almost inevitable. The agricultural use of chemical synthesis pesticides in Mexico is the result of the adoption of the technological paradigm of modernization in agriculture, known as "Green Revolution," since the 1940s. This

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paradigm proposes an intensive industrial agriculture carried out in dependent monocultures external inputs, such as seeds, fertilizers, pesticides, agricultural machinery, and water supply [2].

Despite the benefits that pesticides represent for pest control, serious health risks associated with them from the exposure of farmers when mixing and applying pesticides or working in treated fields and from residues on food and in drinking water for the general population have been raised [3, 4]. The inappropriate and indiscriminate use of these substances has caused a number of accidental poisonings, and even the routine use of pesticides can pose significant short- and long-term health risks to farmers.

In Mexico, farmers face great risks of exposure due to the use of pesticides that are banned in other countries, incorrect application techniques, poorly maintained or totally inappropriate spraying equipment, inadequate storage practices, and often the reuse of old pesticide containers for food and water storage [5].

In addition to the above, the environmental and biological monitoring of pesticide residues is not carried out periodically, which represents a serious public health problem, since the real levels of these substances are not known, while the health of a large part of the population most exposed to these substances continues to deteriorate.

2 Classification of Pesticides Used in Mexico

183 active ingredients are authorized in Mexico as highly dangerous in different uses (agricultural, domestic, gardening, industrial). Regarding the characteristics of its danger to human health, it is known that almost a third, 63 active ingredients, have a high acute toxicity (34.43%), according to WHO classification 1A and 1B, plus those that can be fatal by inhalation and are not included in the above classification. Considering the chronic toxicity, 43 probable human cancer-causing pesticides (23.50%) are authorized according to the United States Environmental Protection Agency (US-EPA), plus others classified by other organisms; 35 pesticides considered endocrine disruptors (19.13%) according to criteria of the Global Harmonized System accepted by the European Union; 21 pesticides that are toxic to reproduction (11.48%); and two that are mutagenic [6].

Taking into account the environmental toxicity of highly dangerous pesticides authorized in Mexico, it stands out that about half (44.81%) have a very high toxicity in bees and can cause death at doses greater than 2 micrograms per bee according to the US-EPA. Regarding those authorized in international environmental conventions, the largest number [15] is included in Annex III of the Rotterdam Convention due to the toxicity of its formulations or because they are prohibited in other countries; three pesticides

(DDT, endosulfan, and pentachlorophenol) are included in the Stockholm Convention on Persistent Organic Pollutants; and only one pesticide, the fumigant methyl bromide, is included in the Montreal Protocol on substances that destroy the ozone layer [6].

2.1 Legal Framework for the Use of Pesticides

The manufacture, commercialization, and application of pesticides, among other processes, are regulated and controlled by the federal authorities of the country. The official legal instruments that govern these activities are the general health laws (General Health Law) [7], agriculture (Federal Law of Plant and Animal Health) [8, 9], and ecological (General Law of Ecological Balance and Environmental Protection) [10], which in turn lead to Official Mexican standards, which are of scope throughout the national territory. Likewise, there is the regulation of the Inter-secretarial Commission for the Control of the Process and Use of Pesticides and Toxic Substances, which establishes the bases for an adequate management of these compounds and their formulations [11].

2.2 Pesticides Applied in Mexico

Pesticides used in Mexico are classified according to their composition and the use for which they are intended. Chemical formulations are used for agricultural, domestic, forestry, industrial, gardening, livestock, and urban purposes. The most frequently used chemical groups are arsenicals, organochlorines, triazines, organophosphates, benzoylureas, carbamates, neonicotinoids, dinitrocompounds, organometallic, thiocarbamates, pyrethroids, and derivatives of coumarin and urea. In addition to chemicals, microbial, biochemical, and botanical pesticides are also authorized [6].

In the management of agricultural pests, there is a wide range of products, and the user freely decides which of all to use according to successful experiences in past cycles, as well as recommendations from professionals, suppliers, and producers. The criteria for the selection of formulations are based on practical aspects and safety in handling, but above all the economic value for the user.

Table 1 presents the 30 active ingredients of the highly dangerous pesticides with the highest number of current authorized registrations in Mexico. It includes any of the uses of these ingredients (agricultural, livestock, domestic, urban, industrial) that together account for more than two-thirds (69.20%) of the total authorized registrations of highly dangerous pesticides in our country at the end of 2016. In the first place, the insecticides parathion methyl, chlorpyrifos ethyl, cypermethrin, malathion, permethrin, mancozeb, chlorothalonil, glyphosate, atrazine, and deltamethrin stand out, accounting for almost 41.16% of the total of highly dangerous pesticides authorized in Mexico. Pesticide category I and II can cause death if they are ingested, by contact with the skin, or if they are inhaled, while those of category III are toxic by ingestion, by

Table 1
Active ingredients of the highly dangerous authorized in Mexico

	Active ingredient	Type	Toxicological category	Classification
1	Methyl parathion	Insecticide	II	Organophosphate
2	Chlorpyrifos ethyl	Insecticide	III	Organophosphate
3	Cypermethrin	Insecticide	III	Pyrethroid
		Acaricidal		
4	Malathion	Insecticide	IV*	Organophosphate
5	Permethrin	Insecticide	IV	Pyrethroid
6	Mancozeb	Fungicide	IV	Dithiocarbamate
7	Chlorothalonil	Fungicide	IV*	Aromatic
				Polychlorinated
8	Glyphosate	Herbicide	IV*	Phosphonomethyl glycine
9	Atrazine	Herbicide	IV	Triazine
10	Deltamethrin	Insecticide	III	Pyrethroid
11	Methamidophos	Insecticide	II	Organophosphate
		Acaricidal		
12	Dimethoate	Insecticide	III	Organophosphate
13	Dichlorvos	Insecticide	II	Organophosphate
14	Diuron	Herbicide	IV*	Derived from urea
15	Imidacloprid	Insecticide	IV	Imida
16	Cupric hydroxide	Fungicide	IV	Inorganic
17	Carbofuran	Insecticide	II	Carbamate
		Nematicide		
18	Endosulfan	Insecticide	II	Organochlorine
		Acaricidal		
19	Bromadiolone	Rodenticide	I	Coumarin
20	Abamectin	Insecticide	II	Pentacycline
		Acaricidal		
21	Methomyl	Insecticide		Carbamate
22	Monochrome	Insecticide	II	Organophosphate
		Acaricidal		
23	Bifenthrin	Insecticide	III	Pyrethroid
		Acaricidal		
24	Lambda	Insecticide	III	Pyrethroid
	Cyhalothrin			

(continued)

Table 1
(continued)

	Active ingredient	Type	Toxicological category	Classification
25	Tetramethrin	Insecticide	IV*	Pyrethroid
26	Propoxur	Insecticide	III	Carbamate
27	Fenvalerate	Insecticide	III	Pyrethroid
28	Carbendazim	Fungicide	IV*	Benzimidazole
29	Trifluralin	Herbicide	IV*	Nitrosamine
30	Acefate	Insecticide	IV	Organophosphate

contact with the skin, or if they are inhaled. The categories with the lowest acute toxicity are IV and V [12].

2.3 Highly Dangerous Pesticides Authorized in Mexico and Prohibited in Other Countries

Table 2 shows 42 of the main pesticides authorized in Mexico, but prohibited in other countries. One of the main drawbacks represented by this list of compounds is that a large part of Mexico's agricultural production is exported to the United States and Europe, where the legislation is very strict regarding residual levels of pesticides, and if the products do not comply with the permissible limits, cannot be marketed. Recently, training programs have been implemented with farmers (good agricultural practices) to allow their products to be marketed; However, the lack of resources on the part of the government and the lack of awareness of many producers have avoided these programs from having good results.

In an inventory of pesticides carried out in two of the main growing areas of the country, it was observed that the most used pesticides were chlorpyrifos ethyl, methamidophos, malathion, atrazine-amethrin, cyromazine, pymetrozine, zeta cypermethrin, lambda-cialothrin, and cyalothrin. Likewise, the presence of paraquat, 2-4-D, and glyphosate were detected, which have been banned in many countries due to their harmful effects on the environment and health [13]. On the other hand, in a rural development district located in the municipalities of Hermosillo, San Miguel de Horcasitas and Carbó (Sonora, Mexico), Silveira-Gramont et al. (2018) reported 23 pesticides used for the protection of wheat, vine, chickpea, walnut, alfalfa, pumpkin, orange, watermelon, sorghum, and safflower [14]. The most important compounds were chlorothalonil, 2,4-D, methyl parathion, endosulfan, paraquat, glyphosate, malathion, chlorpyrifos, methyl azinphos, and mancozeb; all of them with different adverse health effects reported by the International Program on Chemical Safety of the World Health Organization [15]. Meanwhile Ortega-Martínez et al. (2014) described more than 10 families of compounds used in greenhouse plants in Chignahuapan (Puebla,

Table 2
Main pesticides authorized in Mexico, but prohibited in other countries

	Pesticide Active ingredient	Criterion Highly dangerous FAO-WHO	Criterion Pesticide Action Network (international PAN)	Number of prohibited Countries
1	Endosulfan	1	1	75
2	DDT	1	1	71
3	Captafol	1	1	64
4	Pentaclophenol and salts	1	1	62
5	Monochrotophos	1	1	60
6	Methyl parathion	1	1	59
7	Aldicarb	1	1	56
8	Carbofuran	1	1	49
9	Phosphamidon	1	1	49
10	Methamidophos	1	1	49
11	Alachlor	1	1	48
12	Dicofol			45
13	Carbosulfan		1	40
14	Triazophos	1	1	40
15	Azinfos-methyl	1	1	39
16	Disulfoton	1	1	38
17	Paraquat			38
18	Pentachloronitrobenzene			38
19	Atrazine		1	37
20	Phorato	1	1	37
21	Mevinphos	1	1	37
22	Methoxychlor		1	36
23	Methyl bromide	1	1	35
24	Chloropicrin		1	34
25	Metidathion	1	1	34
26	Terbufos	1	1	34
27	Amitraz			33
28	Benomyl	1	1	33
29	Carbaryl	1	1	33
30	Phonophos			33
31	Vinclozolin	1	1	33

(continued)

Table 2
(continued)

	Pesticide Active ingredient	Criterion Highly dangerous FAO-WHO	Criterion Pesticide Action Network (international PAN)	Number of prohibited Countries
32	Zineb		1	33
33	Dichlorvos	1	1	32
34	Ometoate	1	1	32
35	Trichlorfon		1	32
36	Acetafe		1	31
37	Cadusaphos	1	1	31
38	Edifenfos	1	1	31
39	Maneb	1	1	31
40	Quinalphos		1	31
41	Simazine			31
42	Vamidothion	1	1	31

Mexico), of which the most common were copper compounds (cupric hydroxide, copper sulfate, and cymoxanil), carbamates (propamocarb hydrochloride, carbofuran, and methomyl), dithiocarbamates (mancozeb, maneb, and metam sodium), and pyrethroids (betaciflutrin and cypermethrin) [16]; 38 and 90% of the floriculturists interviewed indicated the use of methyl parathion and carbofuran, respectively, whose danger is extreme (IA) in the case of methyl parathion and high (IB) for carbofuran, according to the IPCS classification—WHO (2010) [15].

A different approach is used in the area of vector-borne diseases (dengue, malaria, scorpion, Chagas disease, chikungunya, Zika, among others) because the health authorities, federal and/or state, are responsible for its prevention and control using inputs that do not represent a high risk of exposure for the inhabitants of urban areas. The National Center for Disease Prevention and Control (CENAPRECE) evaluates insecticides that will be used exclusively for these purposes under specific technical and operational standards [17].

3 Routes of Exposure to Pesticides

The main source of exposure to pesticides is agricultural activity, it is estimated that approximately 50% of the pesticides applied are distributed in air, water, and soil. This distribution will depend on

the prevailing climatic conditions, such as wind speed, and direction, solar radiation, temperature, relative humidity, the physical state of the applied product (liquid, solid, gas), and will also depend on the technique used for the application (aerial, terrestrial) [18].

Human exposure to pesticides may occur through occupational exposure in the case of agricultural workers in open fields and greenhouses, workers in the pesticide industry, and in the control of domestic pests [3, 4, 19]. Obviously, the groups of people with the highest risk of exposure and acute poisoning are workers who mix, load, transport, and apply formulated [20]. Usually, exposure to pesticides can occur from accidental spills of chemicals, leakages, faulty spraying equipment, or lack of training to handle these substances.

Exposure of the general population to pesticides occurs mainly through eating food and drinking water contaminated with pesticides and also can occur when living close to a workplace that uses pesticides or even when workers bring home contaminated tools [21]. Exposure affecting the general population is ubiquitous and it tends to be chronic.

The types of pesticides are diverse that in prolonged periods, from multiple sources and at low doses, enter the body using different routes. The main sources of exposure in the population are foods of plant origin (fruits, vegetables, cereals, legumes) or animal (beef, pork, and its derivatives, fish, dairy products, egg, etc.), and to a lesser extent water, air, land, and contaminated fauna and flora [22, 23].

In this sense, it is important to mention that in order to avoid intensive exposure of farmers and the general population, training programs have been implemented in some States of the republic, to obtain accreditation of production units, in the application of Good Use and Management of Agrochemicals (BUMA, for its acronym in Spanish) [24].

4 Pesticide Residues Monitoring in Mexico

Analysis of pesticides in environmental and biological samples has undergone rapid development in recent years. The methodologies implemented have focused mainly on the organophosphate and organochlorine pesticides [25–28], synthetic pyrethroid insecticides [29, 30], triazines [31, 32], chloroacetanilides [33, 34], carbamates herbicides [31, 35], and chlorophenols [36]. As well as the metabolites of some pesticides such as organophosphate pesticides and specific metabolites of chlorpyrifos, malathion, diazinon, and methyl parathion. Other pesticide metabolites frequently monitored in biological samples are p, p′-DDE, atrazine mercapturate, 2-isopropoxyphenol, and 3-phenoxybenzoic acid [36].

In general, the methodology for the analysis of pesticides includes the following stages: (a) sample pretreatment with the aim of separation, preconcentration, and sometimes derivatization of component(s) of interest; (b) instrumental analysis of the treated sample; and (c) interpretation of results. These procedures have been applied to diverse biological samples such as serum, plasma, whole blood, umbilical cord, urine, breast milk, and amniotic fluid.

The objective of the treatment of the sample is the extraction of the compounds of interest. In solid samples, pesticides can be extracted by grinding, mixing, stirring, pressing, and pulverizing directly or after drying as enhanced solvent extraction methods, followed by solvent or liquid extraction [37]. Some of these methods generally require concentrating the analytes and a cleanup step, prior to chromatographic analysis. Meanwhile liquid samples can be processed by using pressurized liquid extraction (PLE) [38], microwave-assisted extraction (MAE), [39], ultrasonic extraction (USE) [40], supercritical fluid extraction (SFE) [41] by solvent–solvent extraction methods or sorption methods such as, solid-phase extraction (SPE) [42], solid-phase microextraction (SPME) [43], headspace-solid-phase microextraction (HS-SPME) [44], and stir bar sorptive extraction (SBSE) [45]. More recently, for the extraction of pesticides from solid or liquid samples, a fast and efficient method has been used (QuEchERS), quick, easy, cheap, effective, rugged, and safe [46].

One of the greatest challenges for analytical chemists has been the development of multianalyte methods, which allow the detection of compounds of different vapor pressures, polarities, solubilities, and pK_a [47]; in the same way, sensitive and selective detection systems are required.

Usually, for the analysis of many pesticides, gas chromatography with an electron capture detector (ECD) and coupling to mass spectrometry (MS) or high-resolution mass spectrometry (HRMS) have been used [34], allows highly specific MS analysis, and thereby can improve detection limits by avoiding most of the interferences, especially when analyzing complex matrices such as biological samples. However, many compounds cannot be analyzed by gas chromatography, given their low volatility and/or high polarity; for these reasons, the use of liquid chromatography coupled with mass spectrometry has proven to be a good technological alternative for the detection of pesticides in complex matrices [48].

In the last two decades, liquid chromatography has been widely used for the analysis of biological and environmental samples. Coupling to soft ionization interfaces has allowed the study of a great variety of labile molecules with high sensitivity (on the order of pg). Among the main sources of ions is the electrospray ionization (ESI). ESI works well with moderately polar molecules and is thus well suited to the analysis of many metabolites, xenobiotics, and peptides [49]. The ionized analytes are then transferred into

the high vacuum of the mass spectrometer via a series of small apertures and focusing voltages. The ion source and subsequent ion optics can be operated to detect positive (ESI^+) or negative (ESI^-) ions, and switching between these two modes within an analytical run can be performed.

Molecules with molecular weights (usually less than ≈ 500 Da) with a single functional group capable of carrying electrical charge give predominantly singly charged ions. Another soft ionization alternative for small molecules that are not well ionized by ESI is the atmospheric pressure chemical ionization (APCI). A corona discharge takes place near the tip of the capillary, initially ionizing gas and solvent molecules present in the ion source. These ions then react with the analyte and ionize it via charge transfer. A feature of APCI and singly charged ions dominate [50, 51].

Recently, the use of triple quadrupole (QqQ) and time of flight (Q-TOF) has been very popular, given its precision and high resolution. In both cases, the confirmation of the compounds is carried out through the use of multiple reaction monitoring mode (MRM) and quantification by LC-MS-MS [52].

It is important to note that despite the fact that methodologies and infrastructure have improved in Mexico, few laboratories and institutions carry out continuous monitoring of pesticide residues in environmental samples, human beings, and food products. In large part due to the high operating costs that this implies and the lack of public policies aimed at caring for the health of the population. It is also important to mention that most of the studies that have been carried out in the republic have focused on the evaluation of organochlorine pesticides in human beings.

5 Current Status of Pesticide Levels Reported in Mexico in Humans

Despite the large number of pesticides used in Mexico, there are few studies that provide evidence of the real levels of residues of these substances in environment and in humans. The main findings reported by some authors in the last 30 years are described below.

5.1 Breast Milk

The analysis of organochlorine pesticide residues has been a priority in the research on human exposure to environmental pollutants in Mexico. Breast milk is the ideal sample for detection due to its high fat content, for which organochlorine pesticides have a great affinity. The first reports were made by Albert et al. 1976, 1977 [53]. The total DDT levels (sum of pp′-DDT, pp′-DDD, and pp-′-DDE) found in breast milk were 0.266 mg/kg in 1976 and 0.306 mg/kg in 1977–1978. These concentrations exceeded those obtained in Chile, Guatemala, Argentina and were two or three times higher than those found in the USA and Canada [53]. Subsequently, Waliszewski et al., 1996, obtained higher levels

of these compounds in Veracruz, Mexico (Mean Σ-DDT = 6.440 mg/kg) [54]. The high concentrations of DDT in human samples are due to chronic exposure caused by the application of the insecticide in agriculture and especially in the National Campaign for the Eradication of Malaria, initiated in 1956 in vast malarious areas of the Mexican Republic [55]. Meanwhile Lara et al., 2000, analyzed the metabolite pp′-DDE, in nursing mothers, finding very high values in rural localities in the state of Morelos (13.320 mg/kg), followed by Cuernavaca (4.280 mg/kg) and Mexico City (2.490 mg/kg) [56]. This compound remains mainly in the environment and in food; however, the magnitude of exposure has decreased due to the ban on the use of DDT at the end of 1999 [55]. Waliszewski et al., 2009, reported lower pp′-DDE and pp′-DDT concentrations compared to previous studies (Mean: 1.807 and 0.528 mg/kg, respectively) [57]. Meanwhile, in Guerrero state, median concentrations of pp′-DDE = 0.760 mg/kg and pp′-DDT = 0.045 mg/kg were reported by Chávez et al., 2018 [58].

Other organochlorine pesticides have been detected in breast milk. For example, in Yucatán state, located southeast of the republic, significant amounts of Σ-chlorobenzenes (0.677 mg/kg lipid), Σ-HCH (0.754 mg/kg lipid), Σ-chlordanes (0.975 mg/kg lipid), Σ-drins (0.404 mg/kg lipid), endosulfan II (0.277 mg/kg lipid), and mirex (0.295 mg/kg lipid) were reported [59]. For his part, Waliszewski et al., 2009, reported concentration levels for β-HCH of 0.0049 mg/kg per day in milk from mothers from Veracruz, Mexico [57]; these concentrations were similar to those found by Chávez et al., 2018, in milk from mothers from Guerrero, Mexico (β-HCH (0.004 mg/kg lipid) [58].

5.2 Serum and Urine

Human urine and serum can be used in a wide variety of populations, and it can be in children and/or adults, and of both sexes, due to the ease of obtaining it, which is not possible when analyzing breast milk, which it is limited only to breastfeeding mothers.

In a study carried out in the state of San Luis Potosi, Mexico, in urinary samples from children and adolescents, six metabolites of organophosphate compounds called dialkylphosphates were determined [60]. The samples were collected in seasons of low exposure (LE) and high exposure (HE) of pesticides. Concentrations (μg/L) were higher in the high exposure season: dimethyl phosphate (LE = 0.8, HE = 2.3), dimethyl thiophosphate (LE = 15, HE = 23), dimethyl dithiophosphate (LE = 0.4, HE = 0.7), diethyl phosphate (LE = 4.0, HE = 7.6), diethyl thiophosphate (LE = 1.4, HE = 2.3), and diethyl dithiophosphate (LE = 0.4, HE = 0.4).

Other studies carried out in people occupationally exposed to pesticides, as in the case of flower growers from Morelos, Mexico, found this same class of metabolites in urine [61]. The highest

concentration was dimethyl phosphate, with 80.6 μg/g creatinine, and in lower concentrations of dimethyl thiophosphate, dimethyl dithiophosphate, diethyl phosphate, diethyl thiophosphate, and diethyl dithiophosphate, with 29.7, 11.2, 16.7, 10.2, and 6.7 μg/g creatinine, respectively. Meanwhile, Lopez-Galvez et al., 2018, determined the presence of organophosphate and pyrethroid pesticide metabolites [62]. These compounds were detected in more than 70% of the urine samples collected from farmers in Sonora, Mexico. The organophosphate metabolites 3,5,6-trichloro-2-pyridinol and para-nitrophenol had higher concentrations (3.56 and 1.63 μg/g creatinine, respectively) and 3-phenoxybenzoic acid (1.83 μg/g creatinine).

In the other hand, organochlorine compounds have been analyzed in blood serum. Lindane (Mean = 3.947 mg/kg) and pp′-DDE (1.702 mg/kg) were detected in more than 85% of infants from nine states of the Mexican Republic ($n = 229$) [63]. Likewise, in adults from Veracruz, Mexico (serum and lipids), higher concentrations of pp′-DDE (13.120 mg/kg) were reported [64]. This difference between the values of children and adults is mainly due to the time of exposure to DDT, which increases in older people. Finally, other pesticides other than DDT were determined in adults from the Soconusco Region, Chiapas, Mexico γ- and β-HCH (1.9 and 4.6 μg/L, respectively), heptachlor (2.9 μg/L), β-endosulfan (3.1 μg/L), and endrin aldehyde (2.9 μg/L) [65].

6 Risk Assessment to Human Health in Mexico

Risk assessment of pesticide impact on human health is not an easy task because of differences in the periods and the levels of exposure, type of pesticides used in the field, and the geographic and meteorological characteristics of the agricultural areas where pesticides are applied [66, 67]. One of the main difficulties is the degree of training of the people who prepare the mixtures in the field, the pesticide sprayers, pesticide storage facilities, greenhouses, or open fields. Considering that human health risk is a function of pesticide toxicity and exposure, a greater risk is expected to arise from high exposure to a moderately toxic pesticide than from little exposure to a highly toxic pesticide. Despite the evidence on the risk posed to human health by exposure to pesticides, dietary exposure of the population to pesticide residues found on food and drinking water consists of a potential threat to human health and is still the subject of great scientific controversy [68].

The study of health risks due to exposure to contaminants through food is useful to generate evidence that allows the establishment of regulations and control mechanisms for the protection of the consuming population. Cow's milk and its derived products are frequent foods in the diet, but in turn they can contain

organochlorine pesticides due to their high amount of fat. In a study conducted in the Central region of Chiapas, 36 milk samples from four organic farms were analyzed [69]. The concentrations of total DDT and the α- and β-HCH isomers were below the maximum residual limits of the FAO/WHO *Codex Alimentarius* (0.05 and 0.10 mg/kg). Lindane and aldrin plus dieldrin also presented levels lower than those established, which are 0.01 mg/kg for lindane and 0.006 mg/kg for aldrin plus dieldrin. Meanwhile, the concentrations of β-HCH, pp′-DDT, and pp′-DDE detected in milk from Veracruz, Mexico, exceeded the limits of the Codex Alimentarius (FAOSTAT 1999) [70]; these results were totally opposite, so the authors recommended that it is necessary to monitor the levels of these contaminants in milk to improve food safety and to know their trend over time.

On the other hand, in the analysis of meat products, Aguayo et al., 2020, found variable frequencies and concentrations of organochlorine pesticides in 120 specimens of four species of fish (*Sphyraena ensis, Centropomus robalito, Diapterus brevirostris, and Scomberomorus sierra*) consumed in Nayarit, Mexico, located in the southeastern part of the Gulf of California [71]. In the absence of a comparison with respect to the maximum residual limits of these products established by regulatory agencies in Mexico, the authors used the limits established by the US Food and Drug Administration (FDA). The concentrations of pesticides did not exceed these limits, so the consumption of these products does not imply risks to human health. For its part, the carcinogenic risk due to the consumption of snapper fish (*Lutjanus spp.*) was studied in the Laguna San Ignacio-Macapule-Navachiste complex, in Sinaloa, Mexico [72]. In general, the levels of organochlorine pesticides were lower than the limits of the US-EPA, but some samples tested had carcinogenic risk concentrations if 0.229 kg portions are consumed from once or twice a month (Σ-Chlordane), three times a month (S-HCH), and more than four times a month (-Σ-Heptachlor). Likewise, Waliszewski et al., 1996, collected samples of adipose and muscle tissue and viscera from cattle for the analysis of organochlorine pesticides in the municipal slaughterhouse of Veracruz [73]. The compounds detected were HCB, β-HCH, γ-HCH, and pp′-DDE, of which only β-HCH (0.149 mg/kg) slightly exceeded the levels established by the US-EPA regulation (0.100 mg/kg). Finally, Pardío et al., 2012, estimated the daily intakes of organochlorine pesticides by analyzing beef at different times of the year, rainy and dry season [74]. During the rainy season, the estimated daily intakes were increased for γ-HCH and Σ-DDT (3.35 and 1.22 μg/kg bw/day), exceeding between 0.7 and 0.12 times the acceptable daily intake (FAO/WHO 2009).

Meanwhile in vegetable products, Pérez et al., 2016, reported levels of pesticides in a species of cactus Xoconostle (*Opuntia*

joconostle), which is of commercial importance for its medicinal and culinary uses in the central region of Mexico. Heptachlor, aldrin, heptachlor epoxide, and the pp′-DDE metabolites had the highest concentrations (Mean = 0.516, 0.490, 0.295, 0.455 ng/g). However, there is no data in the Codex Alimentarius on the maximum permissible limits in this vegetable to make an objective comparison. Therefore, it is necessary to gather enough scientific evidence to establish safety levels, both for this fruit and for other foods for human consumption [75].

Also, Ramírez-Bustos et al., 2018, in a study carried out in the state of Morelos, Mexico, detected the presence of chlorpyrifos 0.309 mg/kg, dimetomorf I 0.029 mg/kg, malathion 0.155 mg/kg, omethoate 0.032, carbendazim 0.090 mg/kg, and imidacloprid 0.058 mg/kg, in nopal vegetable (*Opuntia ficus-indica*), and it should be noted that all concentrations were below the MRL established for this type of product [5].

7 Conclusions

In Mexico, there are relatively few studies that reveal the real levels of pesticide residues in the environment, food, drinking water, and in human beings, which generates great uncertainty in the population, which, as the authorities responsible for caring for health, do not has a defined strategy to mitigate this impact.

The main challenge that Mexico faces in managing the proper use and control of the pesticides used has to do in part with the current legislation, which has structural deficiencies. On the other hand, it should be considered that decision-making is strongly influenced by political interests and social pressures, which makes difficult to align common goals in public health and environmental protection. Finally, not enough resources are invested for the constant monitoring of these substances.

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Chapter 7

Residue Analysis of Organochlorine, Organophosphate, and Pyrethroid Pesticides in Human Biological Specimens by Gas Chromatography–Microelectron Capture Detector (GC-μECD)

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Abstract

Organochlorine, organophosphate, and pyrethroid pesticides are extensively used agents, which may result in either chronic or acute intoxication.

Here, multiresidue methods for detecting various components of these pesticide families are presented using different extraction/cleanup techniques and GC-μECD detection. These methods are used for many sample matrices, namely plasma, breast milk, and umbilical cord blood.

Key words Organochlorine pesticides, Organophosphate pesticides, Pyrethroid pesticides, Plasma, Breast milk, Umbilical cord blood, GC-μECD, Liquid–liquid extraction, Solid-phase extraction

1 Introduction

Pesticides are widely used for agriculture, insect control, forestry, and building protection. In addition, vector control, through the use of pesticides, performs a crucial role in the prevention and control of infectious diseases such as malaria and typhus [1].

These pollutants had received considerable attention in the last century with regard to their persistence, bioaccumulative characteristics in ecosystems, biomagnification up the food chain, and long-term toxic effects to humans and animals [2, 3]. Therefore, pesticides such as aldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), dieldrin, endrin, heptachlor, hexachlorobenzene, and mirex have been removed or their production and use have been restricted in the Stockholm Convention in 2001.

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Risk assessment of organochlorine, organophosphate, and pyrethroid pesticides is of great concern, due to possible adverse health effects, such as endocrine disruption, oxidative stress, learning/developmental disorders, brain and nervous system disorders, and immune system disorders [4].

The availability of validated exposure data is a critical component for assessing the causal relationships between exposure to pesticides and health effects. Therefore, fast and robust methods are necessary that can measure pesticides and untargeted molecules, such as their metabolites, in biological matrices.

This chapter aims at giving current analytical methods available in the Analytical Toxicology Advisory Laboratory (CENATOXA) for determination of various families of pesticides (organochlorine, organophosphate, and pyrethroid pesticides) in plasma, breast milk, and umbilical cord blood specimens, focusing on a small amount of matrix and sample pretreatment (extraction, cleanup, and concentration).

Plasma samples are analyzed using a method adapted from that described by Dale et al. [5]. The procedure utilizes a simple liquid–liquid extraction and cleanup with potassium carbonate to determine organochlorine, organophosphate, and pyrethroid pesticides (*see* **Note 1**).

Extraction of pesticides from breast milk specimens is performed according to a procedure modified from the one described by Hovander et al. [6], with a mixture of ethyl ether:hexane (1:1), and cleanup with activated silica gel, silica gel activated and modified with sulfuric acid (44%, w/w), and anhydrous sodium sulfate (*see* **Note 2**).

For umbilical cord blood specimens, we used a solid-phase extraction and cleanup with activated silica gel, silica gel activated and modified with sulfuric acid (44%, w/w), and anhydrous sodium sulfate using a technique derived from the literature [7] (*see* **Note 3**).

The samples are then injected on the gas chromatography microelectron capture detector (GC-μECD) using a dual-column analysis and an oven temperature program.

Specifically, 3,3′,4,4′-tetrabromodiphenyl (PBDE 77) is used to quantify pesticides in breast milk and umbilical cord blood specimens.

The analytical methods described in this chapter allow the detection of pesticide residues in human biological samples at concentrations of ng/mL or ng/g lipid, useful for environmental exposure.

2 Materials

All glassware must be scrupulously cleaned with detergent solution, rinsed thoroughly with distilled water, and then rinsed with the solvent to be used (*see* **Note 4**). Glassware to be used for analysis must be kept separate and must not be used for any other purpose.

2.1 Extraction

2.1.1 Plasma

1. Blank plasma matrix: Bags of plasma are obtained from the local blood bank when they are no longer suitable for use. Each bag is pooled into a lot and analyzed under all methods that the laboratory currently utilizes (*see* **Note 5**).
2. 5% (w/v) Aqueous potassium carbonate solution: Add 5.0 g of K_2CO_3 to a 0.1 L volumetric flask and fill to volume with distilled water. Mix well to dissolve completely. Store at 4 °C into an amber glass flask for up to 1 month.
3. Activated anhydrous sodium sulfate: Add 100.0 g of Na_2SO_4 to a 0.25 L beaker and activate at 100 °C for 2 h. Store at 100 °C, taking care to properly cover the beaker with metalized paper to avoid contamination.

2.1.2 Breast Milk

1. Blank human milk: Breast milk is collected from the donors 10–15 days postpartum. Each sample is pooled into a lot and analyzed under all methods that the laboratory currently utilizes.
2. Activated anhydrous sodium sulfate: Add 100.0 g of Na_2SO_4 to a 0.25 L beaker and activate at 100 °C for 2 h. Store at 100 °C, taking care to properly cover the beaker with metalized paper to avoid contamination.
3. Clean glass wool: Add 10.0 g of glass wool pesticide grade (silanized) to a 0.60 L porcelain capsule and perform sequential washes with methanol, acetone, and hexane. Dry at room temperature and store in a 0.25 L beaker, taking care to properly cover with metalized paper to avoid contamination.
4. 1% (w/v) Potassium chloride: Add 1.0 g of KCl to a 0.10 L volumetric flask. Fill to volume with distilled water and mix well. Store in an amber glass bottle at 4 °C for a month.
5. Activated silica gel: Add 100.0 g of silica gel 60 (0.063–0.200 mm) for column chromatography (70–230 mesh) to a 0.60 L porcelain capsule and dry in a muffle furnace at 280 °C for 24 h. Cool at room temperature and wash with dichloromethane:hexane (1:1). Remove the organic phase and store at 100 °C (3 months) in a 0.25 L beaker, taking care to properly cover with metalized paper to avoid contamination.

6. Silica gel activated and modified with sulfuric acid (44%, w/w): Add 36.0 g of activated silica gel to a 0.05 L beaker and 15 mL of sulfuric acid 98%. Mix well with a glass stirring rod and store at room temperature.

2.1.3 Umbilical Cord Blood

1. Blank umbilical cord blood: After delivery of the newborn, the umbilical cord is clamped, and 5–7 mL of cord blood is collected by gravity into an EDTA-anticoagulated tube from the umbilical vein after cleaning the cord. Each sample is pooled into a lot and analyzed under all methods that the laboratory currently utilizes.
2. Activated anhydrous sodium sulfate: Add 100.0 g of Na_2SO_4 to a 0.25 L beaker and activate at 100 °C for 2 h. Store at 100 °C, taking care to properly cover the beaker with metalized paper to avoid contamination.
3. Clean glass wool: Add 10.0 g of glass wool pesticide grade (silanized) to a 0.60 L porcelain capsule and perform sequential washes with methanol, acetone, and hexane. Dry at room temperature and store in a 0.25 L beaker, taking care to properly cover with metalized paper to avoid contamination.
4. Activated silica gel: Add 100.0 g of silica gel 60 (0.063–0.200 mm) for column chromatography (70–230 mesh) to a 0.60 L porcelain capsule and dry in a muffle furnace at 140 °C for 48 h. Store at 100 °C (3 months) in a 0.25 L beaker, taking care to properly cover with metalized paper to avoid contamination.
5. Silica gel activated and modified with sulfuric acid (44%, w/w): Add 36.0 g of activated silica gel to a 0.05 L beaker and 15 mL of sulfuric acid 98%. Mix well with a glass stirring rod and store at room temperature.
6. SPE columns. Waters, Oasis HLB (60 mg) 3 mL columns: Store in a cool dry area. If a partial bag remains, it is stored in a desiccator.

2.2 Calibrators, Internal Standard, and Quality Controls

1. 1000 μg/mL Stock standard solutions, in hexane: *Organochlorines*: aldrin, α-chlordane, γ-chlordane, dieldrin, endrin, α-endosulphan, β–endosulphan, endosulphan sulfate, HCB, α-HCH, β-HCH, γ-HCH, δ-HCH, heptachlor, heptachlorepoxide, methoxychlor, mirex, o,p′-DDD, o,p′-DDE, o,p′-DDT, p,p′-DDD, p,p′-DDE, p,p′-DDT; *Organophosphates*: chlorpyrifos-ethyl, chlorpyrifos-methyl, and *Pyrethroids*: biphentrin, λ-cyhalothrin, cypermethrin, deltamethrin, fenvalerate, permethrin, and tefluthrin are purchased as solid (Table 1) and stored in the freezer until consumed. To a 10 mL volumetric flask, add 10.0 mg of each solid. Fill to

Table 1
Organochlorine, organophosphate, and pyrethroid pesticides

Compound	Synonyms	CAS	Chemical formula	Presentation (mg)	Solubility
Organochlorine pesticides					
Aldrin	–	309-00-2	$C_{12}H_8Cl_6$	10	Hexane/isooctane
α-Chlordane	Cis-chlordane	5103-71-9	$C_{10}H_6Cl_8$	10	Hexane/isooctane
γ-Chlordane	Trans-chlordane	5103-74-2	$C_{10}H_6Cl_8$	10	Hexane/isooctane
Dieldrin	Exo-dieldrin	60-57-1	$C_{12}H_8Cl_6O$	10	Hexane/isooctane
o,p′-DDE	2,4′-DDE	3424-82-6	$C_{14}H_8Cl_4$	10	Hexane/isooctane
p,p′-DDE	4,4′-DDE	72-55-9	$C_{14}H_8C_{l4}$	10	Hexane/isooctane
o,p′-DDT	2,4′-DDT	789-02-6	$C_{14}H_9Cl_5$	10	Hexane/isooctane
p,p′-DDT	4,4′-DDT	50-29-3	$C_{14}H_9Cl_5$	10	Hexane/isooctane
o,p′-DDD	2,4′-DDD	53-19-0	$C_{14}H_{10}Cl_4$	10	Hexane/isooctane
p,p′-DDD	4,4′-DDD	72-54-8	$C_{14}H_{10}Cl_4$	10	Hexane/isooctane
α-Endosulphan	Endosulphan 1	959-98-8	$C_9H_6Cl_6O_3S$	10	Hexane/isooctane
β–Endosulphan	Endosulphan 2	33213-65-9	$C_9H_6Cl_6O_3S$	10	Hexane/isooctane
Endosulphan sulfate	Thiodan sulfate	1031-07-8	$C_9H6Cl_6O_4S$	10	Hexane/isooctane
Endrin	Hexadrin	72-20-8	$C_{12}H_8Cl_6O$	10	Hexane/isooctane
HCB	Hexachlorobenzene	118-74-1	C_6Cl_6	100	Hexane/isooctane
α-HCH	Alpha-hexachlorocyclohexane	319-84-6	$C_6H_6Cl_6$	10	Hexane/isooctane
β-HCH	Beta-hexachlorocyclohexane	319-85-7	$C_6H_6Cl_6$	10	Benzene
γ-HCH	Lindane	58-89-9	$C_6H_6Cl_6$	10	Hexane/isooctane
δ-HCH	Delta-hexachlorocyclohexane	319-86-8	$C_6H_6Cl_6$	10	Hexane/isooctane
Heptachlor	Heptachlorane	76-44-8	$C_{10}H_5Cl_7$	10	Hexane/isooctane
Heptachlor epoxide A	Heptachlor epoxide endo	28044-83-9	$C_{10}H_5Cl_7O$	10	Hexane/isooctane

(continued)

Table 1
(continued)

Compound	Synonyms	CAS	Chemical formula	Presentation (mg)	Solubility
Methoxychlor	Methoxy-DDT	72-43-5	$C_{16}H_{15}Cl_3O_2$	10	Hexane/isooctane
Mirex	Dechlorane	2385-85-5	$C_{10}Cl_{12}$	10	Hexane/isooctane
Organophosphate pesticides					
Chlorpyrifos	Chlorpyriphos ethyl	2921-88-2	$C_9H_{11}Cl_3NO_3PS$	10	Hexane/isooctane
Chlorpyriphos methyl	Trichlormethylfos	5598-13-0	$C_7H_7Cl_3NO_3PS$	10	Hexane/isooctane
Pyrethroid pesticides					
λ-cyhalothrin	Lambda-cyhalothrin	91465-08-6	$C_{23}H_{19}ClF_3NO_3$	10	Hexane/isooctane
Bifenthrin	Biphenate	82657-04-3	$C_2\ H_{22}ClFO_2$	100	Hexane/isooctane
Cypermethrin	–	52315-07-8	$C_{22}H_{19}Cl_2NO_3$	10	Hexane/isooctane
Deltamethrin	Decamethrin	52018-63-5	$C_{22}H_{19}Br_2NO_3$	250	Hexane/isooctane
Fenvalerate	Phenvalerate	51630-58-1	$C_25H_{22}ClNO_3$	10	Hexane/isooctane
Permethrin	–	52645-53-1	$C_21H_{20}Cl_2O_3$	10	Hexane/isooctane
Tefluthrin	–	79538-32-2	$C_{17}H_{14}ClF_7O_2$	10	Hexane/isooctane

volume with hexane (benzene for β-HCH) and mix well. Store in the freezer at −20 °C (*see* **Note 6**).

2. 10 μg/mL Standard solution, in hexane: To a 10 mL volumetric flask, add 100 μL of each stock solution. Fill to volume with hexane and mix well. Store in the freezer at −20 °C (*see* **Note 6**).
3. 0.10 μg/mL Working calibration standard, in hexane: To a 10 mL volumetric flask, add 100 μL of standard solution (10 μg/mL). Fill to volume with hexane and mix well. Store in the freezer at −20 °C (*see* **Note 6**).
4. 10 ng/mL Working calibration standard, in hexane: To a 10 mL volumetric flask, add 10 μL of standard solution (10 μg/mL). Fill to volume with hexane and mix well. Store in the freezer at −20 °C (*see* **Note 6**).
5. Internal standard for breast milk and umbilical cord blood samples:
   (a) 500 ng/mL Working internal standard solution, in hexane: To a 10 mL volumetric flask, add 100 μL of 3,3′,4,4-′-tetrabromodiphenyl (PBDE 77: 50 μg/mL) (AccuStandard, New Haven-Connecticut, United States). Fill to volume with hexane and mix well. Store in the freezer at −20 °C.
6. Quality controls (QC):
   (a) Blanks: Reagent water and reference matrix blanks are analyzed to demonstrate that they are free of contamination.
   (b) Internal quality control (QCi): From a working calibration standard (10 ng/mL or 0.10 μg/mL) prepared as described in Subheading 2.2, prepare a laboratory control standard that contains each analyte of interest at a concentration of 0.50 ng/mL in plasma (*see* **Note 7**), 0.02 and 0.30 ng/g in breast milk, and 0.20 and 1.50 ng/mL in umbilical cord blood.
   (c) External quality control program: The QCs are obtained from a source external to the laboratory and are used to check laboratory performance with externally prepared test materials (*see* **Note 8**).

2.3 Instrument

1. Dual column analysis:

   GC Capillary Column 1: J&W Scientific DB-XLB, low bleeding and low polarity, 30 m × 0.25 mm I.D., 0.25 μm film thickness.

   GC Capillary Column 2: J&W Scientific DB-1701P, cross-linked and chemically bonded with 14% cyanopropylphenyl

and 86% dimethyl-polysiloxane, 25 m × 0.32 mm I.D., 0.25 μm film thickness.

2. Agilent HP 6890 N gas chromatograph, coupled to a micro-electron capture detector (micro-ECD) and an HP 6890 Series injector.

3 Methods

In the field of clinical and nonfatal forensic medicine, it is extremely important, for therapeutic or legal reasons, to identify and analyze pesticides taken into the body intentionally or accidentally. When pesticide intoxication is suspected, plasma is the most important sample, as the detection of a residual pesticide in a biomedical sample in clinical medicine is conducted to save life.

Hormonal changes that occur during pregnancy and lactation result in the internal mobilization of pesticides many years after initial exposure. Hence, human breast milk is a noninvasive biological matrix that can be used to examine organochlorine residues in human tissues. The organochlorine levels in breast milk reflect maternal burden and can assess possible health risks to breastfeeding infants. In addition, umbilical cord blood is considered an accurate representation of organochlorine levels in human tissues, necessary for the risk assessment of possible adverse health effects in newborns and the identification of vulnerable groups.

3.1 Plasma

Sample Collection, Preservation, and Storage

1. The blood sample collection typically includes a sterile needle, a syringe, and a 17 × 115 mm conical-bottom borosilicate glass centrifuge tube(s).
2. The syringe used contains heparin as anticoagulant. On average, about 5–7 mL of blood will be drawn during the blood collection process. The contents must be homogenized as soon as possible after collection (e.g., tubes should be gently inverted at least three times) to mix the blood with the anticoagulant contained in the syringe in order to avoid clot formation.
3. Centrifuge a heparin-anticoagulated blood sample in a conical-bottom borosilicate glass centrifuge tube, for 10 min at 1200–1500 g.
4. Remove the supernatant plasma carefully with a borosilicate glass Pasteur pipette and store in a conical-bottom borosilicate glass tube (*see* **Note 9**).

Table 2
Calibrator and Internal quality control preparation

Plasma		Breast milk		Umbilical cord blood	
Spike volume (μL)	**Expected concentration (ng/mL)**	**Spike volume (μL)**	**Expected concentration (ng/g)**	**Spike volume (μL)**	**Expected concentration (ng/mL)**
Calibrators		*Calibrators*		*Calibrators*	
10[a]	0.10	5[a]	0.01	10[a]	0.10
25[a]	0.25	10[a]	0.02	25[a]	0.25
50[a]	0.50	25[a]	0.05	50[a]	0.50
10[b]	1.00	50[a]	0.10	10[b]	1.00
25[b]	2.50	10[b]	0.20	25[b]	2.50
50[b]	5.00	20[b]	0.40	50[b]	5.00
Internal quality control		*Internal quality control*		*Internal quality control*	
50[a]	0.50	20[a]	0.04	20[a]	0.20
15[b]	1.50	15[b]	0.30	15[b]	1.50

[a]10 ng/mL working calibration standard
[b]0.10 μg/mL working calibration standard

Sample Preparation

1. 1 mL of specimen is used for the analysis of plasma.
2. 1 mL of distilled water that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standard, and surrogates that are used with samples.
3. 1 mL of blank plasma matrix is treated as a sample in all respects, including exposure to sampling, site conditions, storage, and preservation.
4. Spike calibrators and QCi according to Table 2. Each is prepared by spiking a working calibration solution into 1 mL of blank matrix. The calibration range is 0.10–5.00 ng/mL (*see* **Note 10**). All calibrators and QCi should be vortexed after preparation is complete.

Extraction

1. Pipet samples (calibrators, controls, and unknowns) as described above into 17 × 115 mm conical-bottom borosilicate glass centrifuge tubes with pennyhead stopper (20 mL capacity).
2. Add 1 mL of formic acid (97%), cap, and vortex thoroughly.

3. Add 5 mL of hexane, secure the cap on the tube, and shake (by vortex) vigorously for 1 min.
4. Remove the organic phase with a borosilicate glass Pasteur pipette. This extract is stored in a conical-bottom borosilicate glass centrifuge tube (20 mL capacity). Keep the aqueous phase.
5. Add 5 mL of hexane to the aqueous phase, cap, and vortex thoroughly for 1 min.
6. Remove the organic phase with a borosilicate glass Pasteur pipette.
7. Combine the two hexane extracts, and wash with 2 mL of 5% (w/v) aqueous potassium carbonate solution (*see* **Note 11**). Cap and vortex thoroughly for 1 min.
8. Collect the hexane layer and dehydrate with anhydrous sodium sulfate.
9. Decant the organic layer and carefully transfer the hexane into another conical-bottom borosilicate glass tube.
10. Wash the sodium sulfate with hexane by slowly dripping hexane into the glass tube.
11. Combine the hexane layers, concentrate further to 1000 μL by evaporating the solution under a stream of nitrogen at 60 °C or below, and transfer into a GC autosampler vial ready for determination by GC/ECD.

3.2 Breast Milk

Sample Collection, Preservation, and Storage

1. Breast milk sample is collected from the donors 10–15 days postpartum.
2. The sample milk aliquots (30 mL) are collected by manual expression into a glass container (50 mL Pyrex glass bottle with a Teflon cap), avoiding the use of mechanical breast pumps.
3. The breasts and hands will be kept as clean as possible, and the use of soap should be avoided. If ointment is used on the nipples, it should only be used in the space between milk extractions, and the ointment should be completely removed and the breasts washed before the procedure.
4. The aliquots are frozen in a glass bottle and stored at −20 °C until chemical analysis (*see* **Note 12**).

Sample Preparation

1. 5 g of specimen is used for the analysis of breast milk.
2. 5 g of distilled water that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standard, and surrogates that are used with samples.

3. 5 g of breast milk matrix is treated as a sample in all respects, including exposure to sampling, site conditions, storage, and preservation.
4. Spike calibrators and QCi according to Table 2. Each is prepared by spiking working calibration solution into 5 g of blank matrix. The calibration range is 0.01–0.40 ng/g (*see* **Note 13**). All calibrators and QCi should be vortexed after preparation is complete.

Extraction

1. Weigh samples (calibrators, controls, and unknowns) as described above into 17 × 115 mm conical-bottom borosilicate glass centrifuge tubes with pennyhead stopper (20 mL capacity). Add 50 μL of working internal standard and vortex thoroughly.
2. Transfer samples into 50 mL glass conical separating funnels with glass stopcock. Add 6 mL of 2-propanol and 1 mL of formic acid (85%), cap, and shake thoroughly for 20 s.
3. Add 6 mL of ethyl ether:hexane (1:1), cap, and shake for 5 min.
4. Collect the organic phase into a conical-bottom borosilicate glass centrifuge tube (10 mL capacity). Add 1 mL of potassium chloride (1% w/v), cap, and vortex thoroughly for 1 min.
5. Centrifuge for 5 min at 1008 × *g*.
6. Remove the organic phase with a borosilicate glass Pasteur pipette. This extract is stored in a conical-bottom borosilicate glass centrifuge tube (20 mL capacity). Keep the aqueous phase.
7. Add 3 mL of hexane to the aqueous phase, cap, and vortex thoroughly for 1 min.
8. Remove the organic phase with a borosilicate glass Pasteur pipette.
9. Combine the two organic phases and evaporate under a stream of nitrogen at room temperature.
10. Add 1 mL of H_2SO_4 and 4 mL of hexane, cap, and vortex thoroughly for 1 min. Centrifuge for 5 min at 1008 × *g*.
11. Remove the organic phase with a borosilicate glass Pasteur pipette. This extract is stored in a 20 mL glass vial. Keep the aqueous phase.
12. Add 3 mL of hexane to the aqueous phase, cap, and vortex thoroughly for 1 min. Centrifuge for 5 min at 1008 × *g*.
13. Combine the hexane layers, concentrate further to 500 μL by evaporating the solution under a stream of nitrogen at 60 °C or below.

Cleanup

1. Disposable glass Pasteur pipette. Place a small clean glass wool plug into the broad end of a Pasteur pipette (12 cm × 1 cm Ø) and push it with the narrow end of another Pasteur pipette to the narrow constriction. Add 0.1 g of activated silica gel followed by 1.0 g of silica gel activated and modified with sulfuric acid (44%, w/w) and 0.5 cm of anhydrous sodium sulfate. Tap it gently on the table for uniform packing.
2. Condition the Pasteur pipette columns with 1.2 mL of hexane.
3. Apply the concentrated hexane layer (500 μL) to the columns using a borosilicate glass Pasteur pipette.
4. Each Pasteur pipette column should be washed with 200 μL of hexane and vortexed thoroughly for 30 s. Repeat this procedure one time.
5. Elute with 8 mL of dichloromethane:hexane (1:1) into 26.5 × 50 mm tubular glass vials with stopper (20 mL capacity).
6. Evaporate to 200 μL with nitrogen at room temperature. Vortex thoroughly for 30 s and transfer into a GC autosampler vial.
7. Wash tubular glass vials twice with 100 μL of hexane and combine with the eluate into GC autosampler vials ready for determination by GC/ECD.

3.3 Umbilical Cord Blood

Sample Collection, Preservation, and Storage

1. Health-care professionals should be well-informed about cord blood collection and storage.
2. Umbilical cord blood is collected from the umbilical vein following placental delivery (ex utero).
3. Cord blood collection is performed by dedicated and trained personnel in a separate room. The cord blood is collected by gravity into an EDTA-anticoagulated tube, as soon as possible after delivery of the placenta.
4. The contents must be homogenized as soon as possible after collection (e.g., tubes should be gently inverted at least three times) to mix the blood with the anticoagulant contained in the tube in order to avoid clot formation.
5. Transfer the cord blood sample into a conical-bottom borosilicate glass centrifuge tube and centrifuge for 10 min at 1200–1500 × *g*.
6. Remove the supernatant plasma carefully with a borosilicate glass Pasteur pipette and store in a conical-bottom borosilicate glass tube (*see* **Note 9**).

Sample Preparation

1. 1 mL of specimen is used for the analysis of umbilical cord plasma.
2. 1 mL of distilled water that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standard, and surrogates that are used with samples.
3. 1 mL of blank umbilical cord plasma matrix is treated as a sample in all respects, including exposure to sampling, site conditions, storage, and preservation.
4. Spike calibrators and QCi according to Table 2. Each is prepared by spiking working calibration solution into 1 mL of blank matrix. The calibration range is 0.10–5.00 ng/mL (*see* **Note 13**). All calibrators and QCi should be vortexed after preparation is complete.

Extraction

1. Pipet samples (calibrators, controls, and unknowns) as described above into 16 × 114 mm conical-bottom borosilicate glass centrifuge tubes with pennyhead stopper (10 mL capacity). Add 25 μL of working internal standard and vortex thoroughly.
2. Add 2 mL of formic acid (97%), cap taking care to properly cover with metalized paper to avoid contamination and vortex thoroughly for 20 s.
3. Add 100 μL of acetonitrile, cap, mix for 20 s, and expose to ultrasonic liquid processing (25 °C, 400 W) for 20 min.
4. Condition the SPE columns prior to the addition of the specimens. In order, add the following one at a time to each column:
   (a) Step 1: 2 mL of dichloromethane, 1 mL of toluene, 2 mL of methanol, and 2 mL of water.
   (b) Step 2: 2 mL of water, 2 mL of methanol, 1 mL of toluene, and 2 mL of dichloromethane.
   (c) Step 3: 1 mL of toluene, 2 mL of methanol, and 2 mL of water.
5. Apply specimens to the columns using a borosilicate glass Pasteur pipette (flow 1 mL/min).
6. After the specimen has completely passed through the column, each SPE column should be dried under vacuum, >10 mmHg, for at least 15 min.
7. Elute the specimens with 4 mL of toluene into 26.5 × 50 mm tubular glass vials (20 mL capacity).

Cleanup

1. Disposable glass Pasteur pipette. Place a small clean glass wool plug in the broad end of a Pasteur pipette (12 cm × 1 cm Ø) and push it with the narrow end of another Pasteur pipette to the narrow constriction. Add 0.06 g of activated silica gel followed by 0.24 g of silica gel activated and modified with sulfuric acid (44%, w/w), and 0.5 cm of anhydrous sodium sulfate. Tap it gently on the table for uniform packing.
2. Condition the Pasteur pipette columns with 1.2 mL of toluene.
3. Apply the specimens for SPE extraction to the columns using a borosilicate glass Pasteur pipette.
4. Each Pasteur pipette column should be washed with 200 μL of toluene and vortexed thoroughly for 30 s. Repeat this procedure one time.
5. Elute with 4 mL of toluene into 26.5 × 50 mm tubular glass vials with stopper (20 mL capacity).
6. Evaporate to 500 μL with nitrogen at room temperature. Vortex thoroughly for 30 s and transfer into a GC autosampler vial.
7. Wash tubular glass vials twice with 100 μL of hexane and combine with the eluate into a GC autosampler.
8. Evaporate to 10–20 μL with nitrogen at room temperature. Add 150 μL of hexane, vortex for 30 s, and transfer to autosampler vials ready for determination by GC/ECD.

3.4 Instrument Parameters

1. GC Oven Conditions:
   Column temperature initially set at 80 °C and held isothermal for 1 min immediately after injection, ramped to 190 °C at a rate of 30 °C/min and ramped to 270 °C at a rate of 3.6 °C/min. Run time 71.89 min.
2. Inlet:
   Mode: Splitless (purge flow 20 mL/min at 0.5 min).
   Temperature: 275 °C.
   Carrier gas: Nitrogen, 1 mL/min, constant flow.
   Injection volume: 2 μL.
3. Detector:
   Mode: Constant column + makeup flow.
   Temperature: 300 °C.
   Combined flow: 60 mL/min.

Check for leaks, ensure all parameters are loaded for the analysis, wait for instrument ready, and inject standard solution in hexane, to ensure all retention windows are appropriately set (Table 3). An example chromatogram is shown in Figs. 1 and 2.

Table 3
Pesticide retention time (min)

Compound	DB-XLB Retention time	DB-1701P Retention time
Organochlorine pesticides		
α-HCH	16.029	10.634
HCB	16.282	12.327
γ-HCH	17.328	11.801
β-HCH	18.307	14.799
δ-HCH	19.546	15.109
Heptachlor	19.789	15.780
Aldrin	21.243	17.246
Heptachlor epoxide	23.261	19.706
o,p′-DDD	24.059	23.549
o,p′-DDE	24.202	16.351
α-Chlordane	24.918	17.077
γ-Chlordane	25.004	20.472
α-Endosulphan	25.319	16.903
p,p′-DDE	25.944	17.696
Dieldrin	26.406	22.822
Endrin	27.569	19.180
o,p′-DDT	27.936	19.521
p,p′-DDD	28.399	25.017
β–Endosulphan	28.946	25.470
Endrin aldehyde	29.631	22.935
p,p′-DDT	30.346	21.719
Endosulphan sulfate	31.558	24.323
Methoxychlor	33.287	28.001
Endrin ketone	34.362	26.101
Mirex	38.613	31.466
Organophosphate and pyrethroid pesticides		
Tefluthrin	16.580	10.825
Chlorpyriphos methyl	19.073	12.938
Chlorpyrifos	20.854	14.240
Bifenthrin	31.688	22.723
λ-cyalotrin	36.265	27.428
Permethrin	41.961	28.104

(continued)

Table 3
(continued)

Compound	DB-XLB Retention time	DB-1701P Retention time
Cypermethrin-isomer 1	48.201	32.538
Cypermethrin-isomer 2	49.274	33.200
Cypermethrin-isomer 3	49.785	33.400
Cypermethrin-isomer 4	50.251	33.935
Fenvalerate-isomer 1	59.028	36.988
Fenvalerate-isomer 2	61.471	38.408
Deltamethrin	69.121	42.417

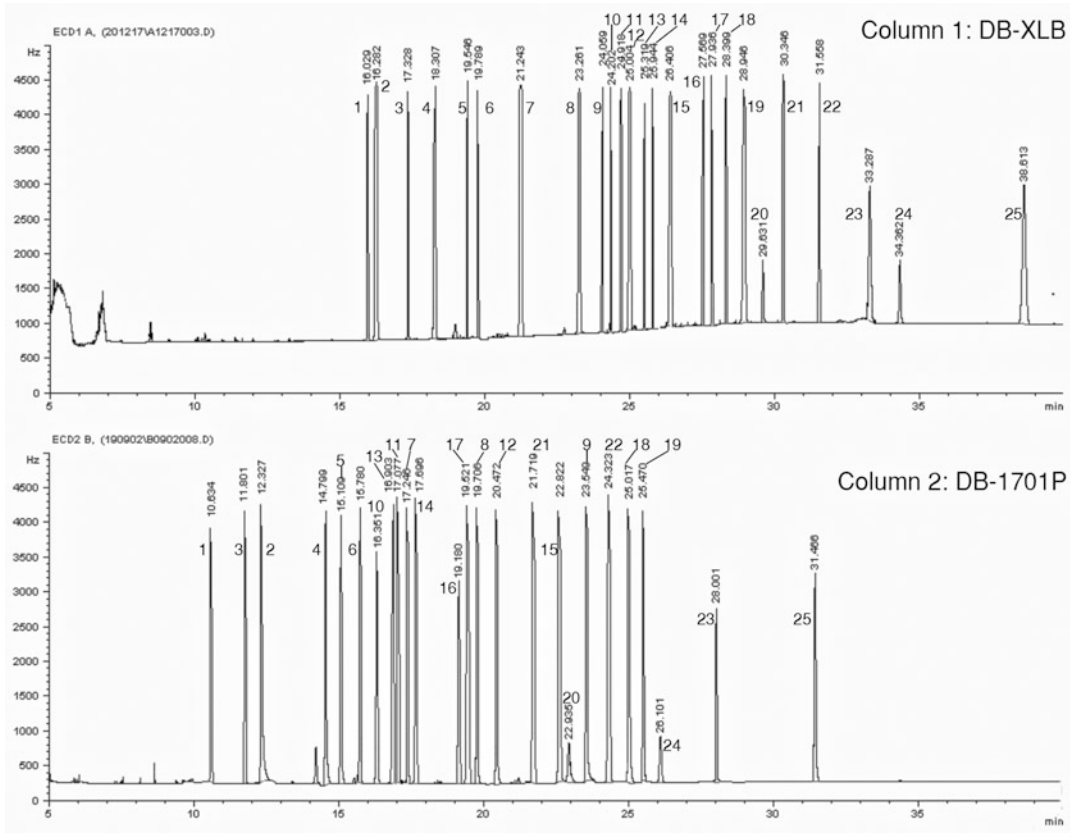

Fig. 1 Chromatograms of a standard solution of 10 ng/mL organochlorine pesticides run by gas chromatography with microelectron capture detector (GC-μECD). α-HCH (1); HCB (2); γ-HCH (3); β-HCH (4); δ-HCH (5); heptachlor (6); aldrin (7); heptachlor epoxide (8); o,p′-DDD (9); o,p′-DDE (10); α-chlordane (11); γ-chlordane (12); α-endosulphan (13); p,p′-DDE (14); dieldrin (15); endrin (16); o,p′-DDT (17); p,p′-DDD (18); β–endosulphan (19); endrin aldehyde (20); p,p′-DDT (21); endosulphan sulfate (22); methoxychlor (23); endrin ketone (24); mirex (25)

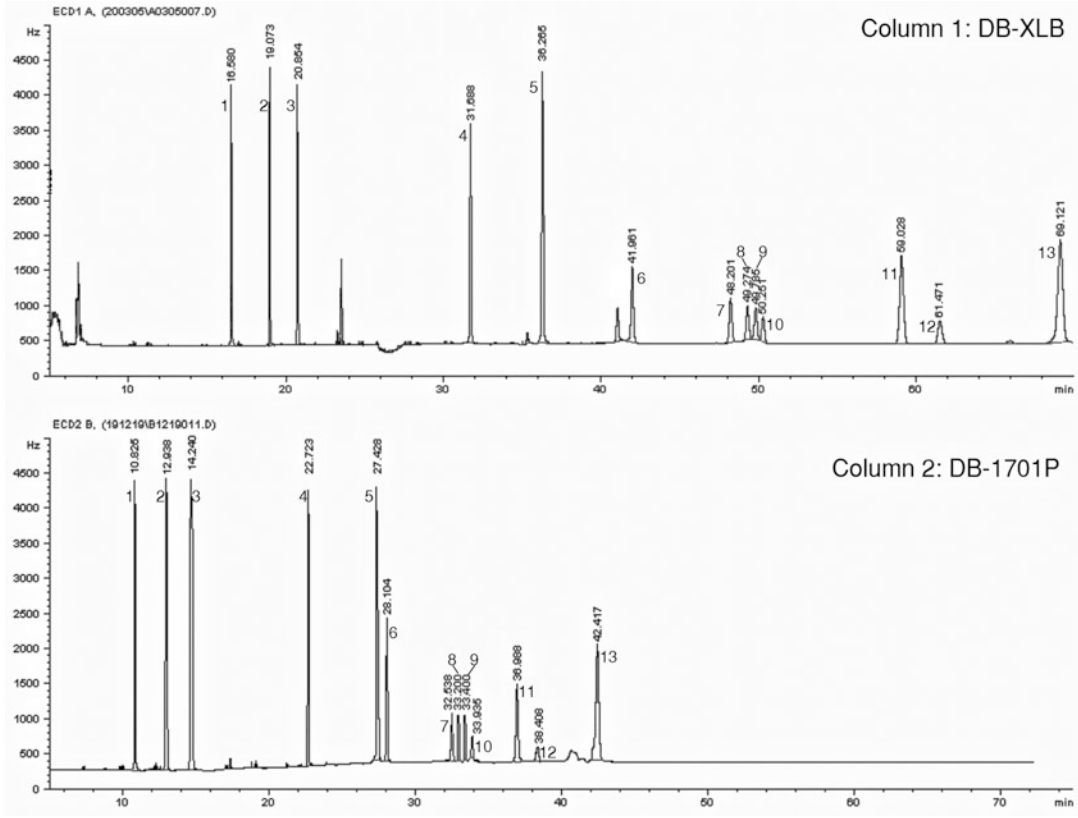

Fig. 2 Chromatograms of a standard solution of 0.10 μg/mL organophosphate and pyrethroid pesticides run by gas chromatography with microelectron capture detector (GC-μECD). Tefluthrin (1); chlorpyriphos methyl (2); chlorpyrifos (3); bifenthrin (4); Λ-cyalotrin (5); permethrin (6); cypermethrin-isomer 1 (7); cypermethrin-isomer 2 (8); cypermethrin-isomer 3 (9); cypermethrin-isomer 4 (10); fenvalerate-isomer 1 (11); fenvalerate-isomer 2 (12); deltamethrin (13)

3.5 Pesticide Identification Protocol

3.5.1 Plasma Samples

1. Determine the absolute retention time (in minutes) in columns A and B for each single component analyte to three decimal places.

The absolute retention time of the analytes chromatographic peak shall not differ by more than ±0.1 min (whichever is smaller) from that of the same substance in a spiked sample, analyzed contemporaneously.

3.5.2 Breast Milk and Umbilical Cord Blood Samples

1. Determine absolute retention times in columns A and B for each single component analyte to three decimal places.
2. The absolute retention time of the analytes chromatographic peak shall not differ by more than ±0.1 min (whichever is smaller) from that of the same substance in a spiked sample, analyzed contemporaneously.

3. Determine the relative retention time in columns A and B, as described below:

$$\text{Relative retention time}: \frac{\text{Analyte absolute retention times}}{\text{Internal standard absolute retention times}}$$

4. The relative retention time shall not differ by more than ±0.01 from that of the same substance in a spiked sample, analyzed contemporaneously.

3.6 Pesticide Quantification Protocol

The CENATOXA adopted an external standard calibration for pesticides quantification in plasma and an internal standard calibration for pesticides quantification in breast milk and umbilical cord blood. Given the large number of target compounds addressed, reagents, solvents, and SPE columns consumed in these methods, and according to the United States Environmental Protection Agency (EPA) recommendation [8], the average calibration factor (CF) is used in place of a calibration curve. This CF is verified on each working day by the injection of a calibration standard.

3.6.1 External Standard Calibration for Pesticide Quantification in Plasma

$$\text{Calibration factor (CF)}: \frac{\text{Area response for the analyte to be measured}}{\text{Calibrator concentration (in ng/mL)}}$$

The following sections describe the calculations necessary to obtain the concentrations of analytes in the original sample, based on its volume.

$$\text{Concentration as ng/mL}: \frac{A_s \times V_{ic} \times C_c \times D}{A_c \times V_{is}}$$

Sample concentration by volume (ng/mL), where:

A_s = Area of the analyte in the sample aliquot introduced into the instrument.

A_c: Area of the analyte in the calibrator aliquot introduced into the instrument.

C_c: Calibrator concentration (in ng/mL).

V_{is} = Volume of sample extract injected (in μL). The nominal injection volume for samples and calibrators must be the same.

V_{ic}: Volume of calibrator extract injected (in μL). The nominal injection volume for samples and calibrators must be the same.

D: Dilution factor, if sample was diluted prior to analysis. If no dilution, $D = 1$. Always dimensionless.

3.6.2 Internal Standard Calibration for Pesticide Quantification in Breast Milk and Umbilical Cord Blood

$$\text{Calibration factor (CF)}: \frac{(A_s \times C_{is})}{A_{is} \times C_s}$$

where:

A_s = Response for the analyte to be measured.

A_{is}: Response for the internal standard.

C_s: Calibrator concentration (in ng/g or ng/mL).

C_{is} = Internal standard concentration (in ng/g or ng/mL).

The following sections describe the calculations necessary to obtain the concentrations of analytes in the original samples.

$$\text{Concentration as ng/g or ng/mL} : \frac{(A_s/A_{is}) \times V_{ic} \times C_c \times D}{(A_s/A_{is}) \times V_{is}}$$

Sample concentration by weight (ng/g) or volume (ng/mL), where:

A_s = Area of the analyte in the sample aliquot introduced into the instrument.

A_c: Area of the analyte in the calibrator aliquot introduced into the instrument.

A_{is}: Area for the internal standard.

C_c: Calibrator concentration (in ng/g or ng/mL).

V_{is} = Volume of sample extract injected (in μL). The nominal injection volume for samples and calibration must be the same.

V_{ic}: Volume of calibrator extract injected (in μL). The nominal injection volume for samples and calibration must be the same.

D: Dilution factor, if sample was diluted prior to analysis. If no dilution, $D = 1$. Always dimensionless.

3.6.3 Comparison Between Results from Different Columns or Detectors

When sample results are confirmed using two dissimilar columns, the agreement between the quantitative results should be evaluated after the identification has been confirmed. Large differences in the numerical results from the two analyses may be indicative of positive interferences with the higher of the results, which could result from poor separation of target analytes, or the presence of a nontarget compound. However, they may also result from other causes. Thus, in order to ensure that the results reported are appropriate for the intended application, the analyst should make a formal comparison, as described below.

$$\text{Relative percent difference} : \frac{\imath C_1 - C_2 \imath}{C_1 + C_2/2} \times 100$$

where:

C_1: Concentration on column 1.

C_2: Concentration on column 2.

Vertical bars in the numerator indicate the absolute value of the difference.

If one result is significantly higher (e.g., >20%), check the chromatograms to see an obviously overlapping peak or examine the baseline parameters established by the instrument data system (or operator) during peak integration.

If no anomalies are noted, review the chromatographic conditions. If there is no evidence of chromatographic problems, it may be appropriate to report the lower result.

3.7 Method Application

Pesticide investigations in human plasma from different regions of Argentina are scarce; however, a study conducted in the CENATOXA [9] evaluated the organochlorine and metabolites levels in 681 plasma samples (315 women and 366 men) analyzed during 2005–2018. Results showed that DDT group appeared most frequently (58%), followed by HCH group (45%), heptachlor group (21%), and HCB (10%). Maximum values of 57.7 and 4.6 ng/mL were registered for *pp*-DDT and β-HCH, respectively. In addition, results showed a significant decrease in the β- and γ-HCH isomers and *pp*-DDT, banned in Argentina from the 1970s to 1980s. Worldwide comparison of organochlorine residues in human serum of the general population is presented in Table 4.

Organochlorine residue levels in umbilical cord blood samples have been shown to be good biomarkers of transfer rate of these pesticides from mothers to newborns, and it may adversely affect the growth and development of the newborn. In this context, analysis of umbilical cord blood samples from 40 normal healthy women with full-term pregnancy was performed in CENATOXA [25]. Results showed that DDT group appeared most frequently (33%), followed by chlordane group (15%), γ-HCH (15%), and endosulphan group (13%). The median concentration of DDT group was 0.25 ng/mL (Range: no detectable–2.47) and γ-HCH group was 0.22 ng/mL (Range: no detectable–0.45). A worldwide comparison of organochlorine residues in umbilical cord blood is presented in Table 5.

4 Notes

1. The developed method results in a simple, fast, highly sensitive, and less-expensive procedure to determine the target analytes, with only 1000 μL of sample analyzed. The lower limits of quantification ranged from 0.10 to 0.20 ng/mL for organochlorine, 0.13 ng/mL for organophosphate, and 0.10 to 0.15 ng/mL for pyrethroid pesticides. These limits are considered satisfactory, especially when compared to those obtained by other authors [35–40].
2. This analytical method is highly sensitive and shows better recoveries, in comparison with the previously published

Table 4
Worldwide organochlorine residues in human blood of the general population

Country/region	Sample	#	Instrument	Unit	α-HCH	β-HCH	Σ HCH	*pp*′-DDE	*pp*′-DDT	Σ DDT	HCB	β-Endosulphan	Reference
Argentina	Plasma	681	GC-ECD	ng/mL	0.08	0.30	0.13	1.29	0.11	0.31	0.09	0.06	[9]
Romania	Serum	142	GC-MS-ECNI	ng/g lipid	31	923	1114	1975	339	2420	30	–	[10]
Slovakia	Serum	1038	GC-ECD	ng/g lipid	–	52.4	–	1755	49.3	–	829	–	[11]
United Kingdom	Serum	154	GC-HRMS	ng/g lipid	0.48	12	15	100	2.9	100	11	–	[12]
Spain	Blood	220	GC-ECD	ng/mL	–	–	–	5.18	3.64	–	3.88	–	[13]
Argentina/ Cordoba	Plasma	167	GC-ECD	µg/L	–	0.44	–	0.68	–	–		–	[14]
Greece/Attika	Serum	61	GC-MS-ECNI	ng/g lipid	0.04	30.6	–	379	7.89	–	38.2	–	[15]
India/Dibrugarh	Blood	169	GC-ECD	µg/L	150	119	348	67	241	417		–	[16]
Italy/Sicily	Serum	101	GC-HRMS	ng/g lipid	4.14	4.26	–	175.0	4.37	–	18.63	–	[17]
Bolivia	Serum	112	GC-ECD	ng/g lipid ng/mL	– –	– –	– –	267.4 1.2	13.0 0.1	– –	22.1 0.1	– –	[18]
Mexico/Chiapas	Plasma	60	GC-ECD	µg/L	–	2.60	–	46.54	19.9	–		5.35	[19]
Mexico/Veracruz	Serum	300	GC-ECD	mg/kg lipid	–	4.83	–	13.12	2.47	14.71		–	[20]
India/Punjab	Serum	50	GC-ECD	ng/mL	–	–	–	15.26	–	23.70		4.08	[21]
Korea	Serum	90	GC-HRMS	ng/g lipid	–	37.3	–	329	20.2	–	13.9	–	[22]
China / Beijing	Plasma	100	GC-MS/MS	ng/mL	3.65	2.83	9.9	4.89	2.53	8.79	2.23	0.25	[23]
Algeria	Plasma	207	GC-MS	ng/g lipid	–	–	–	131.6	–	–	6.09	–	[24]

GC-MS-ECNI: gas chromatograph-mass spectrometer detector-electron capture negative ionization mode; GC-ECD: gas chromatograph-electron capture detector; GC-HRMS: gas chromatography-high-resolution mass spectrometry detector; GC-MS/MS: gas chromatograph-triple quadrupole tandem MS system; GC-MS: gas chromatography-mass spectrometry detector

Table 5
Worldwide organochlorine residues in umbilical cord blood of the general population

Country/region	#	Instrument	Unit	*pp*′-DDT	*pp*′-DDE	Σ DDT	β-HCH	HCB	Reference
Argentina/ Buenos Aires	40	GC-ECD	ng/mL ng/g lipid	0.13 91.9	0.01 4.6	0.25 107.6	0.01 5.8		[25]
Poland	21	GC-MS ECNI	ng/g lipid	19.6	365	385	2.6	21.0	[26]
India	68	GC-ECD	ng/mL	1.03	3.08		7.23		[27]
Mexico/ Veracruz	70	GC-ECD	ng/mL ng/g lipid	1.1 5.9	1.3 6.9	1.3 7.2	5.5 28.0		[28]
USA/Texas	35	GC-ECD	ng/mL	0.01	0.30	0.31	0.02		[29]
Spain/Asturias	308	GC-ECD	ng/mL ng/g lipid	0.08 33.3	0.46 175		0.05 16.9	0.13 49.5	[30]
China	60	HRGC-HRMS	ng/g lipid	5.4	116.1	146.0	67.6	65.1	[31]
China	972	GC-MS	ng/mL	4.31	2.01	6.48	0.68		[32]
Korea	117	HRGC-HRMS	ng/g lipid	3.65	74.5	77.5	6.04	10.9	[33]
China/Shanghai	102	GC-ECD	ng/mL ng/g lipid	0.01 2.4	0.98 102	0.98 389	0.34 124	0.18 68	[34]

GC-MS-ECNI: gas chromatograph-mass spectrometer detector-electron capture negative ionization mode; GC-ECD: gas chromatograph-electron capture detector; HRGC-HRMS: high-resolution gas chromatography–high-resolution mass spectrometry detector; GC-MS: gas chromatograph-mass spectrometer detector

methods [41–43]. The lower limits of quantification ranged from 0.06 to 0.10 ng/mL for organochlorine pesticides. Extraction efficiency ranged from 74% to 113%.

In addition, this method can be used for the determination of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in breast milk.

3. The assay was validated based on its recovery (70.0–110%). In view of the low limits of detection (ranged between 0.007 and 0.018 ng/mL) and lower limits of quantification (0.021 and 0.060 ng/mL), the developed method can serve as a less expensive and more ecologically friendly alternative to the previously published methods [30, 44, 45].
4. Wash glassware with Extran liquid alkaline detergent (Merck, Darmstadt, Germany) and water by brushing the inside with a brush. If the shape or size of the material does not allow for brushing, shake vigorously. Make sure that all inside parts of the item are rinsed. Expose to ultrasonic liquid processing (25 °C) for 60 min. Use tap water as a preliminary rinse to flush all internal surfaces of the glassware and rinse with

distilled water ten times. Then rinse three times with each solvent: methanol followed by acetone (remove any residual water) and finally rinse with hexane. After glassware are cleaned and dried, cover with aluminum foil, dull side toward the clean glassware surface.

Solvents used in all parts of the procedure must be certified as suitable for pesticide residue analysis. All solvent rinsing should be done in a fume hood and dispose of the used solvents in a waste container marked "nonchlorinated solvent waste."

Glassware coming in contact with standard solutions should be decontaminated with chromic sulfuric acid mixture for 24 h and continue with the previous protocol.

5. The blank plasma matrix is stored in 50 mL screw-top containers. Approximately 1 g of sodium fluoride is added to each 50 mL tube to prevent degradation. The containers are then stored at −20 °C until needed.
6. All standard solutions shall be analyzed within 48 h of preparation and on a monthly basis thereafter for signs of degradation. Standards will remain acceptable if the peak area remains within ±15% of the area obtained in the initial analysis of the standard.
7. To a 50 mL volumetric flask, add 25 mL of plasma and 250 μL of 0.10 μg/mL standard solution. Mix well for 10 min and fill to volume with plasma. Mix for 10 min and dispense 1.5 mL into autosampler vials. Cover with metalized paper to avoid contamination and store in the freezer at −20 °C.
8. Ministerio de Desarrollo Productivo, Argentina. Instituto Nacional de Tecnología-Red Argentina de Laboratorios Lácteos de Calidad Asegurada (INTI-REDALAC). Annual proficiency testing program for interlaboratory comparisons. Determination of pesticide residues.
9. Organochlorine levels in plasma frozen at −20 °C are unchanged over a period of 6 months. Organophosphate pesticides and pyrethroids in plasma are less stable, therefore samples are kept at 4 or − 20 °C and should be processed immediately.
10. 10% of the samples analyzed are controls. This can be achieved by extracting two 0.05 μg/mL QCi and dispersing them throughout the instrument run to achieve this 10%. Only two controls are extracted but each is injected repeatedly throughout the run.
11. Aqueous potassium carbonate is a nondestructive cleanup for some pesticides, consumes less time, and reduces the matrix effect.

12. Store samples at 4 °C for 72 h or at −20 °C for 6 months. If it is not possible, add a potassium dichromate (K_2Cr2O_7) tablet for chemical sterilization of breast milk.
13. 10% of the samples analyzed are controls. This can be achieved by extracting two each of 1.0 and 5.0 ng/mL QCi and dispersing them throughout the instrument run to achieve this 10%. Only four controls are extracted but each is injected repeatedly throughout the run.

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Chapter 8

Identification of Pesticides in *Postmortem* Samples: Applicability in Forensic Toxicology

Suzana Fonseca, Mário Barroso, Francisco Vale, Nuno Gonçalves, Suzel Costa, Fernando Castanheira, and João Franco

Abstract

Pesticides play an important role in clinical and forensic toxicology, associated mainly with high acute exposures, as causes of suicidal, homicidal, and accidental poisonings due to misuse by operators. Pesticide analysis in postmortem samples is difficult; it must be used a sensitive method, due to the high toxicity of those compounds; versatile, due to the high variety of samples and robust, to be easily incorporated in the routine flow.

The present work describes a simple and successful method that allows the screening and qualitative confirmation of more than 43 pesticide active substances in *postmortem* samples, using a solid-phase extraction method and GC/MS analysis. This method is routinely applied to different postmortem matrices. Sample preparation, analytical parameters, controls, and acceptance criteria are fully presented.

Key words *Postmortem* samples, Toxicology, Pesticides, GC/MS, Solid-phase extraction, Confirmation

1 Introduction

"Pesticide" encompasses a wide variety of substances used to destroy unwanted life forms, mainly applied in agriculture for crop protection and pest control, and in human and animal hygiene. More than a thousand pesticides are widely used in the world today, in spite of the strict control legislation in developed countries and the alerts launched by international organizations. In addition, several compounds that are no longer legally produced still remain in people's houses or available in the black market [1–3].

Pesticides play an important role in clinical and forensic toxicology, associated mainly with high acute exposures, as causes of suicidal, homicidal, and accidental poisonings due to misuse by operators. The risk of intoxication by pesticide residues in

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commercially traded foodstuffs is very low because they are monitored to fulfil the international standards and legislation. Pesticide intoxications are more prevalent in rural areas, where they are widely available and known for its toxicity, being frequently used for suicidal purposes [4].

Pesticides are commonly classified as a single class of chemicals, but in fact there are several different types of compounds with different uses, mechanisms of action, and toxic effects. They can be classified accordingly with the target species they act on: insecticides, herbicides, fungicides, and rodenticides, etc. These four subclasses are the more used and important for postmortem toxicology [1].

Pesticides are important analytes in *postmortem* toxicology. Insecticides like dimethoate, chlorpyrifos, chlorfenvinphos, quinalphos, and azinphos-ethyl are still the substances with more prevalence. Banned substances are also very often found, such as parathion-ethyl or strychnine [4, 5]. Deaths are mostly due to accidental or voluntary acute intoxication, by oral ingestion. Concentrations are usually higher enough to be detected in blood, although sometimes it is also necessary to analyze stomach content. In cases with hospital treatment before death, like stomach cleaning, it is impossible to detect pesticides on traditional samples, and it is important to perform the screening in organs and tissues, like liver and kidneys. These alternative samples are not easy to analyze due to their complex and diverse composition. Different matrices with a wide range of concentrations can be a big challenge to the forensic toxicologist. Therefore, an adequate sample preparation procedure is deemed necessary to obtain the analytes in a suitable extract for chromatographic analysis. Quantitative analysis is usually not relevant, as these substances are not intended for human consumption and they are lethal at very low concentrations, generally below the limit of detection of the common analytical methods, considering the sample volume available and used. Furthermore, the acute lethal doses of many pesticides in humans remain unknown [4].

The method herein described for the screening and confirmation of pesticides in *postmortem* samples is simple, sensitive, selective, and appropriate to detect a wide group of different substances in several biological matrices. It can be routinely applied to *postmortem* blood, gastric content, and other biological samples like liver or kidneys. In sample preparation, a broad spectrum of analytes can be extracted using a simple solid-phase extraction procedure. The extract analysis is then carried out by gas chromatography/mass spectrometry, with selected ion monitoring. Negative and positive controls are analyzed in the same batch with previously defined acceptance criteria. The limits of detection are between 25 and 100 ng/mL for most of the analytes.

2 Materials and Reagents

All reagents are certified gradient grade or better, unless otherwise stated. All reagents were stored at room temperature.

Water was deionized in a 185 Simplicity™ MilliporeSigma™ Water Purification System (Millipore SAS, Molsheim, France), with purity criteria 18.2 MΩcm^{-1}. Methanol (gradient grade) was purchased from Honeywell (Darmstadt, Germany).

Phosphate buffer pH 7 was purchased from Supelco (Darmstadt, Germany).

Solid-phase extraction Oasis HLB extraction cartridges (3 cc/60 mg) purchased from Waters (Milford, MA, USA) are stored in a dry area at room temperature. If a partial bag remains, it is stored in a desiccator.

Washing solution preparation: Dilute 50 mL of methanol in a 1 L volumetric flask. Fill to volume with deionized water. Mix and store in a 1 L flask, at room temperature.

Pestanal® certified standards are purchased in the solid state from Riedel-de Haën, Sigma-Aldrich, Germany; Dr. Ehrenstorfer certified standards are purchased in the solid state from LGC, Germany (*see* substances in Table 1).

Pesticide standard stock solutions are prepared at 1 mg/mL. Preparation: Weigh accurately about 10 mg of base substance in a 10 mL volumetric flask. Fill to volume with methanol. After mixing, split in 2 mL flasks and store below 0 °C. Ethion was used as internal standard [6] (*see* **Note 1**). Working standard mixtures and internal standard solutions are diluted in methanol at a concentration of 10 μg/mL. Standards solutions and mixtures are stored in the freezer, at approximately −20 °C.

Blood matrix is obtained by donation from the national blood bank of the discarded blood bags, when they were no longer suitable for use. Each batch is validated as blank blood after being analyzed under all routine methods in the laboratory with a negative result and no relevant interferents. Blank blood is split in 30 mL screw-top containers and stored at approximately −10 °C.

3 Equipment and Chromatographic Conditions

Chromatographic analysis is performed using an *Agilent Technologies* GC-MS (6890 gas chromatograph with a 5973 N mass-selective detector) equipped with a capillary HP5-MS column (30 m × 0.32 mm i.d. × 0.25 μm film thickness of 5% phenyl–95% methylpolysiloxane stationary phase) (*see* **Note 2**). Helium was used as the carrier gas in the constant flow mode at 1.1 mL/min. Injection volume of 2 μL in the split injection mode with a ratio 10:1 (*see* **Note 3**). The temperatures of the injection port and

transfer line were set at 280 °C. The GC oven chromatographic conditions were as follows: initial temperature at 150 °C held for 1 min, followed by an increase of 5 °C/min to 290 °C, which was held for the final 8 min (*see* **Note 4**). The mass spectrometer is operated simultaneously in the selected ion monitoring (SIM) and SCAN modes (*see* **Note 5**) using a 70 eV electron impact ionization. The detector parameters are presented in Table 1, as well as the detection limits obtained for each of the 44 compounds.

Table 1
GC-MS parameters for each of the 44 compounds: Retention time (RT); ionic fragments in SIM mode (*m/z*) and limit of detection (LOD)

Compound	RT[1]	SIM ions (*m/z*)	LOD (ng/mL)
IS-ethion	15.6	231, 153	–
2,4-D ME	5.3	199, 175, 234	25
Atrazine	6.9	200, 215, 173	20
Azinphos-ethyl	20.9	132, 160, 77	25
Bendiocarb	5.9	151, 126, 166	25
Bitertanol	21.8	170, 112, 337	25
Bupirimate	14.5	208, 273, 166	25
Chlorfenvinphos	12.2	267, 323, 295	25
Chlorpyrifos ethyl	10.8	197, 199, 258	25
Chlorpyrifos methyl	9.2	286, 288, 125	25
Diazinon	7.7	137, 179, 304	25
Dimethoate	6.6	87, 93, 125	25
Dimethomorph	28.9	301, 165, 387	100
DNOC	5.2	198, 121, 168	50
Endrin	14.5	81, 243, 281	50
Ethoprophos	5.3	158, 126, 200	25
Fenamiphos	13.6	303, 154, 288	25
Fenarimol	20.6	139, 219, 330	25
Fenthion	10.7	278, 169, 153	25
Fenvalerate (2 isomer)	25.9–26.4	125, 167, 181	100
Imidacloprid	13.0	211, 126, 099	100
Lindane	7.2	219, 181, 109	25
Malathion	10.5	173, 125, 158	25
Metalaxyl	9.6	206, 160, 249	20
Methidathion	12.7	145, 085, 125	50

(continued)

Table 1
(continued)

Compound	RT[1]	SIM ions (*m/z*)	LOD (ng/mL)
Methiocarb	10.0	168, 153, 109	25
Mevinphos	3.2	127, 192, 109	25
Myclobutanil	14.2	179, 150, 206	25
Nuarimol	17.1	107, 139, 314	25
Oxyfluorfen	14.4	252, 300, 361	25
Parathion ethyl	10.9	291, 139, 155	25
Pendimethalin	11.8	252, 281, 162	25
Pentachlorophenol	7.1	266, 270, 230	25
Phosalone	19.7	182, 184, 367	25
Pirimicarb	8.5	166, 072, 238	20
Pirimiphos methyl	10.2	290, 276, 305	20
Propoxur	5.1	110, 152, 209	50
Pyraclostrobin	25.7	132, 164, 111	50
Quinalphos	12.2	146, 157, 298	25
Strychnine	29.3	334, 120, 162	50
Sulfotep	6.0	322, 202, 266	25
Terbuthylazine	7.3	214, 173, 229	20
Thiacloprid	25.1	101, 126, 251	50
Vinclozolin	9.2	212, 285, 198	25

4 Methods

This method was based and adapted from those by Raposo et al. [6] and Pereira et al. [7].

4.1 Sample Preparation

All samples are stored at approximately −10 °C. Whole blood and other liquid samples are allowed to reach room temperature in a rotation/inversion homogenizer. Solid samples need a pretreatment before extraction.

For solid samples, weigh approximately 0.5 g of sample for a 2 mL plastic tube adequate for use in the Precellys® 24 Tissue homogenizer (Bertin Technologies, France). Add 6 inox spheres (bearings) and 800 μL of deionized water. Homogenize using three cycles of 30 s at 3500 × *g*. Repeat if necessary. Centrifuge at 6700 × *g* for 10 min. Repeat if necessary. Use a 200 μL aliquot of the homogenate supernatant in the sample preparation step [7].

Table 2
Preparation of negative and positive controls

Control		Blank Blood	IS solution	Std Mix 10 μg/mL
RB	Reagent blank	–	20 μL	–
NC	Negative control	500 μL	20 μL	–
CT1	100 ng/mL control	500 μL	20 μL	5 μL
CT2	500 ng/mL control	500 μL	20 μL	25 μL
CT3	1000 ng/mL control	500 μL	20 μL	50 μL

The analysis uses 500 μL aliquots of whole blood sample or 200 μL aliquots for other samples (gastric content, urine, and supernatant obtained in the pretreatment of solid samples).

4.2 Sample and Control Preparation

Each sample aliquot is diluted with 5 mL of pH 7 phosphate buffer and spiked with 20 μL of internal standard working solution. Prepare and dilute negative and positive controls as presented in Table 2. Homogenize the mixture by rotation/inversion movements for 5 min and centrifuge at 1400 × *g* for 15 min.

4.3 Extraction Procedure

Oasis HLB extraction cartridge is conditioned with 2 mL of methanol and 2 mL of deionized water. The supernatant obtained after sample preparation is allowed to pass through the column by gravity, which is afterwards washed with 2 mL of 5% methanol in water. Cartridges are then dried under full vacuum for at least 30 min. The analytes are eluted with 2 mL of methanol to a glass tube. The elution product is evaporated to dryness under a gentle stream of nitrogen in a bath at the temperature of 35 °C, to prevent analyte losses, using a TurboVap® Evaporator. The dry extract is reconstituted with 75 μL of methanol, using a vortex for 10 s, and transferred to a GC autosampler vial with a 300 μL insert.

5 Report and Results Analysis

The following criteria are applied for acceptance of each analytical batch:

- Reagent blank: Absence of chromatographic signals at the RT of the analytes.
- Negative control: Negative and no interfering peaks at the RT of the analytes.

- Positive controls: At least one control must to be positive for each substance, according with its limit of detection. Chromatographic signals for three diagnostic ions must be present at the retention time of the substance.

Screening is made by visual observation of the SIM report. If the chromatographic signal for each of the three qualifier ions is present at the retention time of the substance, a confirmation analysis is then required. Additionally, the screening in SCAN mode can also be made (*see* **Note 5**).

The screening report for a control sample with the 44 substances is partially presented in Fig. 1. More substances can be detected by this method (*see* **Note 6**).

5.1 Confirmation Analysis

Confirmation analysis is a second independent analysis of the same sample, repeating all the procedure of sample treatment, extraction, and instrumental analysis (*see* **Note 7**). For a successfully and unequivocally identification of the presence of a substance in the sample, a comparison of the ion ratios and relative retention time obtained in the positive control analyzed contemporaneously must be made. Confirmation criteria are as follows [8]:

RTT: Relative retention time of the analyte in the sample has maximum variation of 1% when compared to the value obtained in the quality control.

Signal/noise > 3 for all diagnostic ions detected.

Ion ratios: Relative intensities of the three diagnostic ions selected for the analyte should not differ by more than a tolerated amount from those generated in the quality control sample. That is, 10% of absolute tolerance for ion ratios higher than 50%; 20% of relative tolerance for ion ratios between 25% and 50%; and 5% of absolute tolerance for ion ratios less than 25%.

6 Notes

1. It is possible to use Promazine as internal standard [9, 10]. Retention time and diagnostic ion are in Table 3.
2. HP5-MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness of 5% phenyl–95% methylpolysiloxane stationary phase) can also be used, correcting the small differences in retention times in Table 1.
3. Holding time at initial temperature can be modified in order to maintain the retention times when changing to a new capillary column, when it is necessary to cut its length or with the loss of performance over time.

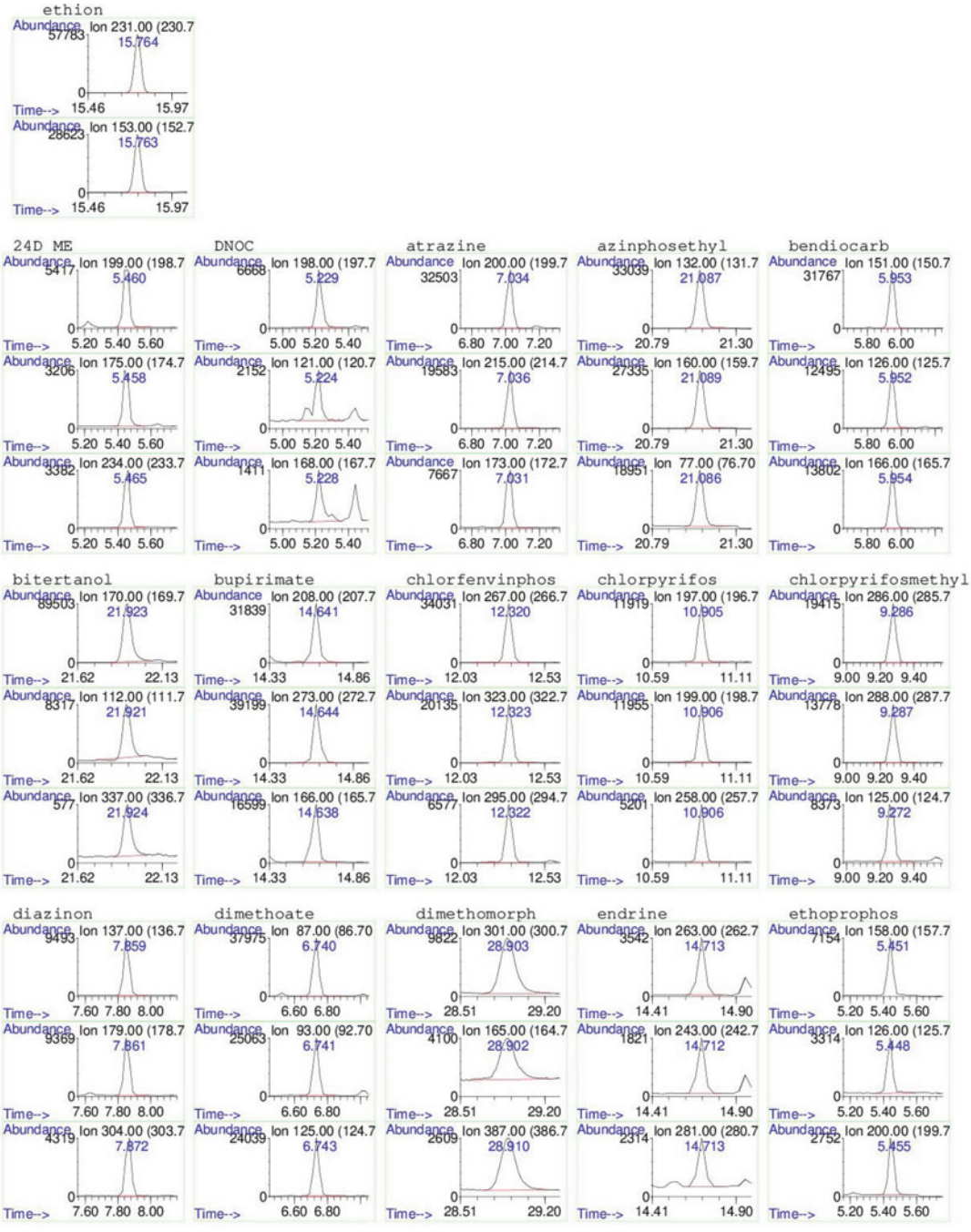

Fig. 1 Screening report for a control sample

Table 3
GC-MS parameters for additional compounds that can be analyzed by the method: Retention time (RT); ionic fragments in SIM mode (*m/z*) and limit of detection (LOD)

Compound	Aprox RT	SIM ions (*m/z*)	LOD (ng/mL)
Acetamiprid	19.0	221, 166, 152	[a]
Alpha-cypermethrin	22 (3 peaks)	163, 181, 209	500
Deltamethrin	24 (2 peaks)	181, 253, 208	1000
Endosulfan	12.8	195, 237, 277	500
Omethoate	5.5	156, 110, 126, 141	100
Promazine (IS)	18.7	284	–
Tetrachlorvinphos	12.7	240, 329, 333	100
Triclopyr	6.0	210, 269, 271	[a]

[a]Not tested

4. Injection volume and split ratio can be modified as well. For instance, samples with high concentrations, with overload of the chromatographic peak, can be reanalyzed using an injection volume of 1 μL and a split ratio > 10 (50 or 100, if necessary). In order to obtain a good repeatability of retention times, the split ratio should not be lower than 10, under the chromatographic conditions of this method.
5. The SCAN result is routinely verified using ChemStation autointegration parameters and Library Search Report. The libraries used are the laboratory in-house library (match >80%) and the commercial PMW2_TOX.l (match >50%) and wiley7n.l (match >50%). The SCAN screening can be important for broadening the group of substances analyzed. If the presence of a substance is identified by a library comparison, the SIM mode confirmation analysis is needed, as described in the method section. It can be used other commercial or free access libraries.
6. Other substances than the listed in Table 1 can also be detected using this method, but the limit of detection is higher (Table 3).
7. Toxicological confirmation should be performed in a second independent analysis and also in a different matrix, if possible, to prevent and detect sample manipulation errors. Positive

samples have high concentrations very often, namely in the gastric content. In the confirmation analysis of those samples, it is advisable to use a lower volume of sample or dilute it previously. If necessary, change the injection volume and the split ratio (*see* **Note 3**).

7 Case Analysis

The method presented in this chapter is routinely used in our lab.

Pesticide cases are mostly prevenient from rural areas, with scarce or none circumstantial information. All the *postmortem* positive cases are accidental, suicidal, or homicidal acute oral intoxications. The color and smell of the gastric content is usually the first indicator of a possible pesticide case, but also generic autopsy findings such as strange coloration of soft tissues, pulmonary edema, and generalized congestion of the organs.

Analysis of gastric content or blood is worthless in some cases with hospitalization, when medical treatment includes stomach washing, charcoal administration, and hemodialysis procedures. In those situations, alternative samples like liver should be analyzed.

Blood concentrations are usually low due to the high toxic potential of these active substances. Contrariwise, stomach content or organs have so high concentrations that is often necessary to dilute the extract to have a good chromatographic response, enabling to obtain a full MS spectrum using SCAN parameters in addition to the SIM detection (*see* Fig. 2).

Occasionally, the law enforcement officers deliver nonbiological samples, like flasks or glasses with unknown content, that were found in the crime scene, in the victim, or in suspect's home. They are very useful to identify the possible substance and to compare with the biological samples. That can be made by diluting the content in water (for SPE extraction procedure) or in methanol (for direct injection). The volume of dilution should be chosen accordingly with the type and available amount of sample. Be aware that the commercial products have usually very high concentration.

Complex *postmortem* cases also happen to be positive for pesticides. For instance, homicide with suicide simulation (hanging or fire), car accident, traumatic cutting injuries, or cases with the presence of other toxic substances. It is generally accepted that the toxicological confirmation of the presence of pesticide in blood, using this method, leads to intoxication as the main cause of death. Therefore, quantitative analysis is not necessary.

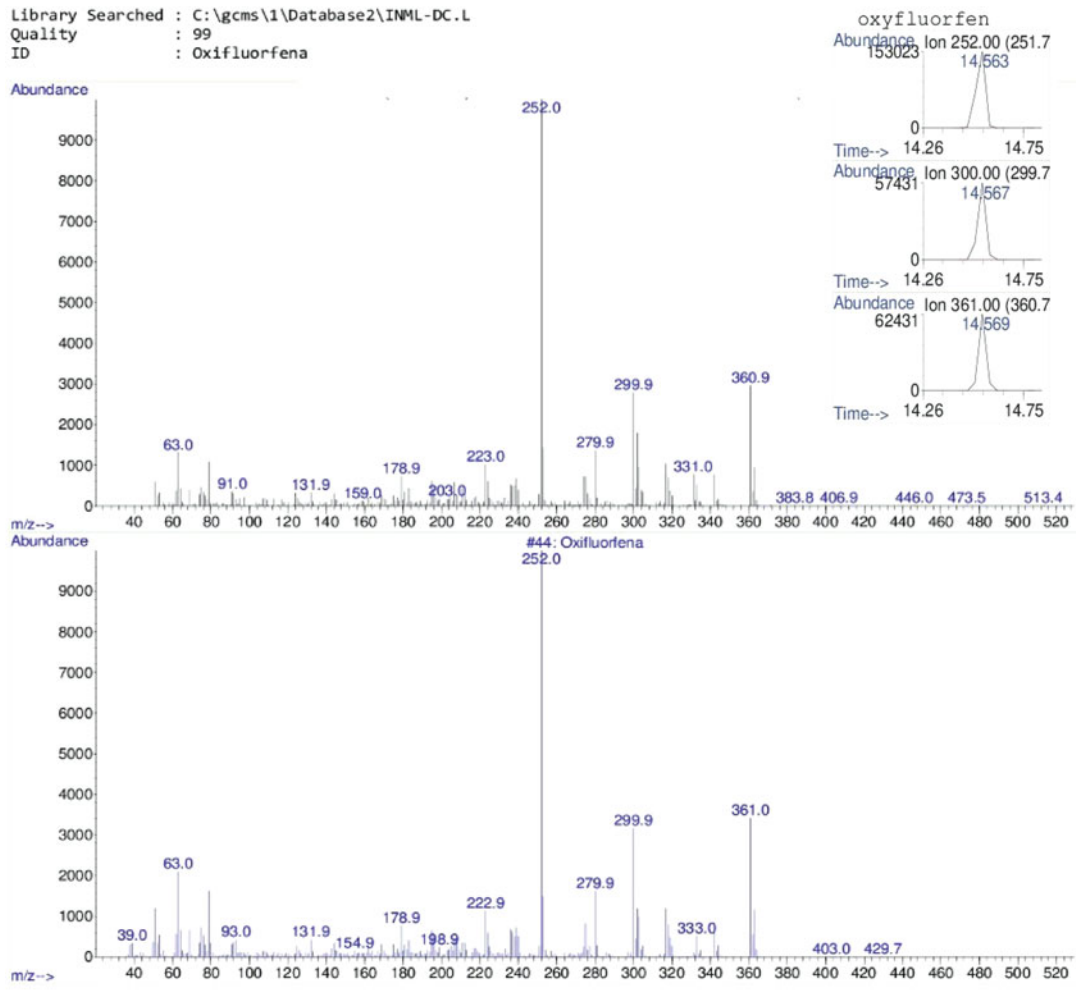

Fig. 2 Full MS spectrum using SCAN parameters with a match >90%, in addition to the SIM detection, of oxyfluorfen obtained in the analysis of a gastric content of a routine case

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Chapter 9

A Modified Micro-QuEChERS Approach for the Determination of Pesticides Found in Forensic and Clinical Blood Samples Using LC-MS/MS

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Abstract

In many developing countries, pesticides are frequently related to suicide and homicide attempts, non-intentional poisonings, especially in children and other criminal acts, such as the sacrificing of domestic animals. Here, a new methodology for the determination of nine common pesticides found in forensic blood samples is described. This new approach uses a modified micro-QuEChERS sample preparation approach combined with LC-MS/MS for analysis. This new method can be applied to different sample matrices in forensic cases, such as plasma, serum, and postmortem blood.

Key words Pesticides, Micro-QuEChERS, LC-MS/MS, Microextraction, Forensic toxicology, Clinical toxicology

1 Introduction

Pesticides are chemicals intended for use in production, storage, and processing of agricultural products, in order to serve as plant protection avoiding damage caused by living beings considered harmful. However, pesticides are also one of the most common substances implied on suicide and homicide attempts, nonintentional poisoning, especially in children and also used for other criminal acts such as the sacrificing of domestic animals. According to the World Health Organization (WHO), in 2016, unintentional poisoning caused 106,683 deaths, and about 20% of global suicides are due to pesticide poisoning, mainly in rural agricultural areas in low- and middle-income countries, and in many countries, poisoning is one of the main causes of emergency attendance at hospitals [1]. The class of these pesticides routinely detected in clinical and

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forensic toxicology includes insecticides (mainly carbamates and organophosphates) and herbicides [2, 3].

The Campinas Poison Control Center carried a monitoring program for cases involving poisoning by ingestion. The study showed an increased use of insecticides containing acetylcholinesterase inhibitors as well as an alarming increase of suicide attempts. It was reported that 55% of pesticides intoxications in Brazil between 2007 and 2012 were attributed to suicide attempts [2]. Therefore, it was necessary to develop a fast and efficient method for pesticides determination in human biological samples.

The use of pesticides as means for suicide or homicide in developing countries with high population densities in rural areas is justified due to people's easier access to these types of highly toxic products. The existing short time between intoxication and medical care is a factor that contributes to the evolution of poisoning cases [1]. The treatment of patients acutely intoxicated by pesticides is, at first, symptomatic. The correct identification of the active substance responsible for the intoxication is fundamental when monitoring patients as practitioners can, for example, determine the amount of antidotes to be used as well as to outline treatment period. This has led analytical toxicology laboratories to develop methodologies that allow the identification and quantification of pesticides in biological samples, both for forensic toxicology and emergency toxicology analysis.

To accurately detect and quantify poisonous substances present in biological fluids, several sample pretreatment steps must be executed. The goal is to separate the analytes of interest from other compounds present in the sample, which may be incompatible with the instrumentation used for identification and quantification. In addition to this, samples go through a concentration step for detection of the substances present at trace concentration levels. Current trends in analytical chemistry have shown a growing interest and implementation of novel sample preparation techniques. Particularly, methods based on microextraction processes have seen an evolution as alternatives to classic extraction methods, such as liquid–liquid extraction and solid-phase extraction, which are faster, more efficient, automatable, and environmentally friendly [4, 5].

The micro-QuEChERS sample preparation technique is a fast methodology that requires low sample volumes and is suitable for compounds with different physiochemical properties [6–8], while being environmentally friendly due to its low solvent consumption requirements.

The study presented in this chapter describes a methodology based on a modified micro-QuEChERS approach and the use of

liquid chromatography tandem mass spectrometry (LC-MS/MS) for the quantitative determination of nine pesticides (selected based on the mostly common agricultural crops in Brazil) in whole blood samples.

2 Materials

2.1 Extraction Materials and Preparation Procedure

Pesticide standards used: Carbamates [aldicarb, aldicarb sulfone (aldicarb main metabolite in blood), carbofuran]; organophosphates (chlorpyrifos, fenthion mevinphos); herbicides (atrazine, 2,4-D); and phenylpyrazole (fipronil) were available as powder and were purchased from Merck KGaA (Darmstadt, Germany).

Analysis Materials Preparation:

1. Blood matrix: Blank blood samples (whole blood, serum, and plasma) were donated by local blood bank when they are no longer suitable for use. Each bag can be pooled into a lot and documented as negative by the laboratory for routine testing.
2. 1 mol/L ammonium formate: Add 6.306 g of ammonium formate (≥99.0%, LCMS grade) to a 100 mL volumetric flask and fill to volume with ultrapure water (LCMS grade). Store at room temperature.
3. Individual pesticides stock solutions (1 mg/mL): Add 10 mg of the pesticide to a 10 mL volumetric amber flask and fill to volume with methanol (LC grade). Store at −20 °C in amber glass vial.
4. Working standards solutions: Pesticides working solutions can be prepared by diluting the corresponding stock solutions in volumetric amber flask (using methanol LC grade as dilution solvent), in order to achieve concentrations between 50 ng/mL and 50,000 ng/mL. Store at −20 °C in amber glass vial.
5. Internal standard working solution: (IS, diazepam-d_5 200 ng/mL). Diazepam-d_5 is purchased as 100 μg/mL solution (Cerilliant, Round Rock, TX) and is stored in the freezer until consumed. To a 25 mL volumetric flask, add 0.05 mL (50 μL) of the stock solution. Fill to volume with methanol (LC grade) and mix well. Store at −20 °C in amber glass vial.
6. QuEChERS extraction salt mixture: Weigh 4 g of anhydrous magnesium sulphate (≥99%, reagent grade) and 1 g of anhydrous sodium acetate (≥99%, reagent grade) to a mortar. Gently homogenate with a pestle. Store in clean glass vial, at desiccator and room temperature.

2.2 Instrumentation Configuration

1. Liquid chromatograph coupled to tandem mass spectrometer: Our laboratory utilized a Nexera X2 UFLC chromatographic system coupled to a LCMS8060 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) for the analysis.
2. Analytical Column: Restek Raptor Biphenyl (100 mm × 2.1 mm, 2.7 μm).
3. Mobile phase A (MP-A): Ultrapure water containing 0.1% formic acid and 2 mmol/L ammonium formate. The mobile phase A can be prepared every week by adding 1 mL of formic acid (≥98%, LCMS grade) and 2 mL of 1 mol/L ammonium formate aqueous solution to a 1 L volumetric flask and filling to volume with ultrapure water (LCMS grade). Mix well.
4. Mobile phase B (MP-B): Methanol containing 0.1% formic acid and 2 mmol/L ammonium formate. The mobile phase B can be prepared every week by adding 1 mL of formic acid (≥98%, LCMS grade) and 2 mL of 1 mol/L ammonium formate aqueous solution to a 1 L volumetric flask and filling to volume with methanol (LCMS grade). Mix well.
5. Autosampler rinsing solution: A mixture of methanol and ultrapure water (70:30, v/v) was utilized as the autosampler needle cleaning solution. With a 1 L measuring cylinder, measure 700 mL of methanol and transfer to a 1 L autosampler glass bottle. With a 1 L measuring cylinder, measure 300 mL of ultrapure water and transfer to the same 1 L autosampler glass bottle. Mix well. Tables 1 and 2 display the LC and MS/MS conditions.

3 Methods

This method was validated following the recommendations of the Scientific Working Group for Forensic Toxicology for quantitative analyzes (SWGTOX). The calibration curves were linear from 5 to 1000 ng/mL (except for 2,4-D, evaluated from 25 to 5000 ng/mL), achieved a linear regression coefficient $r > 0.98$, with all standards quantifying within ±15% of target except ±20% at the limit of quantification. No endogenous or exogenous interferences were observed. Method imprecision and bias were less than 20%, and matrix effect was greater than 38% for all compounds. No carryover was observed in blank samples after analysis of a 5000 ng/mL sample, and neither endogenous (analysis of 10 different blood samples) nor exogenous interferences (common pharmaceuticals and drugs of abuse) were documented.

Table 1
LC-MS/MS parameters for the analysis of pesticides in whole blood by micro-QuEChERS and LC–MS/MS

Parameters	Set value
Column temperature	40 °C
Autosampler temperature	10 °C
Mobile phase flow	0.400 mL/min
Mobile phase elution gradient	0–0.5 min: 5% MP-B 0.5–9.0 min: 100% MP-B 9.0–12.0 min: 100% MP-B 12.0–12.1 min: 5% MP-B 12.1–15 min: 5% MP-B
Total run time	15 min
Injection volume	2 μL
Rinse mode	Before and after aspiration
Needle rising volume	2000 μL
Needle rising speed	35 μL/s
Rinsing deep time	5 s
MS acquisition mode	MRM (2 transitions/compound)
Ion spray voltage	4.0 kV (+) and 3.0 kV (−)
Heat block temperature	400 °C
Nebulizer gas (N_2) flow	3 L/min
Desolvation line temperature	250 °C
Drying gas (air) flow	10 L/min
Heating gas (N_2) flow	10 L/min
CID gas pressure (Ar)	270 kPa

3.1 Sample Preparation Protocol

1. Calibrator preparation: Transfer 225 μL of blank whole blood to a polypropylene tube. Add 25 μL of pesticides working solution to achieve the target calibrator concentration (from 5 ng/mL to 5000 ng/mL). Use the calibrators with the extraction described below.
2. Quality control (QC) preparation: Transfer 225 μL of blank whole blood to a polypropylene tube. Add 25 μL of pesticides working QC solution to achieve the target QC concentrations (from 5 ng/mL to 5000 ng/mL). Use the QCs with the extraction described below.
3. Negative control preparation: Transfer 225 μL of blank whole blood to a polypropylene tube. Add 25 μL methanol. Use the negative controls with extraction described below.

Table 2
Mass spectrometer acquisition parameters, analyte retention times, and deuterated internal standard used for the analysis of whole blood samples by modified micro-QuEChERS and LC–MS/MS

Pesticide	Precursor ion (*m/z*)	Product ion[a] (*m/z*)	Collision energy (eV)	Ionization mode	Retention time (min)
Aldicarb sulfone	240	<u>148</u> 86	−15 −22	positive	3.77
Mevinphos	225	<u>193</u> 127	−8 −16	positive	5.74
Aldicarb	208	<u>89</u> 116	−18 −9	positive	5.80
Atrazine	216	<u>132</u> 174	−23 −17	positive	6.65
Carbofuran	222	<u>165</u> 123	−13 −22	positive	6.66
2,4-D	219 221	<u>161</u> 163	13 11	negative	6.77
Fipronil	437	<u>320</u> 332	23 15	negative	7.45
Diazepam-d_5 (IS)	290	<u>154</u> 198	−28 −34	positive	8.40
Fenthion	279	<u>247</u> 169	−18 −14	positive	8.60
Chlorpyriphos	350	<u>162</u> 198	−38 −20	positive	9.11

[a]Underlined ions are used to quantification

3.2 Micro-QuEChERS Extraction Protocol

1. Transfer 250 μL of whole blood sample (*see* **Notes 1** and **3** for further details) to a polypropylene tube.
2. Add 25 μL of internal standard working solution.
3. Add 500 μL of ice-cold acetonitrile, cap the tube, and vortex for 10 s.
4. Let sample equilibrate for 5 min.
5. Add 100 mg of QuEChERS salt mixture (magnesium sulphate and sodium acetate, 4:1 m/m).
6. Homogenize in a multibead shaker (*see* **Note 2**) at 7 m/s for 20 s (three cycles). Centrifuge at 18,000 × *g* for 10 min.

7. Transfer 125 μL of supernatant to an autosampler vial. Dilute with 375 μL of MP-A. Cap the vial and vortex for 10 s.
8. Inject 2 μL into the liquid chromatography–tandem mass spectrometry (LC-MS/MS) system.

3.3 Instrument Preparation Procedure for Analysis

1. Pump each mobile phase component at 5 mL/min for 3 min with the instrument purge valve open to flush lines.
2. Purge the autosampler rinsing solution for 3 min.
3. Check for leaks and turn on the entire system to allow for the column, autosampler, and mass spectrometer to equilibrate at the set parameters for at least 15 min before start the analysis. Monitor system back-pressure ensures the chromatographic system is ready before sample injections. *See* Tables 1 and 2 for instrument parameters.

4 Notes

1. Human plasma and serum samples were analyzed successfully with this method without the need for adjustments in the sample preparation and micro-QuEChERS extraction procedure.
2. For regular samples (not-clotted blood, serum, plasma), the multibead shaker homogenization process can be replaced by a 10 min vortex mixing process.
3. If the blood is clotted (e.g., *postmortem* blood), use the multibead shaker with stainless steel balls (2–3 balls, 3 mm i.d. each) to grinding the clot before applying the micro-QuEChERS extraction. Grind the clot in a multibead shaker at 7 m/s for 25 s (three cycles).
4. Analyte identification criteria to be considered: (a) a symmetrical chromatographic peak with retention time within ±2% of the average calibrator retention time, (b) signal/noise ratio higher than 3 for both qualifier and quantifier ions, and (c) the ratios of the two transitions within a maximum of ±30% of those established by the calibrators, varying more for those with low intensity for the major transition.
5. Figure 1 shows a chromatogram of a spiked sample with eight pesticides.

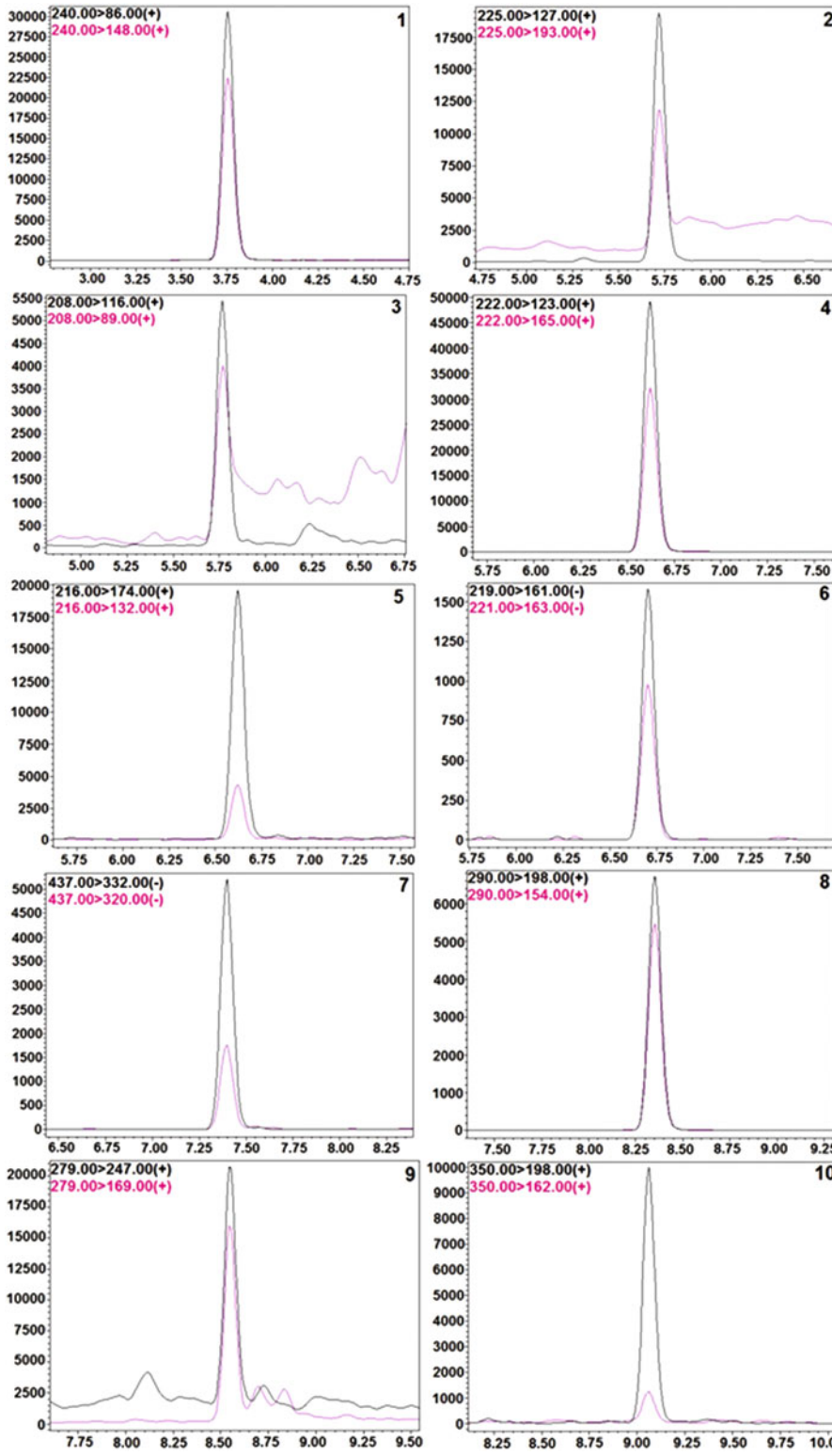

Fig. 1 Extracted ion chromatograms from a blood sample spiked with eight pesticides at 5 ng/mL and 2,4-D at 25 ng/mL (LOQ), and the internal standard (diazepam-d5 200 ng/mL) Legend: 1-aldicarb sulfone; 2-mevinphos; 3-aldicarb; 4-carbofuran; 5-atrazine; 6–2,4-D; 7-fipronil; 8-diazepam-d5 (internal standard); 9-fenthion; 10-chlorpyrifos

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Chapter 10

Dried Urine Spots as a Sampling Approach for the Determination of Organophosphorus Insecticides by Gas Chromatography Tandem Mass Spectrometry

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Abstract

There are numerous methods described for the identification and quantification of organophosphorus insecticides, and these involve sample preparation techniques such as solid-phase or liquid–liquid extraction. However, the demand for more efficient and productive approaches by laboratories continues to increase. As an improvement over the existing procedures, we describe herein a new method for the determination of five organophosphorus insecticides (diazinon, chlorpyrifos, parathion-ethyl, chlorfenvinphos, and quinalphos) in low volume urine specimens, using the dried urine spots (DUS) sampling approach and gas chromatography (GC) coupled to tandem mass spectrometry (MS/MS) analysis. The studied compounds were chosen for their incidence in intoxications, and ethion was used as internal standard. The novelty of using the DUS approach for the extraction of these compounds guarantees greater simplicity and sensitivity compared to conventional extraction techniques and can be applied routinely in scenarios of clinical and forensic toxicology.

Key words Organophosphorus insecticides, Urine samples, Dried urine spots, GC-MS/MS

1 Introduction

Pesticides are a family of chemical compounds used in most countries worldwide, especially in rural areas, to control and protect agricultural crops against insects, fungi, weeds, and other pests. They can be classified as insecticides, herbicides, rodenticides, fungicides, etc., according to the target organism [1]. Organophosphorus pesticides are the most used insecticides in agriculture, and diazinon, parathion-ethyl, chlorpyrifos, chlorfenvinphos, and quinalphos are some examples [2].

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Although these compounds are beneficial for crop production, their extensive use can lead to serious and deleterious consequences to the human health. Intoxication cases occur mainly in suicidal or accidental scenarios, and death often follows. The World Health Organization (WHO) estimated that each year there are 3,000,000 cases of acute pesticide poisonings, from which more than 250,000 are suicidal; 220,000 deaths are attributed every year to intoxication by insecticides [3].

Accidental poisoning or attempted suicide with organophosphorus compounds are among the most important forms of acute poisoning worldwide, affecting over 1,000,000 people each year, with around 100,000 deaths [4]. Most of these deaths occur in rural regions of the developing world, where easy access, excessive exposure, or inappropriate use turn many impulsive acts of self-poisoning into suicide [5].

Human exposure to organophosphorus insecticides can occur via ingestion, inhalation, or dermal absorption. Once they have entered the human body, these compounds are absorbed and distributed rapidly throughout the organism; however, due to their rapid biodegradation, they do not accumulate as occurs with organochlorine compounds as DDT. After distribution, they are metabolized in the liver, and active metabolites may originate for some compounds (e.g., parathion), while others can be eliminated without metabolism. Active metabolites, as well as unchanged forms, are mostly excreted in urine, and to a lesser extent in the feces and expired breath [6].

After exposure to these insecticides, signs of intoxication may appear early within a few minutes or hours, depending on the degree of exposure and the physical and chemical properties of the agent. The toxic effects occur by inhibition of acetylcholinesterase. This enzyme exists in both insects and humans, and although these compounds show preferential toxicity to the former, they are also toxic to humans, presenting a great risk of intoxication [7].

Briefly, due to the action of these substances, acetylcholine accumulates in synapses of the autonomic nervous system, central nervous system, and neuromuscular junction, and this leads to an overstimulation of muscarinic and desensitization of nicotinic receptors, provoking a cholinergic crisis; typical symptoms include miosis, hypersalivation, bronchorrhea, muscle fasciculation, failure of respiratory musculature, and toxic effects in the central nervous system. Peripheral and central respiratory paralysis will cause death in severe cases, unless the patient is artificially ventilated. In most situations, when individuals attend emergency services at hospital units, the toxic agent is already fully distributed or in the excretion phase [8].

For all these reasons, it is of great interest to develop a method that allows rapid detection and quantification of this type of compounds in biological specimens. Different sample preparation protocols have been used to determine these compounds in urine, including solid-phase microextraction (SPME) [9], solid-phase extraction (SPE), [10] and liquid–liquid extraction (LLE) [11]. LLE and SPE techniques were routinely used for many years; nevertheless, these need considerable volumes of organic solvents, which are expensive and increase the operator's exposure to toxic vapors, and therefore there is a growing trend to develop and use "green" extraction procedures [12].

Microextraction techniques have the advantages of using particularly low volumes of sample and organic solvents and usually offer the possibility of reusing the extraction device several times. The large amount of potential interferents and the incompatibility of the biological samples' matrix with analytical instruments are the main reasons for research efforts [13].

Recently, new sample preparation techniques have emerged, for instance, dried urine spots (DUS). This is a noninvasive sampling approach based on spotting urine samples onto dried spot collection cards, followed by air-drying before analysis. It is characterized by a simple extraction procedure, and low volumes of urine are used. After drying the biological sample on the paper, it is possible to proceed with the extraction of the retained compounds with small volumes of organic solvents, fulfilling the WHO environmental sustainability goals [14].

The most common instrumental methods involve some sort of chromatography, usually liquid (LC) or gas chromatography (GC) coupled to different detectors. Organophosphorus compounds are quite volatile, and therefore GC is the most used instrumentation for samples analysis. Several types of detectors may be used, and tandem mass spectrometry (MS/MS) is one of them [15].

The use of a gas chromatography system coupled to tandem mass spectrometry (GC-MS/MS) has a number of advantages. Indeed, its high-resolution power ensures the identification of compounds with great accuracy, thus allowing the analysis of more complex samples. In addition, this instrumentation usually provides low limits of detection and quantification when compared to other methods, since the use of the MS/MS detector increases sensitivity and selectivity [16].

The aim of this work is to use the DUS sampling approach and GC-MS/MS for the determination of a number of organophosphorus insecticides in urine samples.

2 Materials

2.1 Preparation of Working Standards

All reagents were of HPLC or analytical grade:

- Standard working solutions were prepared by properly diluting the starting solutions with methanol to the final concentration of 50 μg/mL for all analytes.
- Internal standard working solution was prepared in methanol to a final concentration of 1 μg/mL.
- All working solutions were stored in the absence of light at 4 °C.

2.2 Supplies and Analytical Equipment

- Agilent gas chromatography system (model HP 7890A).
- Agilent triple quadrupole mass spectrometer (model 7000B).
- MPS2 autosampler and a PTV injector from Gerstel.
- J&W Scientific capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) with 5% phenylmethylsiloxane (HP-5MS).
- Whatman™ Human ID Bloodstain Card BFC180.

3 Methods

3.1 Sample Preparation (See Note 3)

- Prepare the working calibration standards for the analytes with internal standard. Prepare the unknown sample for application.
- Apply 50 μL of urine fortified with calibration standards and 50 μL of the unknown urine to each of the spots on the Whatman™ Human ID Bloodstain Card BFC180 cards (*see* **Note 1**).
- Dry the cards for 12 h.
- Cut out the spots and place them in glass tubes.
- Add 3 mL of methanol and 50 μL of internal standard (1 μg/mL) (*see* **Note 2**).
- Agitate the samples for 25 min at room temperature.
- Centrifuge the samples for 5 min at 1260 × *g*.
- Remove the cards and evaporate the extracts to dryness.

3.2 Analysis

- Reconstitute the extracts with 50 μL of methanol.
- Transfer the extracts to autosampler vials and inject a 2 μL aliquot in the GC-MS/MS system.
- Define the chromatographic method to the following parameters:
  - The initial oven temperature is maintained at 100 °C for 4 min, then increases to 200 °C at 10 °C/min. Finally, the

Table 1
Analytical and detection conditions for organophosphorus pesticides and internal standard (*see* Note 6)

Analyte	Retention time (min)	Quantifier transition (*m/z*)	Qualifier transitions (*m/z*)	Collision energy (eV)
Diazinon	15.40	136.6–84.1	178.3–137.1	10 (15)
Chlorpyrifos	16.73	196.6–168.9	313.1–257.8	15 (15)
Parathion-ethyl	16.75	290.8–109.0	290.8–81.0	10 (20)
Chlorfenvinphos	17.19	266.1–159.0	322.0–266.9	15 (15)
Quinalphos	17.24	145.8–118.1	156.3–129.1	10 (10)
Ethion[a]	18.40	230.6–128.9	–	10

[a]Internal Standard

temperature is increased to 270 °C at 24 °C/min (maintained for 8 min), giving a total run time of 25 min.

- The injection inlet temperature is set at 240 °C.
- The detector temperature is set at 280 °C.
- The sample is introduced in the splitless injection mode.
- The helium flow (carrier gas) is 1.0 mL/min at a constant flow rate.
- The mass spectrometer operates with a filament current of 35 μA and electron energy 70 eV in the positive electron ionization mode.
- The nitrogen is used as collision gas at a flow rate of 2.5 mL/min.
- Data is acquired in the multiple reaction monitoring (MRM) mode using the MassHunter WorkStation Acquisition Software rev. B.02.01 (Agilent Technologies).

- Table 1 shows the mass spectrometer parameters for each of the compounds and the internal standard (ethion).
- Figure 1 shows the chromatograms of organophosphorus pesticides and internal standard (ethion) obtained after extraction of the spiked samples (*see* **Note 5**).

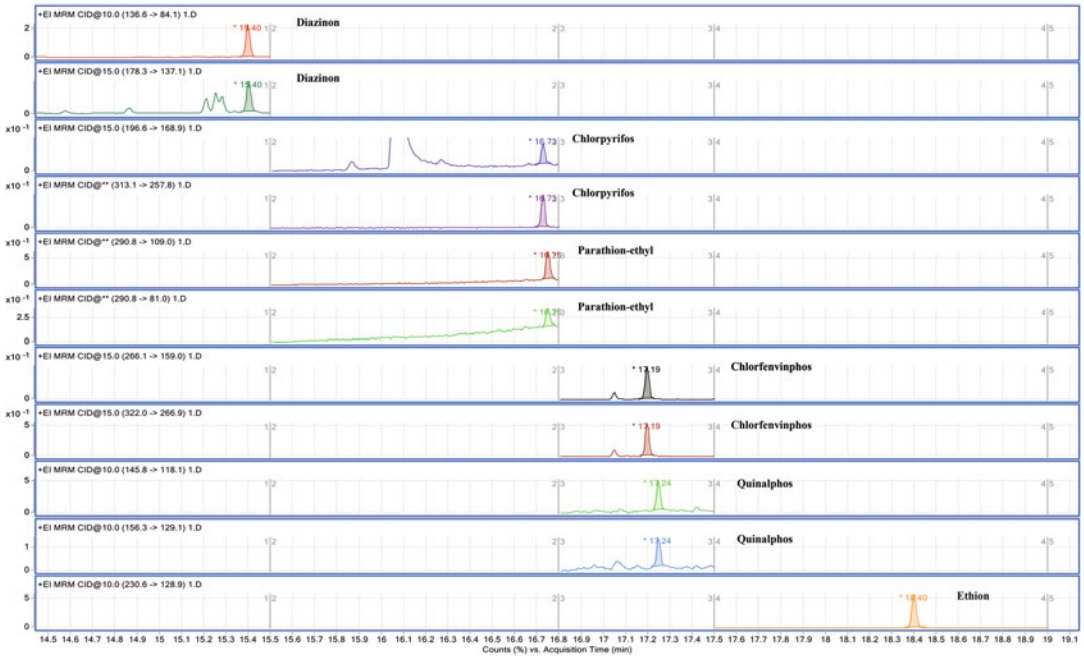

Fig. 1 Chromatograms of selected fragments of organophosphorus pesticides (diazinon, chlorpyrifos, parathion-ethyl, chlorfenvinphos, and quinalphos) and internal standard (ethion) obtained after extraction of the samples spiked at the LOD

4 Notes

1. Urine samples, depending on the time of analysis, can be stored frozen at −20 °C or at 4 °C.
2. The choice of ethion as internal standard is justified by its similarity to the analyzed compounds in this method, belonging to the same family, and by the fact that it is not commercialized in Portugal, which makes it unlikely to be present in real urine samples. For this reason, ethion may not be the best choice for all laboratories worldwide, and analysts should bear this in mind in order to choose adequately their internal standard.
3. To optimize the extraction process of biological samples, a univariable study was carried out to choose the extraction solvent, and methanol was chosen for its stable performance, for the considerable areas of the compounds and for the CV values below 20%. An experimental design study (DOE) was carried out to define multivariate parameters such as the drying

time of the spots, solvent volume, and extraction time. With the results obtained (using Minitab 17) from the pareto effects, main effects plot, and interaction plot, it was possible to define 12 h of drying time, 3 mL of solvent volume, and 25 min of extraction time. The remaining parameters (biological sample volume, agitation temperature for extraction, and centrifugation time) were defined based on the experience of the group that has developed several studies with the application of dried matrix spots as sampling approach.

4. For the study of absolute recovery, in order to understand the percentage of compounds that can be extracted from the spots using this sampling approach, recoveries were calculated at six different concentration levels (25 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL, and 5000 ng/mL). One group of samples was fortified after extraction, while the other group was fortified before extraction. Recovery results were obtained by comparing relative peak areas of the samples from the second group with those of the samples from the first group. The results are shown in Table 2.
5. The LOD value for each compound under study was determined as 25 ng/mL for chlorpyrifos, chlorfenvinphos, and quinalphos and 100 ng/mL for diazinon and parathion-ethyl, by the analysis of ten replicates of spiked samples with CV values between 1.12 and 19.90%. These limits were established as the lowest concentrations that exhibited a discrete peak undoubtedly distinguishable from a blank sample and a signal-to-noise ratio of at least 3.
6. All of the pesticides analyzed in this method must be detected both in the quantifier and in the qualifier transitions for their unambiguous identification. However, the parameters of mass spectrometry vary between instruments and, therefore, must be optimized for each laboratory.

Table 2
Absolute recoveries of organophosphorus pesticides ($n = 3$) from urine samples (*see* Note 4)

Analyte	Recovery[a] (%)					
	25 ng/mL	100 ng/mL	250 ng/mL	500 ng/mL	1000 ng/mL	5000 ng/mL
Diazinon	n.d.	0.36 ± 0.02	0.59 ± 0.11	1.18 ± 0.14	2.57 ± 0.24	7.91 ± 0.88
Chlorpyriphos	0.22 ± 0.04	0.32 ± 0.02	1.30 ± 0.17	2.45 ± 0.18	3.22 ± 0.22	8.85 ± 0.87
Parathion-ethyl	n.d.	0.16 ± 0.00	0.41 ± 0.01	1.04 ± 0.19	2.30 ± 0.32	6.15 ± 1.17
Chlorfenvinphos	0.18 ± 0.03	0.32 ± 0.02	0.88 ± 0.14	3.30 ± 0.13	3.89 ± 0.13	7.16 ± 1.41
Quinalphos	0.55 ± 0.08	1.03 ± 0.04	3.91 ± 0.49	10.44 ± 0.65	13.88 ± 0.57	32.37 ± 2.70

[a]Mean values ± standard deviation; n.d.: not detected

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Chapter 11

Detection of Organochlorine Pesticides in Water and Biological Matrix

Eleuterio F. Umpiérrez and Daniela Ariadna Díaz

Abstract

The continuous introduction of new technologies and new instruments in the analytical field drives the laboratories to try to purchase fancy instruments that most of the cases are oversized for the needs of the laboratory. Identifying and quantifying organochlorine compounds is a normal task nowadays but it is always challenging when the matrix involved has environmental factors or complex biological matrix.

Herein we describe step-by-step the protocols used in our lab for sample preparation and accurate quantification of organochlorine compounds in drinking water, surface water, underground water, and biological samples using gas chromatography with electron capture detector (GC-ECD) or gas chromatography coupled to mass spectrometry (GC-MS).

Key words Organochlorines, POPs, GC-ECD, GC-MS, Water, Drinking water, Urine, Blood, Biological samples

1 Introduction

The determination of persistent organic compounds is very well documented and standardized (by EPA, ISO, etc.) [1–3]. The presence of these compounds in soil, water, and food has made it important to seek for their presence in these matrices, and also to quantify them.

Some of the first doubts that usually arise when someone starts to work in this field is which methodology will be the most suitable for routine assays, namely gas chromatography with an electron capture detector (hereinafter GC-ECD) or gas chromatography coupled to a mass spectrometer (GC-MS), or even make a leap in detectability and investment, and use hyphenated techniques such as gas chromatography coupled to tandem mass spectrometer

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(GC-MS/MS), liquid chromatography coupled to tandem mass spectrometer (LC-MS/MS), gas chromatography coupled to a high-resolution mass spectrometer (GC-HRMS), or liquid chromatography coupled to a high-resolution mass spectrometer (LC-HRMS).

The technique of GC-ECD is a simple technique with a simple and very specific equipment to detect organochlorine compounds but only allows us to "identify" them per retention time. The why of the "identify" quotes is because the correct use allows detecting the presence of persistent organic pollutants (POPs) if one knows the matrix very well and suspects that there should be no other substance that could coincide in the retention time and possess a halogen moiety. That is, if the global Identification Points criteria are taken, it does not meet these identity requirements. But the rules continue to allow it for matrices where there is a control of its reasonable production. This type of detector also presents another inconvenience or virtue, because as it detects all halogenated compounds, it also detects the metabolites, thereof, although we do not know what they are. And it is almost always the maximum limits allowed for POPs are referred to the active compounds and very few include their metabolites or degradation products. And what happens is that it still remains a lot to investigate the health effects of the metabolites thereof.

The technique of GC-MS is more complex and more expensive, but it allows following the identification criteria point to make a correct identification of the substance being quantified; furthermore, if another signal appears at the same retention time, it can be separated according to mass, provided that a correct choice of ions was made to monitor. Unknown substances can also be detected by using interchangeable global libraries, which can be introduced in both qualitative and quantitative methods, provided that their certified standard is commercially available.

If the substances to be monitored are present at concentrations lower than ng/mL in the extract that is injected, more sensitive equipment will be necessary, and there enter the mass/mass instruments that have quantification limits of the order of ng/L (at least for some substances). And the most robust instruments for this purpose are still triple quadrupoles. Now if what is desired is to detect new substances at those low concentrations and be able to identify what structures are associated to them, high-resolution mass spectrometers are needed. Next we will describe the sample preparation methods in a simple way to be used in GC-ECD and GC-MS equipment to detect POPs in drinking water and biological matrices.

2 Materials

Milli-Q water.

1 L or 500 mL amber borosilicate bottle fitted with screw caps lined with TFE-fluorocarbon.

10 mL glass vacutainer tubes.

1 L or 500 mL separating funnel with a Teflon stopper.

Hexane (pesticides free).

Sodium chloride ACS reagent.

Anhydrous sodium thiosulfate. ACS reagent.

Vials of 20 mL.

Screw caps with Teflon septa or crimp caps with Teflon septa and vials of 2 mL.

Vortex.

Balance.

**All reagents are reagent-grade unless otherwise stated.*

3 Methods

3.1 Sample Collection

3.1.1 Water

Collect the water sample in 1 L amber borosilicate bottle fitted with screw caps lined with TFE-fluorocarbon. If residual chlorine is suspected to be present, add 80 mg of sodium thiosulfate per liter of sample to the sample bottle prior to collecting the sample. Samples must be iced or refrigerated at 4 °C from the time of collection until extraction.

3.1.2 Blood

Collect the blood sample in 10 mL glass vacutainer using 21-gauge stainless steel needles This process and the use of glass blood collection tubes were used to prevent contamination.

Blood samples were collected directly into plain 10 mL glass vacutainer tubes, allowed to clot, and after 30 min were centrifuged for 10 min at 2000 revolutions per minute (RPM). After serum was separated off, stored in a freezer at −20 °C until the analysis is made.

3.1.3 Urine

Collect the urine (for example the first morning midstream urine) sample into a 500 mL glass amber bottle with Teflon-lined lid. Stored in a freezer at −20 °C until the analysis is made.

3.2 Sample Preparation

Allow samples to reach room temperature, and then place 500 mL of the sample in a 500 mL separating funnel with a Teflon stopper.

Add 1 mL of a solution of pentachloronitrobenzene with a concentration of 2 μg/mL approx. as internal standard (IS).

3.3 Calibrators Preparation

Positive and negative controls must be prepared.

For the positive control, add the internal standard pentachloronitrobenzene as in the samples and add 10 μL of standard solution of organochlorines mix with all the analytes of interest at a concentration of 20 μg/mL approx. in 500 mL of Milli-Q water.

For the negative control, add only the internal standard as in the samples in 500 mL of Milli-Q water.

3.4 Extraction

3.4.1 Extraction of Water Samples

Add to the sample approx. 85.7 g of sodium chloride in the separating funnel. Shake until everything is dissolved. Then 20 mL of hexane free of pesticides should be added, cover and shake vigorously for about 3 min to mix the two phases well.

Uncover and wait, a minimum of 5 min, for the phases to separate.

Discard the aqueous phase and filter the organic phase with a funnel filter paper and 1 g anhydrous sodium sulfate into a 20 mL vial.

Let the solvent evaporate to dryness.

For this manipulation, we have three options:

- Let the sample evaporate at room temperature in a laboratory hood.
- Evaporate under a gentle nitrogen stream.
- Use a centrifugal vacuum concentrator in which you have to control the speed of the centrifugation, the vacuum use, and the temperature of the block; all these parameters depend on the used tube size. If these parameters are not correct, there can be analyte losses (volatile organochlorine compounds).

Take up the residue with 100 μL of hexane, vortex and transfer to an insert in a 2 mL vial with a sealable cap.

Inject 1 μL into the GC-MS or GC-ECD to be analyzed.

3.4.2 Extraction of Biological Fluids

Urine (1 mL) and blood (1 mL) samples are diluted with 2 mL of H_2O, and 1 mL of pentachloronitrobenzene with a concentration of 2 μg/mL approx. as an internal standard is added.

The same procedures as above described for water analysis are then performed.

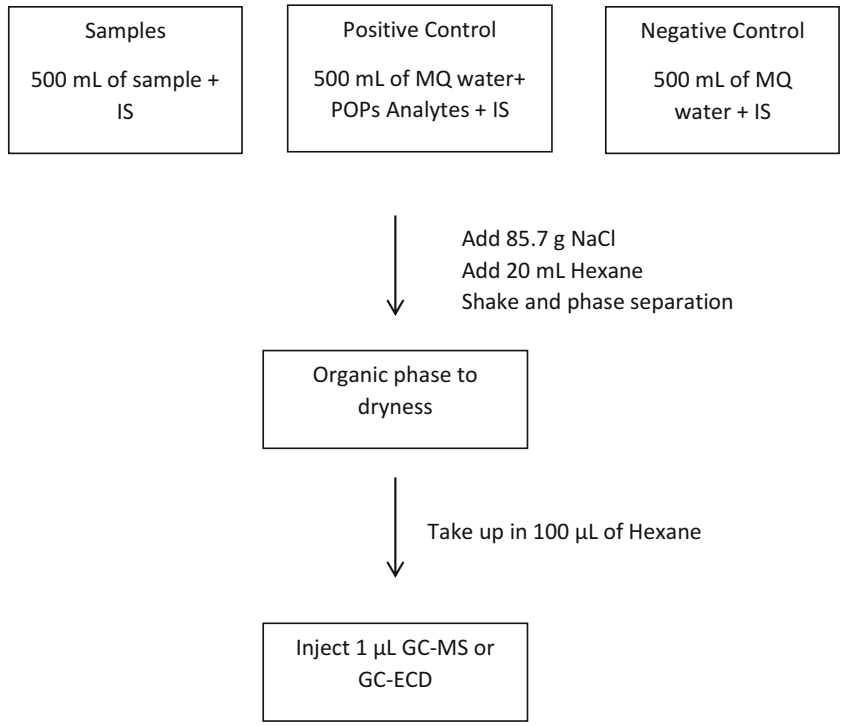
3.4.3 Quality Controls

All batches of samples have their positive and negative controls.

The positive control is at the higher permitted value.

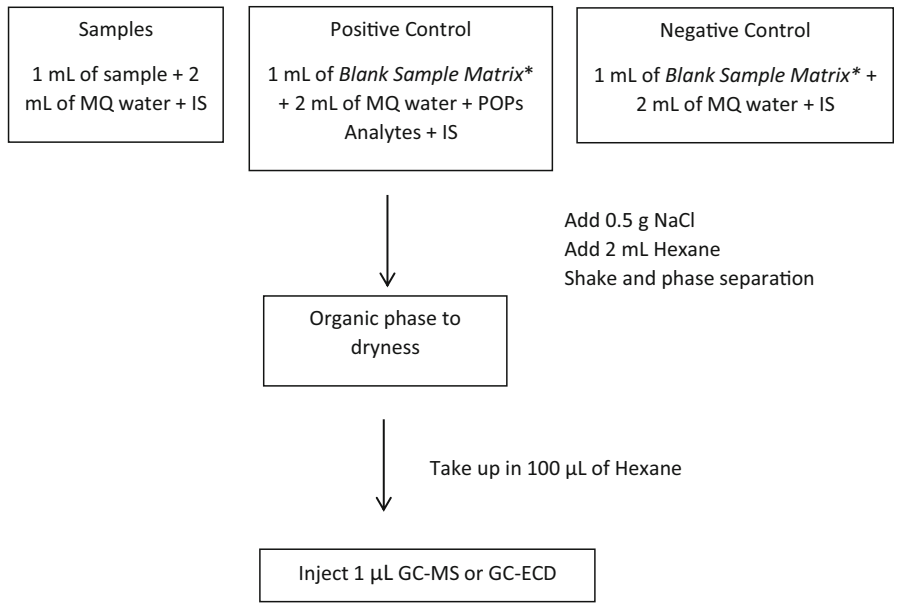
Summary:

3.4.4 Water



Summary:

3.4.5 Biological Samples



Blank Sample Matrix: in case of not having a blank matrix, replace with MQ water.*

3.5 Instrument

3.5.1 GC-ECD

Analysis was carried out on a gas chromatography instrument Agilent 6890 N GC with Electron Capture Detector. The column was an Agilent HP-5MS (30 m length, 0.25 mm i.d., and 0.25 mm film thickness, Agilent Technologies).

Injection in splitless mode, with nitrogen as carrier gas flow of 0.5 mL/min (constant flow).

Nitrogen also as makeup flow of 60 mL/min.

Injection volume: 1 μL.

Injector temperature: 250 °C.

The start oven temperature 60 °C, hold for 1 min, then 160 °C at 20 °C/min with a hold of 3 min, 275 °C at 3 °C/min without hold, and 310 °C at 20 °C/min without a hold.

Detector temperature: 320 °C (*see* **Note 1**) (Figs. 1 and 2).

3.5.2 GC-MS

Analysis was carried out on a gas chromatography instrument Agilent 5890 GC coupled with an Agilent MSD 5973 N. The column was an Agilent HP-5MS (30 m length, 0.25 mm i.d., and 0.25 mm film thickness, Agilent Technologies).

Injection in splitless mode.

With a helium flow of 0.5 mL/min.

Injector temperature: 250 °C.

The start oven temperature 60 °C, hold for 1 min, then 160 °C at 20 °C/min with a hold of 3 min, 275 °C at 3 °C/min without hold, and 310 °C at 20 °C/min without a hold.

Electron ionization is used, and analyses are performed in both scan and selected ion monitoring (SIM) acquisition modes (*see* **Note 2**).

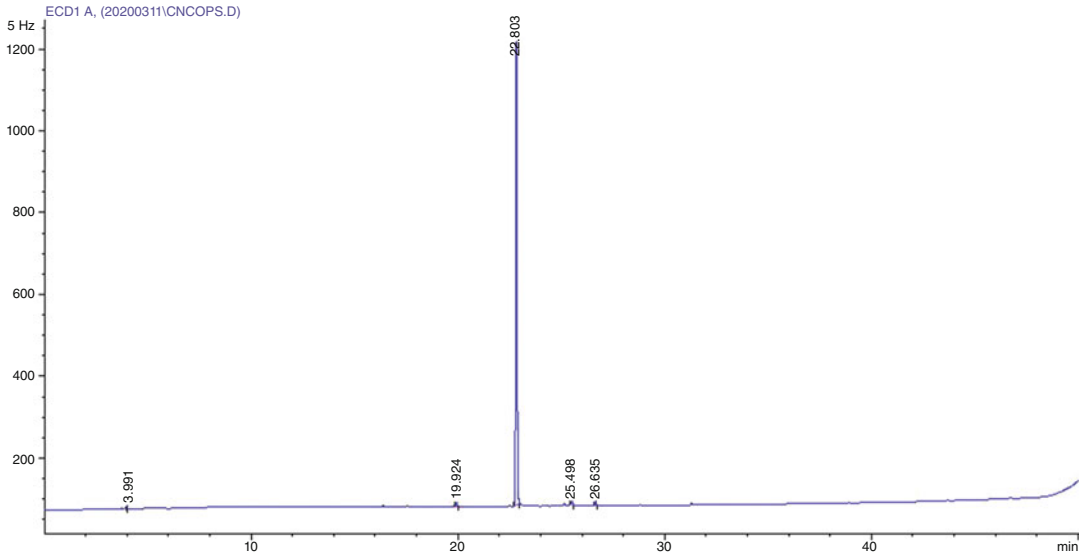


Fig. 1 GC-ECD signal of blank sample

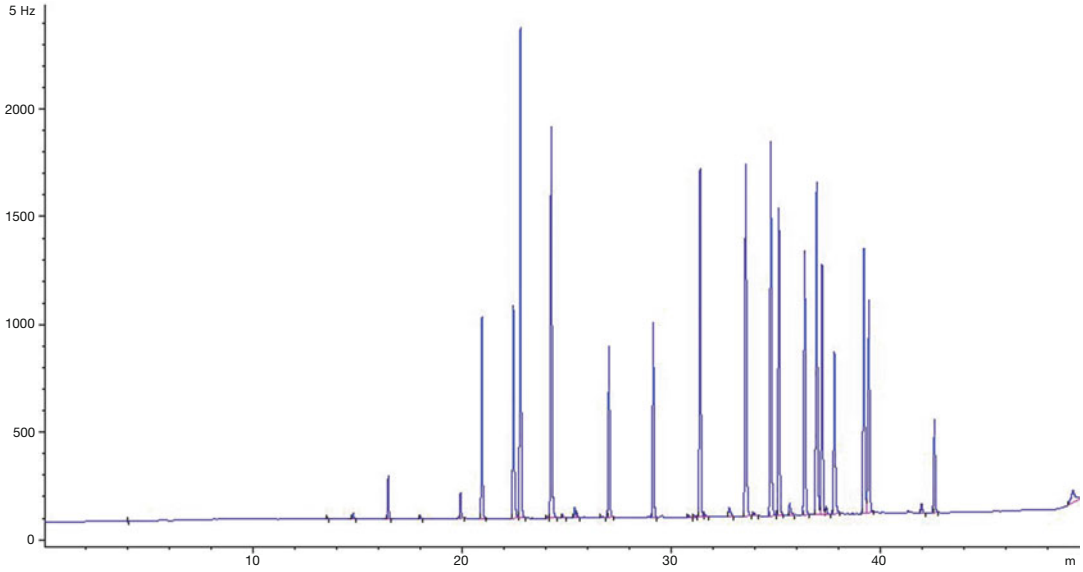


Fig. 2 GC-ECD signal of positive control sample

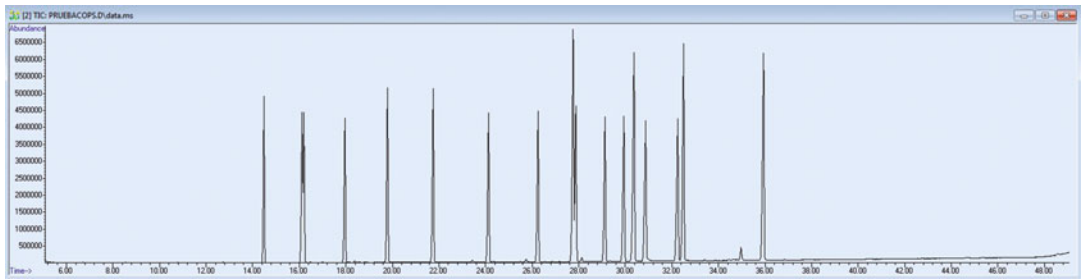


Fig. 3 GC-MS positive control

For scan: 45–350 *m/z*.
The ions of interest are as follows (Fig. 3):

m/z 142, 237	Pentachloronitrobenzene
m/z 284	Hexachlorobenzene
m/z 200	Atrazine
m/z 181, 219	Alpha BHC-lindane
m/z 181, 219	Beta BHC-lindane
m/z 181, 219	Delta BHC-lindane
m/z 181, 219	Gamma BHC-lindane
m/z 160, 188	Alachlor
m/z 263, 293	Aldrin
m/z 373, 375	Cis-chlordane

(continued)

m/z 373, 375	Trans-chlordane
m/z 100	Clomazone
m/z 197, 314	Chlorpyrifos
m/z 163, 183	Cypermethine
m/z 163, 183	Permetrine
m/z 263, 277	Dieldrin
m/z 165, 235	DDD
m/z 246, 318	DDE
m/z 165, 235	DDT
m/z 263, 281	Endrin
m/z 263, 281	Endrin aldehyde
m/z 195, 339	Endosulfan I
m/z 195, 339	Endosulfan II
m/z 386, 272	Endosulfan sulfate
m/z 227	Metoxychlor
m/z 100, 272	Heptachlor
m/z 81, 353	Heptachlor epoxide
m/z 55, 126	Molinate
m/z 186, 201	Simazine
m/z 161, 217	Propanil

When we work with equipment such as GC-ECD, we must be very careful in the search for our analytes, since the only information that we will have to identify them is their retention time (Rt). In the initial stage of the implementation of the methodology in the laboratory, it is vitally important to work ensuring that the identification of the analytes is correct, since many times the analytes of interest are purchased in mixes in order to lower costs, but the problems that this carry is in knowing that each peak corresponds to a single analyte and the order in which they are separated by the chromatographs is what we know from the bibliographic consultation.However, when we use a GC-MS, the problems of superposition of analytes can be solved easily if you choose the correct ions mass/charge (m/z) to increase the selectivity of the signal. Regarding the chromatographic order of the signals, since the GC-MS allows their identification through the mass spectra, they can be compared against any available pesticide library, either paid or with open access.

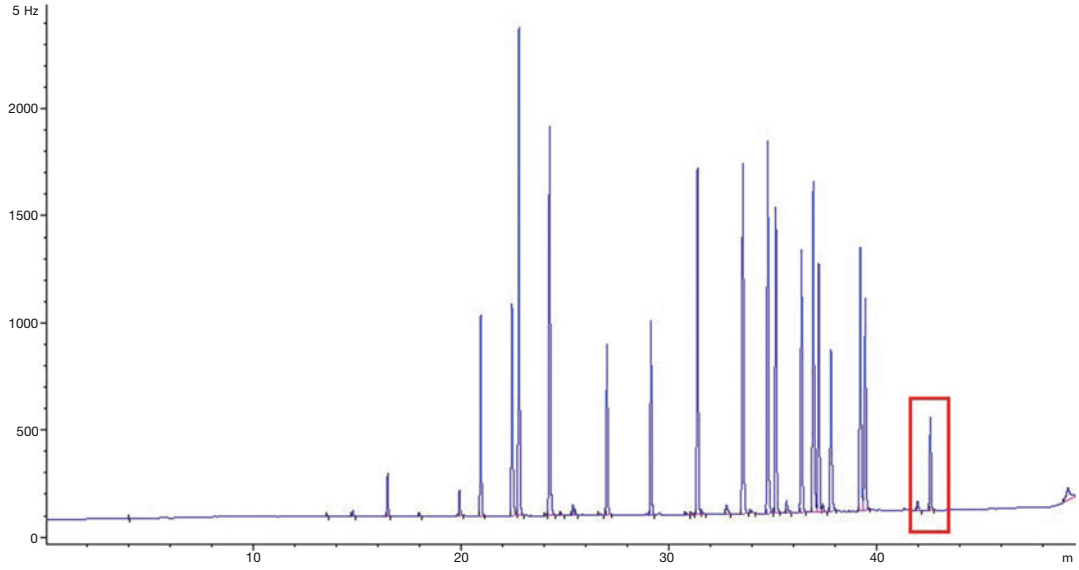


Fig. 4 ECD chromatogram (metoxychlor is signaled)

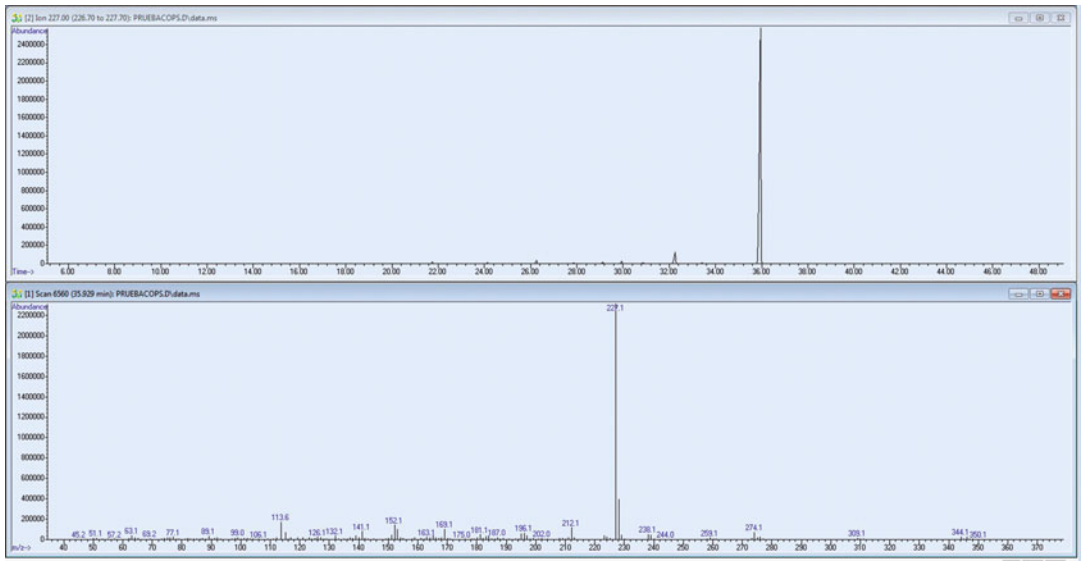


Fig. 5 Ion extracted chromatogram for metoxychlor

Likewise, it should be noted that many analytes are structural isomers of others, so some of their search ions may be the same, but not their full spectra, so they must be known to be able to identify them correctly. In this point, there are several guidelines about mass spectrometry identifications criteria (Figs. 4 and 5).

4 Notes

1. Method modification

   In our laboratory, the methodology established by EPA (EPA 505-1) was used. However, one of the difficulties found was that two analytes (dieldrin and DDE) were not correctly separated because they had the same retention time, and therefore they coelute in one peak. If we think in terms of the instrument, the coelution of two analytes in the same peak does not represent a great challenge for a detector such as the MS, since by correctly selecting the ions we can identify and also quantify them individually. But when we are faced with a detector like the ECD, we do not have the same luck.

   We always have the possibility of running a second method to separate these compounds; this issue must be addressed by the time when the methodological validation begins, and the laboratory has to decide how it is going to solve it or not. For us, it was very important to be able to quantify independent analytes with the use of ECD in the first injection, so various tests were carried out to achieve this, modifying the column's flow rate to obtain a good resolution of the peaks. Subsequently, it was validated and subjected to a proficiency test, obtaining satisfactory results for all analytes.

2. Macros

   On the other hand, taking into account the large number of analytes that must be identified and bulk quantify of samples, arises the need for tools that quickly allow us to determine whether or not our sample contains any of these substances.

   The tool that we use is called Macros in the ChemStation software from Agilent [4, 5]. When the laboratory implemented the methodology and identified all its analytes of interest, having the retention times well identified for the case of ECD; and retention times and ions for the case of the MS, a programming algorithm can be carried out in such a way to generate reports in which they show limited time windows corresponding to each analyte, and for the case of the MS also relying on those ions. Working in this way allows quickly processing of a large number of samples, not waste resources and focusing on those potential positive samples. On a second level, when the identification criteria are fulfilled, automatic quantification can be defined (Figs. 6 and 7).

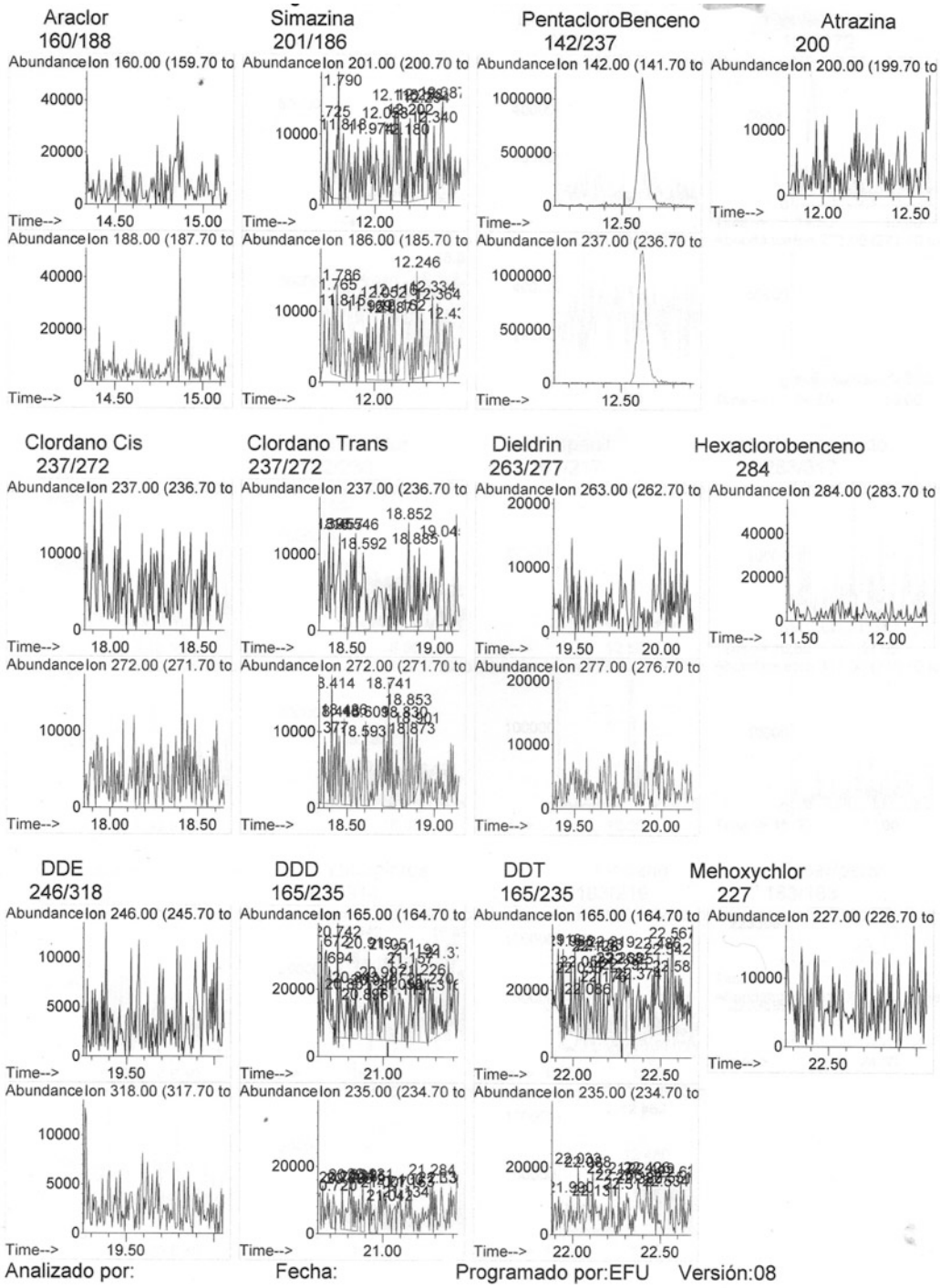


Fig. 6 Macro of total ion chromatogram of a blank sample

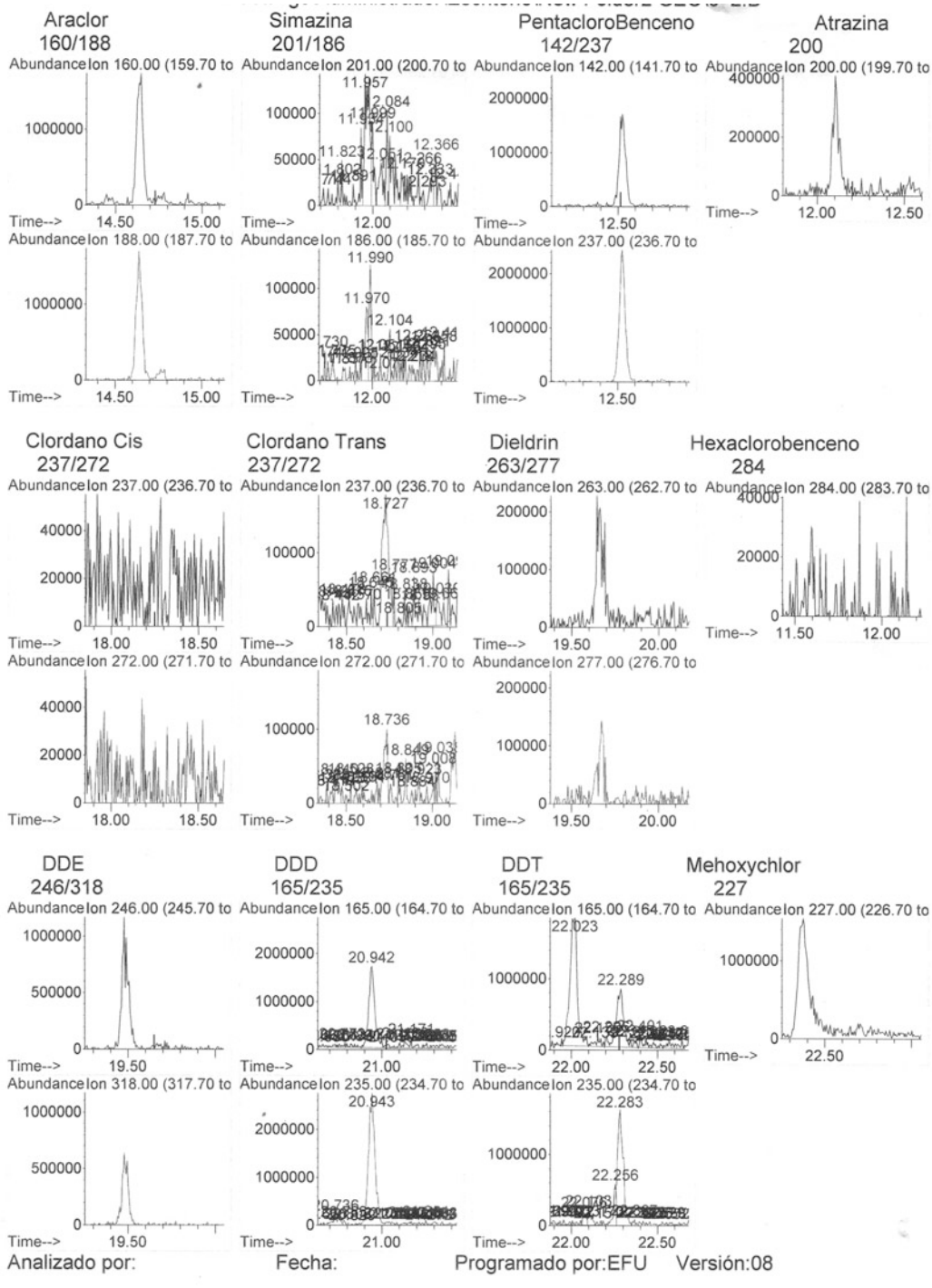


Fig. 7 Macro of total ion chromatogram of a positive control sample

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