Pesticide Residue Analysis in Animal Origin Food: Procedure Proposal and Evaluation for Lipophilic Pesticides

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Additional information is available at the end of the chapter

1. Introduction

Pest control in intensive agriculture involves treatments with a variety of synthetic chemicals generically known as pesticides. These chemicals can be transferred from plants to animals via the food chain. Furthermore, animals and their accommodations can be sprayed with pesticide solutions to prevent infestations. Consequently, both contamination routes lead to bio-accumulation of pesticides in food products of animal origin as meat, fish, fat, offal, eggs, and milk [1].

Persistent organic pollutants (POPs) are mostly organochlorinated pesticides (OCPs) which have been banned for agricultural and domestic uses in Europe, North America and many countries of South America, due to their environmental persistence and potential adverse effect on wildlife and human health, according to the Stockholm Convention in 1980s. However, some OCPs are still used, as the *DDT* (for the control of the outbreak of the mosquito that spread malaria and as and as antifouling agent in some developing countries). Besides, the most commonly used acaricide, *Dicofol*, is made of *DDT*, so its formulated products always contain small amounts of *DDT* [2]. These harmful compounds persist in the environment and they are transported around the world, as Coscollà et al.[3] found in the air of France. OCPs, have a highly stable, low volatile, non-polar, lipophilic nature, and consequently exhibit considerable environmental persistence with a tendency to bio-accumulate, leading to the contamination of foodstuffs, especially those with a high fat content.

Also synthetic pyrethroid pesticides (PYRs) are effective broad spectrum insecticides although with low toxicity for mammalian and short-term environmental persistence, due to their rapid degradation, being ester cleavage the major process [4]. Pyrethroids range from

non-polar to low-polarity lipophilic compounds. Owing to their metabolism in animals, they tend to bio-acumulate in lipid compartments, becoming a potencial source of human exposure through foodstuffs [1].

Organophosphorus pesticides (OPPs), mainly used as insecticides, are esters of phosphoric acid with different substituents. They have a wide variety of physico-chemical properties, as polarity and water solubility. OPPs, less persistent than OCPs, use to be the first choice for treatment because they provide efficacious, safe and cost effective control of a wide range of pests. They can be absorbed by inhalation, ingestion and dermal absorption; and they can also concentrate along the food chain.

Triazines are among the most widely used herbicides in agriculture. They are degraded by chemical and biological processes in their respective hydroxytriazines. Triazines and their degradation products are low polarity compounds, weakly basic, stable in the environment and therefore, persistent. [l].

Other pesticides, such as carbamates, benzoylureas, quinoxalines, amines and fluorides, have been evaluated for analytical purposes in animal origin food.

Most usually studied pesticides in animal origin food are non polar compounds that tend to concentrate and remain in fatty food. Reported studies from literature [1] for products of animal origin, five groups of pesticides (OCPs, OPPs, PYRs, carbamates and triazines) were the most analysed. Few of them have been detected in various animal products (*DDT* and its metabolites *DDD* and *DDE, Hexachlorocyclohexane* α, β and γ isomers, *HCB, Aldrin, Dieldrin, Endrin, Heptachlor* and *HCE, Endosulfan α and β isomers, Chlordane, Simazine, Atrazine, Deltamethrin and Cypermethrin*). The 2009 European Union Report on Pesticide Residues in Food concludes that the majority of animal origin food was free of detectable residues, that 34 different pesticides were found and most of them were rather due to environmental contaminations with persistent pesticides, also *Deltamethrin, Cypermethrin* and *Clorpyrifos* were found in some samples [5]. In another study made in Spain, triazines (*Terbutilazine, Simazine* and *Atrazine*) were detected in 16% of raw cow´s milk samples [6].

The US Food and Drug Administration (FDA) categorize food as fatty food when the fat content is ≥ 2% of weight and non-fatty food when it is < 2 %. Besides that classification, Lehotay divided food into non-fatty (< 2% fat), **low**-fatty (2-20%) and high-fatty (>20%) [2]. In low-fatty samples, lipophilic and hydrophilic pesticides can occur, so analytical methods should cover a wide pesticide polarity range. However, in fatty food samples, occurring pesticides are fat soluble, so analytical methods should focus on non-polar pesticides [7].

For dietary intake purposes, it is desirable to include metabolites and degradation products of toxicological concern, for example the oxygen analogues of organophosphate pesticides that might be produced or persist in processed food. In most of cases, the MRL definition is established as the sum of the parent compound and its relevant metabolites [8].

As it has been said before, some non polar pesticides are fat soluble, facilitating their tendency to bio-accumulate in the food chain and to concentrate in human and animal tissues. The presence of these compounds in food could constitute a serious risk to human and animal health, and also to the environment. Different organizations have established

R (EC) 396/2005. Pesticide EU MRLs.	Analyzed compounds	Fat, Meat and Liver (1)	Milk	Eggs
Alachlor	Alachlor	0.01*	0.01*	0.01*
Aldrin and Dieldrin (Aldrin and dieldrin combined expressed as dieldrin**) (F)**	Aldrin	0.2	0.006	0.02
	Dieldrin			
Bifenthrin (F)	Bifenthrin	0.05* (0.1 fat bovine)	0.01*	0.01*
Chlordane (sum of cis- , trans and oxy-chlrodane) **(F)**	Chlordane-cis	0.05*	0.002*	0.005*
	Chlordane-trans			
Chlorpyrifos-methyl (F)	Chlorpyrifos-m	0.05*	0.01*	0.01*
Chlorpyrifos (F)	Chlorpyrifos	0.05* (chicken)	0.01*	0.01*
Cyfluthrin (cyfluthrin including other mixtures of constituent isomers (sum of isomers)) **(F)**	Cyfluthrin	0.05	0.02*	0.02*
Cyhalothrin-lambda (F)	Cyhalothrin-λ	0.5 (except 0.02* chicken)	0.05	0.02*
Cypermethrin (cypermethrin including other mixtures of constituent isomers (sum of isomers) **(F)**	Cypermethrin	Fat and meat 2 (except 0.1 chicken) Liver 0.2 (except 0.05* chicken)	0.05	0.05*
DDT (sum of p,p´-DDT, o,p´-DDT, p-p´-DDE and p,p´-TDE (DDD) expressed as DDT) **(F)**	DDT-pp	1	0.04	0.05
	DDT-op			
	DDE			
	DDD			
Deltamethrin (cis-deltamethrin) **(F)**	Deltamethrin	0.5 (except 0.1 chicken)	0.05	0.05*
Diazinon (F)	Diazinon	0.05*	0.01*	0.05*
Endosulfan (sum of alpha- and beta-isomers and endosulfan-sulphate expresses as endosulfan) **(F)**	Endosulfan-α	0.05*	0.05*	0.05*
	Endosulfan-β			
	Endosulfan sulphate			
Endrin (F)	Endrin	0.05	0.0008	0.005
Fenthion (fenthion and its oxigen analogue, their sulfoxides and sulfone expressed as parent) **(F)**	Fenthion	0.05*	0.01*	0.01*
Heptachlor (sum of heptachlor and heptachlor epoxide expressed as heptachlor) **(F)**	Heptachlor	0.2	0.004	0.02
	HCE-endo			
	HCE-exo			
Hexachlorobenzene (F)	HCB	0.2	0.01	0.02
Hexachlorociclohexane (HCH), α-isomer**(F)**	HCH-α	0.2	0.004	0.02

R (EC) 396/2005. Pesticide EU MRLs.	Analyzed compounds	Fat, Meat and Liver (1)	Milk	Eggs
Hexachlorociclohexane (HCH), β-isomer **(F)**	HCH-β	0.1	0.003	0.01
Lindane (Gamma-isomer of hexachlorociclohexane (HCH)) **(F)**	Lindane	0.02	0.001*	0.01*
Malathion (sum of malathion and malaoxon expressed as malathion)	Malathion	0.02*	0.02*	0.02*
Methidathion (F)	Methidathion	0.02*	0.02*	0.02*
Parathion (F)	Parathion	0.05*	0.05*	0.05*
Parathion-methyl (sum of Parathion-methyl and paraoxon-methyl expressed as Parathion-methyl)	Parathion-m	0.02*	0.02*	0.02*
Permethrin (sum of isomers)	Permethrin	0.05*	0.05*	0.05*
Pirimiphos-methyl (F)	Pirimiphos-m	0.05*	0.05*	0.05*
Pyrazophos (F)	Pyrazophos	0.02*	0.02*	0.1*
Trifluralin	Trifluralin	0.01*	0.01*	0.01*
Triazophos (F)	Triazophos	0.01*	0.01*	0.01*

Table 1. Analyzed pesticides, MRLs (mg/kg) (Reglament (EC) 396/2005. EU Pesticides database, accessed February 2012).

(F)-Fat soluble, (*)-Indicates lower limit of analytical determination, (1)-MRL or limit of analytical determination in animal meat, fat and liver (swine, bovine, sheep, goat, poultry-chicken)

strict regulation controls on the use of pesticides, handling to minimize the exposure of the population. Maximum Residue Levels (MRLs) are set by the European Commission to protect consumers from exposure to unacceptable levels of pesticides residues in food and feed. *Table 1* presents established MRLs for the studied pesticides in animal origin products, as *Regulation (EC) Nº 396/2005* establish [9]. Individual MRLs have not yet been required for fish. Depending on their water solubility, the maximum allowed levels for food of animal origin (meat, offals, milk, eggs) are regulated either on product or fat, as follow: When pesticides and/or metabolites are water soluble (log Pow < 3), the MRL is expressed as mg/kg of product. When they are fat soluble (log Pow ≥ 3), the MRL is expressed as mg/kg of fat or product [10]. Differences on fat content, type of foodstuffs and the ways to express those results are explained in *Table 2*.

Acording to the European Union legislation, some pesticides can be used for veterinary purposes. *Regulation (UE) Nº 37/2010* [11] establish MRLs for residues of pharmacologically active substances in foodstuffs of animal origin. This regulation includes pesticides used as veterinary drugs, as *Cypermethrin, Deltamethrin, Permethrin, Fenvalerate* and *Diazinon* are. In some cases, these MRLs are different from those from the *Reglament (EC) 396/2005*. *Cypermethrin* in bovine fat is an example, its MRL is 2 mg/kg in R 396/2005 and it is 0.2 mg/kg in R 37/2010.

Traditionally, pesticide residue analysis are carried out in a sequence of several steps, i.e. (i) extraction by organic solvent methods such as liquid-liquid partitioning [12-13], solid phase

micro-extraction [14], matrix solid phase dispersion [15] and supercritical fluid extraction [16]; (ii) clean-up by normal phase liquid chromatography (NPLC) [17] and/or gel permeation chromatography (GPC) [18-19], solid phase extraction (SPE) [20-21], and dispersive solid phase extraction (d-SPE) [22]; (iii) concentration according to analytical technique.
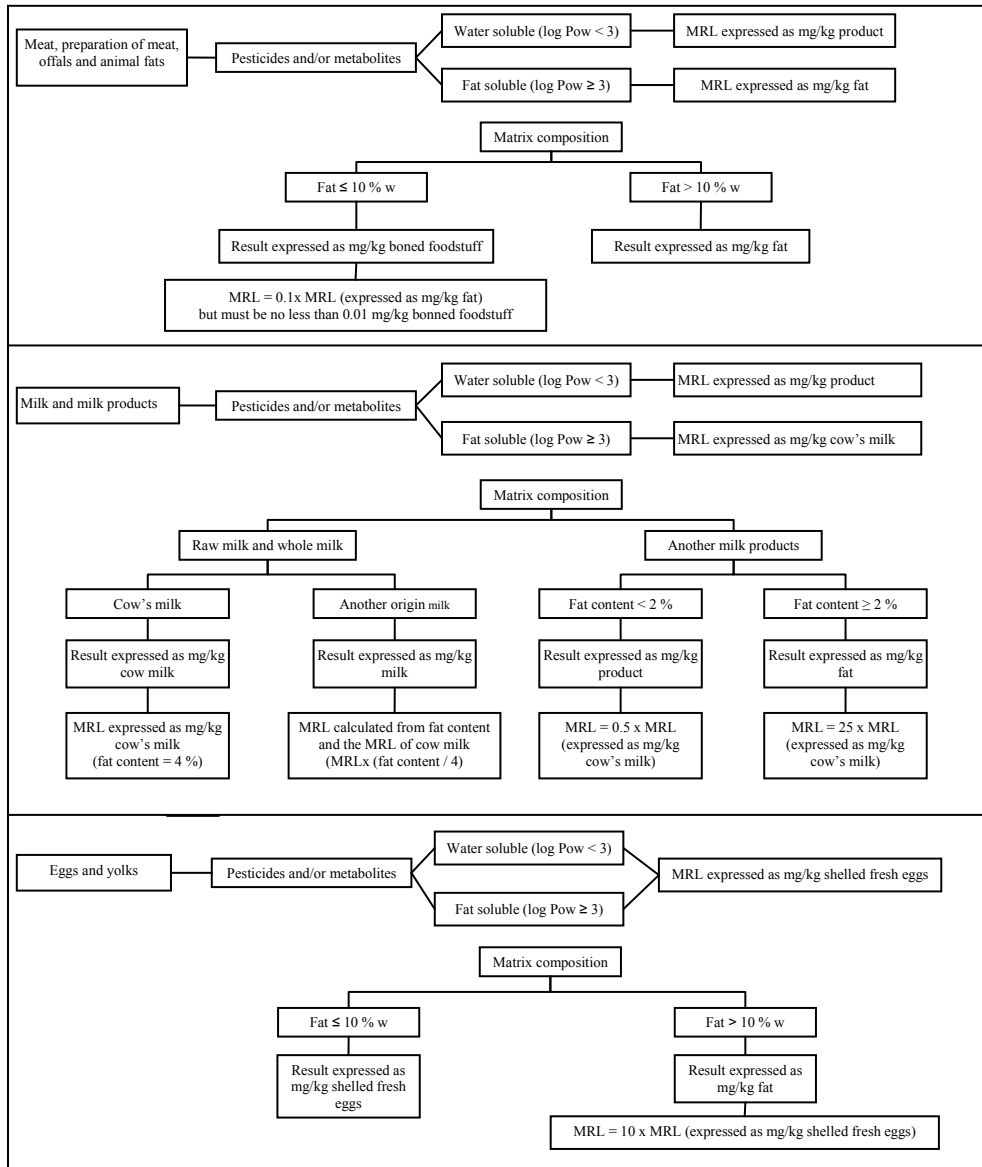
Table 2. Results expression and legal interpretation of MRLs depending on the products and substance properties

In recent years, QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methodology (used for extraction and clean-up steps) has been applied with success on several non-fatty (< 2 %) food matrices such fruits and vegetables [23-24] and low-fatty (2-20 %) food matrices, such milk, egg and avocado [25].

In multi-residue analysis, the sample preparation process cannot be selective enough to remove chemical compounds of the matrix when they have similar properties to analytes. Modifications of QuEChERS method focused on adjusting solvents, salts volumes, water content and clean-up sorbents. Several modifications of the original QuEChERS method are present in literature, to adapt the method to specific applications. Recently modified QuEChERS methodology has been applied on meat based baby-food [26], olive oil, flaxseeds and peanuts [27-28].

The determination of pesticides can be carried out by sensitive analytical methods such as gas chromatography (GC) or liquid chromatography (LC) with a variety of selective detection methods. Chromatographic analysis traditionally used GC with electron capture detection (ECD) and nitrogen-phosphorus detection (NPD) for non-polar pesticides. Nowadays it is preferable to use mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detection in order to verify peak identities. Automated large volume injection (LVI) systems, based on programmable temperature vaporizing (PTV) injection, are employed to improve the limits of detection for pesticides in food matrices [29-30].

In fatty samples, conventional methods for the analysis of pesticide residues usually involve laborious and time consuming clean-up steps. Moreover, analytical problems associated with lipids extraction when using Selected Ion Monitoring (SIM) mode detection are well known due to matrix interferences, they cause signal suppression or enhancement and make their identification difficult. Moreover, small amounts of remaining lipids in final extracts can harm columns, sources and detectors [28-29]. Therefore, to achieve obtained detection limits using GC-ECD, LVI systems are required when GC-MS is used, involving the introduction of a greater amount of co-extracted matrix material that can contaminate the inlet, column and MS source [30]. This fact makes the clean-up more exhaustive and therefore, conventional methods are tedious for routine analysis. When GC-MS/MS is used, smaller amount of sample can be injected on the system, due to its higher selectivity that minimizes interferences [31].

Governmental regulations demand an increasing number of pesticides to be included in the monitoring programmes. These regulations force laboratories to develop effective methods, capable of detecting an increasing number of pesticides with high certainly degree [32].

Last years, instrumentation and software tools have been developed in order to facilitate the analysts work. For example, Automated Mass spectral Deconvolution and Identification System (AMDIS) provided by the National Institute of Standards and Technology (NIST), has demonstrated the capability to detect target pesticides in matrices with high background of interfering compounds. The usual way to extract background spectra is by subtracting spectrum next to the target peak. The resulting component spectrum is compared with

spectra from database and reported if the quality match factor of the spectrum is over a certain pre-set value. Retention time of the target pesticides are locked to match the retention times in the AMDIS database. The software allows to pre-set a threshold level for the match factor. If it is too low or too high, the possibility of reporting false positive or false negative results increases [32].

One of the main problems when full scan working mode is used is the lack of sensibility compared to SIM or Multiple Reaction Monitoring (MRM) working modes, used in MS and MS/MS respectively. However, the main disadvantage of these methods is their restricted scope to target analysis, making necessary an optimization of each compound to obtain the MRM method transition lists. Available softwares facilitate this work by offering wide database libraries, which can be a starting point. One of those libraries is the Dynamic MRM Pesticide Database, which provides over 600 compounds with specific parameters for Agilent 6400 series LC/QqQ mass spectrometers. It allows re-optimization of compounds through the optimizer program and incorporation of the compounds into data acquisition methods for multi-residue analysis [33].

Over 1000 pesticides have been in use over the last century and new pesticides are being developed, so there is a great need to perform both targeted and non-targeted compounds screening in food. High mass resolution spectrometers (Time of Flight (TOF), tandem hybrid Quadrupole Time of Flight (Q-TOF), Magnetic Sector, and Orbitrap afford fast full-spectral sensitivity, at high-mass resolution with mass accuracy [33-35]. These full spectrum techniques offer the advantage of performing retrospective analysis. These tools allow a rapid and accurate screening of target and not-target compounds.

Non-target analysis can demonstrate the presence of not included compounds in the initial scope, leading us to identify those contaminants which can generate food safety problems and asses their inclusion in target lists [36].

Ideally, all compounds would have the same extraction efficiency, the same chromatography and equal response in MS. But actually, extraction efficiency, peak shapes and responses are different for each compound and each matrix. Detection capability must not be confused with the fact that not detected compounds are not present; this can only be done by validation studies showing that the specific method employed on specific matrices can detect the reported compounds as not present at the levels of concern [34,37].

Different platforms may be combined for the development of multi-residue analysis protocols combining the preliminary screening for the initial qualitative identification using full-scan with high resolution spectrometer platform followed by their quantitative MRM confirmation.

This is very interesting on research purposes and for defining future trends. However, from the point of view of the routine work of an official control laboratory, monitoring the presence of certain compounds proposed for the food safety authority is a priority, as well as monitoring the compliance with the MRL for the regulated analytes.

Our research is targeted on the evaluation of sensitive, selective, easy and quick methods for multi-residue analysis of pesticide residues in animal origin food. Also proposes on-going and effective systematic validation for evaluation methods.

The aim of this work focuses on: 1) to propose a multi-residue method for the determination of lipophilic pesticides in animal origin food, 2) the clean-up evaluation, 3) the instrumental analysis optimization, 4) the comparison GC-MS and GC-MS/MS data, 5) to propose of a screening methodology focusing on quantitative method validation for those detected analytes.

2. Determination of lipophilic pesticide residues in animal origin food: Procedure proposal

2.1. Method description

Steps to approach the analysis of lipophilic pesticides in animal origin food are: 1) Sample preparation: fat extraction from samples; extraction and purification of pesticide residues from lipidic fraction and (2) analytical determination.

The studied scope is shown in Table 1. All the included pesticides are classified as lipophilic (log Kow > 3), except *Malathion* (log Kow = 2.7), *Alachlor* (log Kow = 2.9) and *Parathion-m* (log Kow = 2.9).

2.1.1. Standards

Certified pesticide standards are purchased from Dr. Ehrenstorfer. Purity-corrected individual pesticide stock standards (200 µg/mL) are prepared: weighing 10 mg of pure standard, dissolving each compound in acetone, aliquoting, and storing in capped vials in freezer [23]. Adjusted aliquots (depending on the MRL for each pesticide) of the individual stock solutions are diluted with acetone to prepare three standard mixture solutions (organochlorines, organophosphorus, and pyrethroids). Working mixture solution in acetone (between 0.5 and 5 mg/L), spiking solution in n-hexane (between 0.2 and 2 mg/L), hexanic standards solutions (between 8 and 1500 µg/L), a internal standard solution containing *PCB-54* (100 µg/L) and surrogate solution containing *4,4'-DDT-D₈* (100 µg/L) are prepared and stored at -18 °C.

2.1.2. Sample preparation

a. Fat extraction

Animal fat is the most simple and pure matrix, it only needs to be melted and filtered through sodium sulphate. For other matrices, the extraction of the lipidic fraction is required.

Traditionally, this step has been carried out by Soxhlet and Sonication extractions, but Accelerated Solvent Extraction (ASE) allows faster extractions of larger sample series with lower consumption of organic solvents. In our method, the lipidic fraction from meat, offals,

fish, shellfish and dairy products matrices are extracted with ASE; except for eggs, which is extracted by homogenising with solvent, using "Ultraturrax" device. Each sample is spiked with surrogate standard, and some blank samples are spiked with spiking solution for validation and quality control purposes. Two approximations are considered: a) when the results are expressed as mg/kg of fat, the fortification is done in 1 g of melted fat, and b) when the results are expressed as mg/kg of product, the fortification is done in 10 g (5 g of fish) of homogenized sample. For dairy products, the sample volume is reduced by freeze-dried before analysis; for example 25 g of cow milk, corresponding to 1 g of fat, are freeze-dried and used at all. Spiked samples are let for 30 min before the beginning of extraction process.

Samples are mixed with an amount of diatomaceous earth enough to dry completely the sample and obtain a homogeneous powder consistency and dealing in ASE cells as needed. The ASE extraction is carried out using a Dionex ASE 200 and/or 350 with DCM/acetone (1:1) as the extraction solvent at a temperature of 100 ºC and pressure of 1500 psi, with at least two extractions per sample. In the case of eggs, mixed samples are centrifuged to separate the organic fraction from the rest of the matrix. The extracted solvent is reduced in volume under nitrogen stream.

A small part of moisture and powder of diatomaceous earth elute from the sample during the ASE lipid extraction step. In order to remove these impurities, the organic extract is purified by partitioning with hexane/MTBE (9:1) and 0.1M H_3PO_4 with 1% KCl, twice. Purified fraction is evaporated to obtain the lipidic fraction.

b.   Extraction and purification of pesticide residues from lipidic fraction

One gram (when results are expressed as mg/kg of fat) or all (when results are expressed as mg/kg of product) the lipidic fraction is extracted twice with 8 mL of n-hexane saturated acetonitrile (18% (v/v)) each time and centrifuged, collecting the acetonitrile fraction. The acetonitrile extract is subjected to a pre clean-up step by remaining fat precipitation by cooling (4 °C overnight).

Clean-up step is d-SPE based on QuEChERS method for fatty samples [20 – 30, 38]. 8 mL of the defatted extract is introduced in a 15 mL polypropylene centrifuge tube containing 1,200 mg $MgSO_4$, 400 mg end-capped C_{18} and 400 mg PSA. It is mixed by vortex and centrifuged. Finally, 4 mL of cleaned supernatant is evaporated under nitrogen stream until near dryness, and then dissolved in a volume of internal standard solution (0.5 mL for GC-MS or 0.25 mL for GC-MS/MS). Then, the solution is transferred to autosampler vials for analysis [39].

Preparation of matrix-matched standards follows the same procedure. Standard solutions (0.5 mL in GC-MS analysis or 0.25 mL in GC-MS/MS analysis) are added before blank extract evaporation.

c.   Analytical determination

GC-MS analysis is performed on an Agilent 6890 gas chromatograph coupled to a quadrupole mass spectrometer 5973 configured with a PTV inlet. A prototype deactivated borosilicate and multibaffle liner with fritted glass on interior walls purchased from Agilent

is employed. A retention gap of 5 m x 0.25 mm i.d. (deactivated fused silica) was placed between the injector and the analytical column to avoid a rapid analytical system contamination. Chromatographic separation is performed on a 30 m x 0.25 mm i.d. x 0.25 μm Agilent HP-5MS U.I. column. Helium (99.99%) is used as carrier gas at constant flow (1.2 mL/min). The PTV is configured in solvent vent mode. The automatic liquid sampler, in multiple injection mode, makes ten injections of 4 μL each into the PTV inlet with a 20 sec delay between injections to allow solvent evaporation. During the injection, the column head pressure is set to 0 psi, the steady flow of carrier gas through the liner causes the solvent to evaporate and to be swept with the carrier gas out through the split vent. After the ten injections, the column head pressure is restored and the vent flow is turned off. At this point, the inlet temperature is programmed up to 290 ºC to transfer all the analytes into the GC column. During the sample transfer, the oven temperature is held a 42 ºC. The GC temperature program is: initial 42 ºC, hold 5.8 min; rate 20 ºC/min to 80 ºC, hold 1 min; rate 5 ºC/min to 190 ºC, hold 5 min; rate 15 ºC/min to 270 ºC, hold 10 min and rate 25 ºC/min to 290 ºC, hold 10 min. Determination parameters are: Transfer line temperature 280 ºC, quadrupole temperature 150 ºC and ion source temperature 250 ºC. Ionization operates in electronic impact mode (EI) with 70 eV of electron energy. MS calibration is checked daily following the autotune test of sofware. Electron multiplier voltage is set at 400V above autotune. All the analyses are performed in SIM mode monitoring three ions for each analyte (*Table 3*). Data analysis is assured by MSDChemStation software D.02.00.275 (Agilent).

GC-MS/MS analysis is performed on a Trace GC Ultra and Triplus autosampler coupled to triple quadrupole mass spectrometer from Thermo-Finningan (TSQ Quantum). Separation is carried out on a HP-5MS U.I. capillary column (Agilent) 30 m x 0.25 mm i.d., 0.25 μm film. High-purity helium (99.99%) and argon C50 (99.99%) are used as carrier gas (constant flow of 1.2 mL/min) and as a collision gas, respectively.

The GC temperature program is: initial 90 ºC, hold 5 min; rate 25 ºC/min, to 180; rate 5 ºC/min to 200 ºC, hold 10 min; rate 5 ºC/min to 300 ºC, hold 15 min. Splitless injection conditions are as follow: split flow, 40 mL/min; splitless time, 1.00 min; surge pressure, 200 kPa; surge duration, 1.00 min; injection volume, 2 μL. The mass spectrometer operates in EI mode using 50 μA ionization voltage. The ion source temperature and the GC-MS/MS interface are set to 250 ºC and 300 ºC, respectively. MS calibration is verified daily with calibration gas (PFTBA). MRM working mode is used for all analytes. *Table 4* shows the two monitored transitions for each pesticide.

Data processing is performed using the QuanLab forms 3.1 software (Thermo-Finningan) witch allows the use of obtained MRM with different parents to confirm each compound.

3. Method development

3.1. Clean-up optimization

Acetonitrile is used as extraction solvent because: a) It is polar enough to minimize the amount of co-extractive fat, and b) it is non-polar enough to extract with efficiency lipophilic

pesticides from matrix. The addition of a small amount of a highly non-polar solvent as hexane is, improves the obtained recoveries of more lipophilic pesticides without increasing lipids co-extraction.

In our research, 2 classic clean-up procedures (GPC and fat oxidation), as well as some modifications based on QuEChERS procedures, were applied to fat samples and compared. Analytes and lipids quantitation were carried out to evaluate clean-up efficiency. Total lipids were determined by the sulfo-phospho-vanilline reaction followed by colorimetric determination, prior to each injection.

GPC sample treatment was discarded because a) it was observed that overlapping elution profiles from real samples and standard solutions, higher molecular weight pesticides (mainly pyrethoids) co-eluted with lipids, and b) high amount of chlorinated solvent (125 mL of dichlorometane for sample) was used. Those reasons leaded us to continue our studies testing alternative procedures in order to improve recoveries and minimize the use of organic solvents.

Sulfuric acid treatment was described as a good alternative for removing fat from matrix [40], so it was tested with representative chlorinated pesticides. After successive tests, it was concluded that acid removed fat with efficiency but it showed problems because of the partial (e.g., *Heptachlor* to *exo* and *endo HCEs*) or total (e.g., α and β *Endosulfan*) degradation of some analytes, as well as inconsistent recovery values for others; supporting what had been described by Esteve-Turrillas et al. [12] and Przybylski et al. [26] previously.

As a first approach, extract of 1 g of fat was evaporated and purified with 2 mL QuEChERS using constant amounts of PSA (400 mg) and $MgSO_4$ (1,200 mg) and different amounts of end-capped C_{18} (100, 200 and 400 mg). As a second approach, 2 g of fat was extracted and aliquots were taken achieving dilution factor 4 and purified with 15 mL QuEChERS with constant amounts of PSA (400 mg) and $MgSO_4$ (1,200 mg) and different amounts of end-capped C_{18} (100, 200, and 400 mg). Finally, 15 mL QuEChERS were tested with constant amounts of PSA (400 mg), $MgSO_4$ (1,200 mg), and end-capped C_{18} (400 mg), but two different amounts of sample were tested. *Figure 1* shows fat content after clean-up procedures.

When 2 mL QuEChERS were tested, obtained recoveries decreased as C_{18} amount was increased, while differences on the quantitation of remaining lipids were not relevant, but chromatograms without enough cleanliness were obtained. Experimentally, in some cases retention time drifts higher than 0.5% were found, preventing the determination of certain analytes. In addition, the extracts could not be injected into LVI without damaging the column. These facts led us to think that C_{18} retained analytes and the need of a dilution step or minimize matrix interferences [22]. Higher recoveries were obtained when dilution step was included and 15 mL QuEChERS were used, and remaining fat in vial before injection was one half less than what had been obtained previously.

Finally, the use of 1 g of fat sample instead of 2 g, made easier the quantitation, obtaining clearer chromatograms, reducing background noise, reaching the lowest fat content among

tested conditions and achieving required MRLs. This fact can be explained by the reduction of injected matrix that removed most of chromatographic interferences present when 2 g of sample were analyzed.
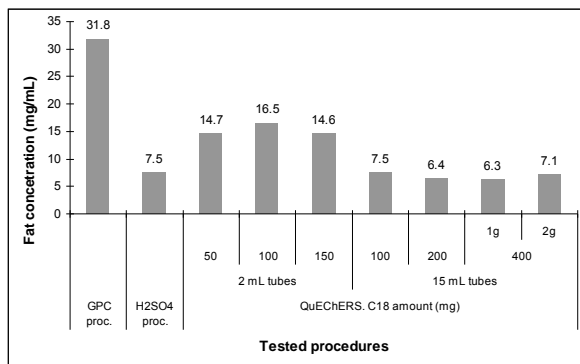
Figure 1. Fat content evaluation after clean-up procedures: GPC procedure, H_2SO_4 procedure, 2 mL QuEChERS and 15 mL QuEChERS.

These results leaded us to choose 15 mL QuEChERS with 400 mg of C_{18} using 1 g of sample and including a dilution step as the better procedure. This technique reached a sample concentration factor similar to the studied on GPC method (0.5 g/mL), obtaining clean extracts that could be injected into LVI (40 µL) without damaging chromatographic system. In addition, this technique consumed smaller solvent amount (13 mL acetonitrile + 3 mL n-hexane). Finally, acceptable recovery values were obtained for all studied pesticides [39].

3.2. Instrument optimization

Chromatographic and spectrometric parameters were optimized with hexanic standard solution and matrix matched standard solution of all pesticides, due to the complexity of matrices. Final delay let to achieve the cleaning up of capillary column and to avoid losses by possible shifts of the retention times. Gradient slope optimization was performed with matrix matched solution. The application of a low gradient slope allows improving separation of co-extractives which could be co-eluted with analytes and allow the separation of isomers such as *HCHs*, as well as *DDD* and *DDTs* [41]. In *Figure 2*, shows two chromatograms where a couple of isomers are represented in both GC systems.

a.   Ion selection in GC-MS system

As general criteria, mass spectrometry conditions were carefully selected to provide a compromise solution between sensitivity, selectivity and structural information, being the base ion chosen for quantitation purposes. However, most abundant ions could not be used in all cases, because of the interferences from matrix. Therefore, more selective and less sensitive ions must be used. The three most selective ions were chosen after the injection of matrix matched standard solution, in full scan mode; except for *Triazophos*, because of the

absence of a third selective ion with sensitivity enough to be detected at the lowest studied level.
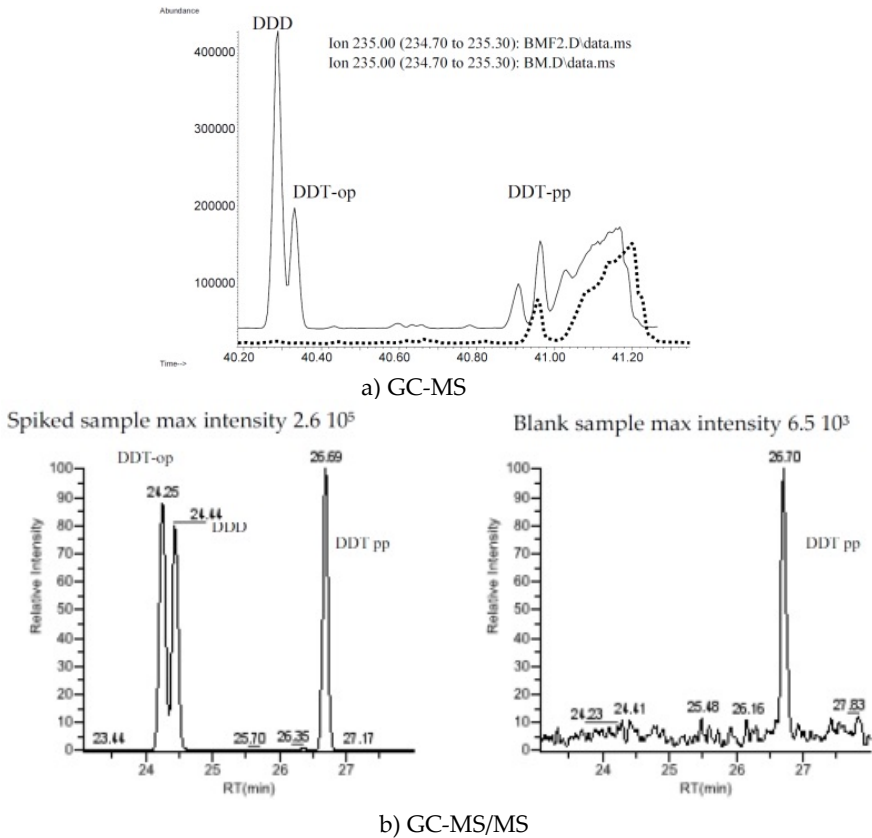
Figure 2. Chromatograms for *DDD, DDT-op* and *DDT-pp* in: a) blank sample (discontinuous trace), spiked sample at 0.05 mg/kg (continuous trace) with GC-MS(SIM) system and, b) blank sample, and spiked sample at 0.02 mg/kg with GC-MS/MS(MRM).

The method was divided into as many time segments as possible, in order to obtain the maximum signal for pesticide that gave the lowest response. Dwell time for each ion was set between 10 and 100 ms, depending on the number of ions monitored in each group and the sensitivity of each ion; so the minimum cycles/sec for each group was higher than 2.5. *Table 3* provides the SIM program used for the GC-MS analysis.

b.   MRM selection in GC-MS/MS system

The use of MRM mode based on QqQ mass analyzer allows low analyte detectability and is one of the most selective approaches at present for trace analysis. However, for multiclass,

multi-residue methods, a precise optimization of MS/MS parameters is needed, in order to maximize the signal for each pesticide.

Group	Pesticide	RT (min)	Q (m/z)	q1 (m/z)	q1 ratio	q2 (m/z)	q2 ratio	Dwell time	Cicles/Seg
1	Trifluralin	28.7	306	264	65.0	290	12.4	25	3.36
	HCH-α	29.1	217	145	29.2	181	137.4	40	
	HCB	29.3	284	286	79.4	249	23.9	25	
2	HCH-β	30.4	221	181	188.6	147	35.8	60	3.09
	Lindane	30.5	181	217	87.6	145	19.6	60	
3	Diazinon	31.3	137	179	93.2	152	67.4	20	3.07
	PCB 54	32.3	292	220	56.0	255	6.5	80	
4	Chlorpyrifos-m	33.5	286	288	69.7	290	15.5	10	3.03
	Parathion-m	33.5	263	264	10.3	233	9.2	10	
	Heptachlor	33.7	272	274	79.7	270	53.7	60	
	Alachlor	33.9	160	188	90.6	146	32.8	20	
5	Pirimiphos-m	35.4	290	276	82.5	305	75.5	10	3.12
	Aldrin	35.8	265	293	57.2	261	95.7	45	
	Malathion	36.0	173	158	56.5	285	8.1	10	
	Fenthion	36.4	278	279	12.7	280	11.5	10	
	Chlorpyrifos	36.5	314	316	75.4	286	41.8	10	
	Parathion	36.6	291	186	19.1	235	16.4	10	
6	HCE-exo	37.7	353	355	79.9	351	52.1	30	3.09
	HCE-endo	37.9	353	355	81.1	263	42.1	70	
7	Chlordane trans	38.5	373	272	18.4	237	19.5	25	2.99
	Methidathion	38.6	145	302	3.7	146	6.7	25	
	Endosulfan-α	38.9	277	265	117.6	339	81.4	45	
	Chlordane-cis	39.0	373	374	13.4	272	17.9	25	
8	Dieldrin	39.6	263	277	81.0	279	77.1	70	3.08
	DDE	39.6	246	318	88.6	316	68.8	30	
9	Endrin	40.1	263	265	67.6	345	23.0	40	2.89
	Endosulfan-β	40.3	277	265	76.1	339	93.1	40	
	DDD	40.5	235	237	64.7	239	11.1	40	
	DDT-op	40.6	235	237	64.7	239	10.9	40	
10	Triazophos	40.9	172	313	31.8			30	3.02
	Endosulfan-sulfate	41.2	387	385	60.3	389	67.1	30	
	DDT-D$_8$	41.2	243	245	62.2	173	31.4	30	
	DDT-pp	41.2	237	235	69.0	212	11.7	10	
11	Bifenthrin	42.3	181	166	26.3	165	26.9	100	3.11
12	Cyhalothrin-λ	43.6	181	197	77.6	208	54.9	50	3.07

Group	Pesticide	RT (min)	Q (m/z)	q1 (m/z)	q1 ratio	q2 (m/z)	q2 ratio	Dwell time	Cicles/Seg
	Pyrazophos	44.0	232	373	70.0	237	58.9	50	
13	Permethrin	44.9	183	184	14.9	163	23.5	100	3.11
14	Cyfluthrin	46.4	165	226	81.9	199	66.2	50	3.08
14	Cypermethrin	46.9	163	181	73.4	209	20.0	50	3.08
15	Deltamethrin	51.5	181	253	92.9	255	45.4	100	3.10

Table 3. GC-MS SIM method parameters used for quantification and confirmation.

Group	Pesticide	RT (min)	Q transition (m/z)	CE (eV)	q transition (m/z)	CE (eV)	IR (Q/q)	ST (s)
1	Trifluralin	11.77	306.00>264.27	10	263.96>159.60	15	30	0.08
2	HCH-α	12.27	216.85>180.93	10	218.86>183.17	10	75	0.05
2	HCB	12.44	283.74>214.04	30	283.74>249.11	20	90	0.05
3	HCH-β	12.90	180.84>144.88	15	218.87>183.15	10	70	0.045
3	Lindane	13.01	180.84>144.88	15	218.87>183.15	10	65	0.045
3	Diazinon	13.26	304.05>179.23	10	179.02>137.00	20	70	0.045
4	PCB-54	14.14	219.94>149.80	30	291.85>219.8	20	100	0.05
4	Chlorpyrifos-m	14.77	285.85>271.12	15	285.85>92.89	20	50	0.05
4	Pirimiphos-m	14.81	262.94>108.85	10	262.94>79.00	20	20	0.05
4	Alachlor	15.00	188.03>160.14	10	188.03>130.06	30	80	0.05
4	Heptachlor	15.05	271.74>237.08	15	271.74>234.96	15	20	0.05
5	Parathion-m	15.77	290.00>233.18	10	275.99>244.43	10	15	0.033
5	Malathion	16.16	172.98>98.90	15	127.00>98.95	10	90	0.033
5	Aldrin	16.39	262.8>228.00	20	262.8>193.06	25	170	0.033
5	Fenthion	16.58	277.95>109.09	20	277.95>169.06	15	90	0.033
5	Chlorpyrifos	16.62	313.89>258.05	15	196.85>169.02	15	85	0.033
5	Parathion	16.69	291.05>109.15	10	291.05>80.85	25	70	0.033
6	HCE endo	18.31	352.78>263.10	15	352.78>282.25	15	25	0.02
6	HCE exo	18.55	182.84>119.02	20	182.84>155.09	15	100	0.02
7	Chlordane trans	19.67	372.75>266.00	20	372.75>264.13	20	65	0.11
7	Methidathion	19.91	144.88>58.26	15	144.88>84.95	15	150	0.11
7	Endosulfan-α	20.46	238.84>204.12	15	240.87>204.12	15	30	0.11
7	Chlordane cis	20.67	372.75>266.00	20	372.75>264.13	20	65	0.11
8	DDE	22.38	245.94>176.14	25	245.94>211.13	20	15	0.15
8	Dieldrin	22.39	276.84>240.57	10	262.81>193.08	30	160	0.15
9	Endrin	24.00	280.96>245.15	10	262.79>192.89	30	260	0.25
10	Endosulfan-β	24.74	240.88>206.05	15	194.84>159.00	10	85	0.13
10	DDD	25.50	235.07>165.09	20	236.95>165.07	20	70	0.13
10	DDT-o,p	25.65	235.07>165.09	20	236.95>165.07	20	70	0.13
11	Triazophos	26.94	256.94>162.18	10	284.97>162.020	10	40	0.07
11	Endosulfan sulfate	27.47	271.73>236.83	15	273.73>239.09	15	55	0.07

Group	Pesticide	RT (min)	Q transition (m/z)	CE (eV)	q transition (m/z)	CE (eV)	IR (Q/q)	ST (s)
	DDT-D_8	27.63	243.03>206.14	15	243.03>172.6	20	35	
	DDT-p,p	27.76	235.07>165.09	20	236.95>165.07	20	70	
12	Bifenthrin	30.93	181.00>165.06	25	181.00>166.18	10	65	0.22
13	Cyhalothrin-λ	33.56	181.00>152.14	20	196.97>141.20	10	55	0.1
	Pyrazophos	34.05	232.07>204.18	10	221.05>193.07	10	175	
14	Permethrin	35.45	182.99>168.16	10	182.99>153.12	15	100	0.22
15	Cyfluthrin	37.05	226.07>206.21	15	162.98>90.98	10	100	0.14
	Cypermethrin	37.23	181.00>152.14	20	162.98>90.98	10	70	
16	Deltamethrin	40.72	252.90>92.76	15	181.00>127.03	25	50	0.2

Table 4. GC-MS/MS(MRM) method parameters used for quantification and confirmation.

The first step was the selection of the parent ions (precursor ions), which were chosen after examining the full scan spectra of each pesticide. This step was performed using a hexanic standard solution. Not always the base peak was preferred (highest intensity), for some pesticides a more selective one was selected. For some pesticides, two parent ions were selected, one for each transition.

Secondly, three transitions were taken for each pesticide after checking the fragmentation of each parent ion, working in product scan mode with a hexanic standard solution. Finally, the MRM collision energy was optimized for each selected transition using matrix matched solutions, and the two most selective transitions were chosen. *Table 4* provides the selected conditions used in MRM program used for GC-MS/MS analysis.

An adjusted scan time (dwell time) was fixed for each transition, taking into account the recommendation that about 12-15 scans are needed across any peak to ensure representative peak shape and area.

GC-MS(SIM) method was optimized with animal fat samples. A LVI procedure based on a PTV injection was employed, in order to improve the detection limits of the target compounds in the analysis, The cleanliness of the obtained extracts with the developed method allows longer working sequences with acceptable drifts and a significantly reduce of instrumental maintenance. However, matrix interferences for some pyrethroids, as well as sensitivity limitations, were observed [39].

MS/MS offers additional specificity with secondary fragmentations, and thus circumvent co-elution problems. Besides, it provides a more definitive tool. Tandem MS also decreases matrix interferences, improves selectivity, achieves higher signal-to-noise ratio, and subsequently improves the detection limit [2, 31].

The optimized GC-MS/MS method achieved higher selectivity, lowers LODs and reduced the influence of the matrix, as for *Deltamethrin* and *Cyhalothrin-λ*, injecting the tenth part of sample that in GC-MS was.

Figure 3a shows the obtained chromatograms for *Permethrin* in a pork fat sample spiked at 0.05 mg/kg by GC-MS and GC-MS/MS analysis. *Figure 3b* shows the obtained
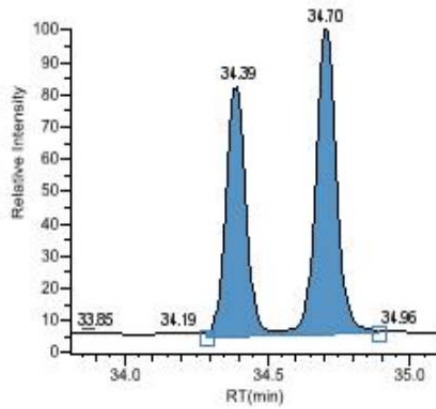
chromatograms for *Deltamethrin* in a pork fat sample spiked at 0.2 mg/kg by GC-MS and at 0.1 mg/kg by GC-MS/MS analysis. The additional MS stage of MS/MS instrument provides enhanced selectivity, reducing the noise from the matrix.

In addition, the reduction of sample volume injected, minimized problems caused by matrix components at the injector/column and detector sites.
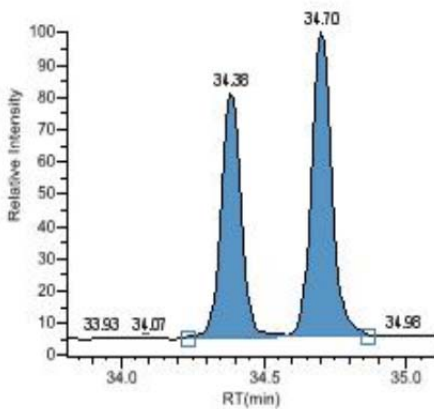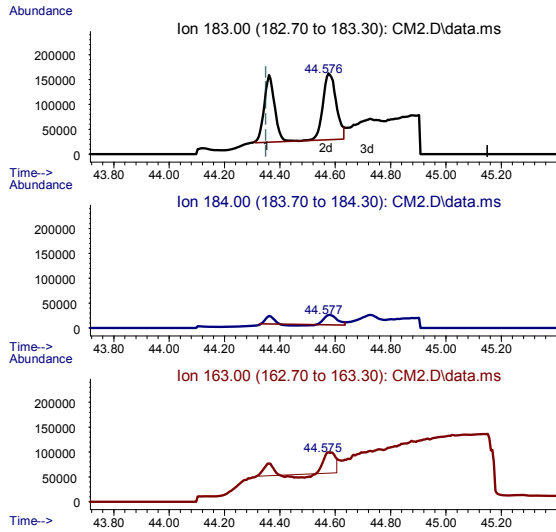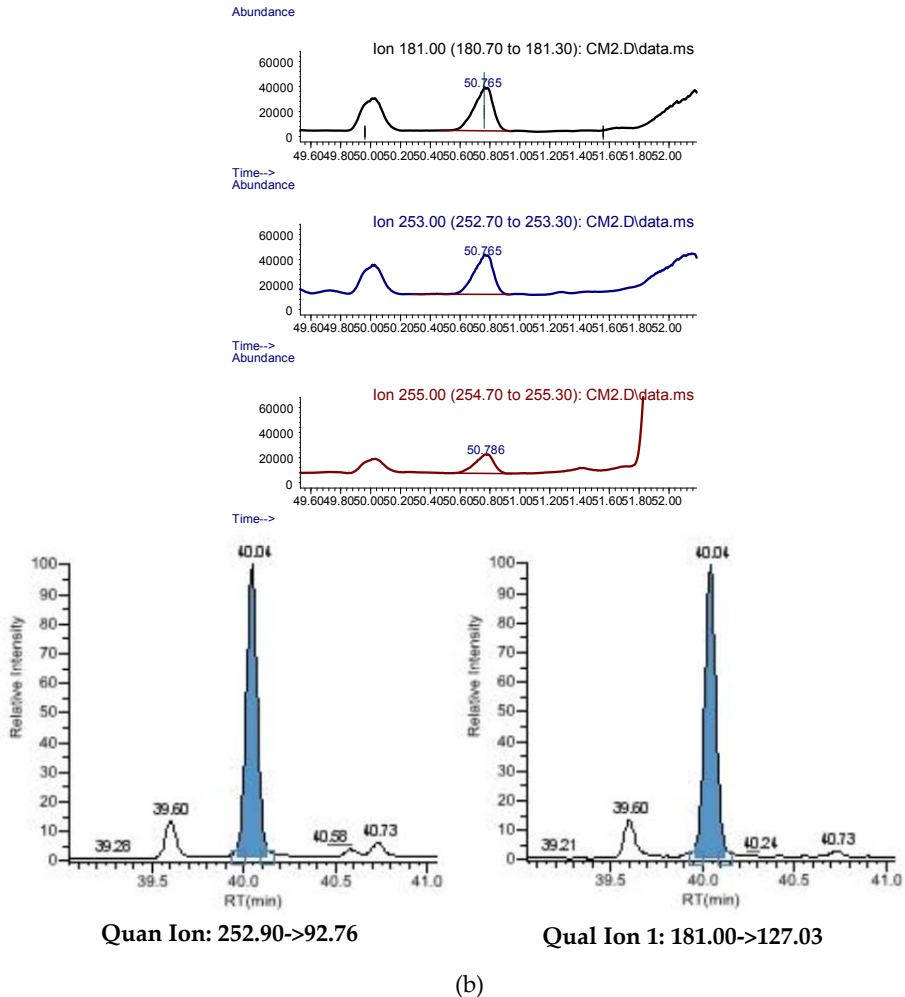

Quan Ion: 182.99->168.16   **Qual Ion 1: 182.99->153.12**

(a)

Quan Ion: 252.90->92.76   Qual Ion 1: 181.00->127.03

(b)

Figure 3. a) *Chromatograms obtained for Permethrin in a pork fat sample at 0.05 mg/kg by GC-MS and GC-MS/MS analysis.* **b)** *Chromatograms obtained for Deltamethrin in a pork fat sample at 0.2 mg/kg by GC-MS and at 0.1 mg/kg by GC-MS/MS analysis.*

4. Method performance

A significant proportion of analyzed samples are free of pesticides in a detectable concentration and the most of the analytes are not detected, so a systematic quantitative study of them is a waste of effort and time.

The use of qualitative methods allow to discriminate samples and analytes where we should focus our efforts, applying quantitative methods only in those samples with previously detected analytes.

Primary sequences are screening sequences, composed of an appropriate number of unknown samples, a negative control and duplicated positive controls, formed by blank samples spiked at reporting level. Screening and confirmation can be performed in a single analysis in which the identification/confirmation criteria are applied to detected peaks. In any case, it is important to choose properly the detection criteria (cut-off): if it is too strict, we find too many false negatives and if it is too loose, we have many tentative detects, needing further following. We use as cut-off-value the obtained in validation studies from the mean response of spiked samples (V) and its standard deviation (S) (cut-off = V – 1.64 x S) [42]. Confirmation criteria, as Relative Intensity (IR) and Retention Time (RT) are adjusted with matrix-matched standards at LOQ level each sequence.

Quantitative sequences allow measuring the concentration of detected pesticides in selected samples as well as their confirmation, when necessary. In this case, multi level matrix matched calibration, adjusted by weighted linear regression, is used with this purpose.

5. Method evaluation

Document SANCO 1495/2011 [37] describes the method validation and analytical quality control requirements to support the validity of data used for checking compliance with maximum residue limits (MRLs), enforcement actions, or assessment of consumer exposure to pesticides.

5.1. Matrix effects

On the analysis of pesticide residues in fat and fatty food of animal origin, low concentration levels of residues in the presence of a great quantity of compounds from the matrix is expected. Practically, no clean-up method completely removes all the matrix components from a crude extract. The matrix components injected into chromatographic system may led to false positive or negative results, low analyte detectability, inaccurate quantitation and decreased method ruggedness [7].

That is the reason why there are several approaches in literature to reduction of matrix effect, as: a) the use of isotopically labeled internal standards, b) an exhaustive clean-up to remove the most of the matrix from the extracts, and c) the use of matrix-matched standard calibration. The first option is the most expensive one, especially in multi-component analysis. The second one has to be reached with selective extraction procedures or with more extensive sample clean-up, leading to time consuming procedures. The third one is the most widely and simple approach used in pesticide residue analysis.

Single MS detectors with low resolution can be not selective enough to distinguish interfering matrix compounds from selected ions. This fact becomes the main problem with compounds with non-specific ions with low m/z ratios. In that case, the use of chromatographic systems with increased sensibility and specificity, as MS/MS are, are a possible solution.
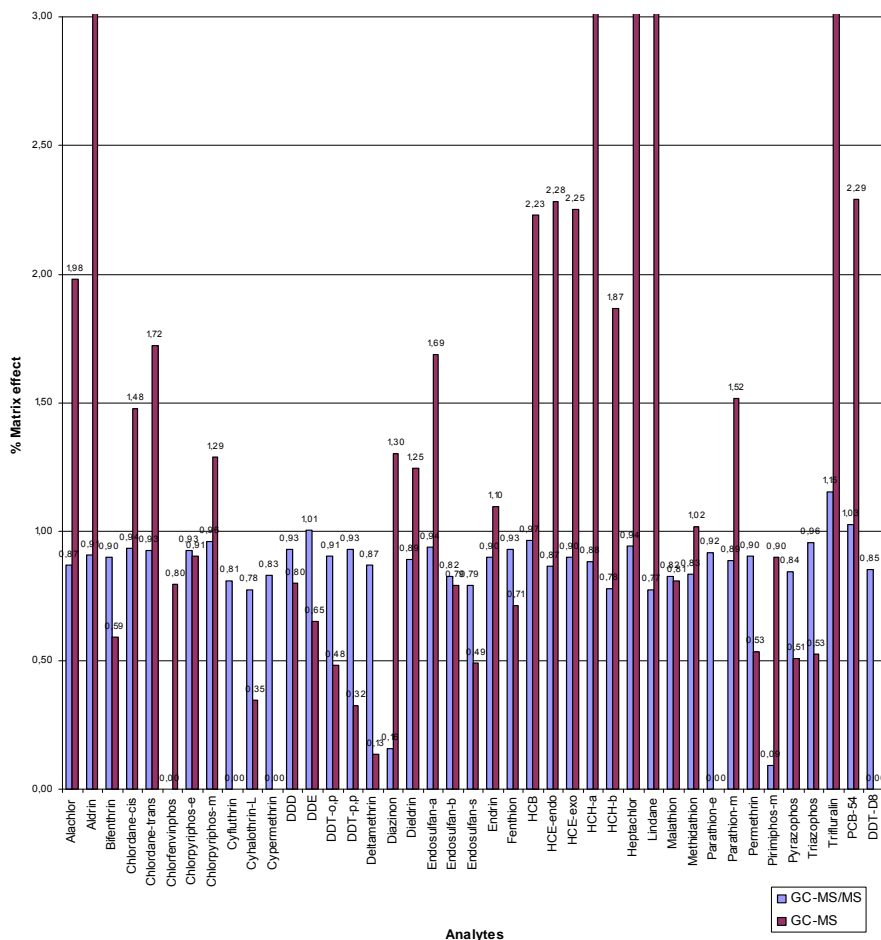
Figure 4. Experimental matrix effect (matrix matched slope/solvent slope) obtained with previous GC-MS and actual GC-MS/MS systems. Values below 1 mean signal suppression and values above 1 mean signal enhancement.

Matrix components can behave like interferences inducing a reduction of the signal corresponding to an analyte, presumably by decreasing ionization potential or also by inducing directly or indirectly its degradation [43-44]. Other pesticides can be greatly protected by the action of the co-extractives as powerful masking agents which block the active sites of analytical system, mainly in the injector or in the column. Mastovska et al. related that the addition of analyte protectants (ethylglycerol, gulonolactone, and sorbitol) on sample extracts and solvents standards corrected the matrix-induced enhancement and suppression [45]. The strength of this effect, can be evaluated by comparison of the calibration curve slopes obtained with solvent-based standards and with matrix-matched standards. An increased response represents a sensitivity enhancement, due to matrix, compared to the standard solution in solvent.

On this work, matrix-matched calibration standards were prepared adding standard solutions to blank extracts of a pork fat sample. Linearity was studied analyzing those matrix-matched standards. Matrix effects were evaluated by comparison of the solvent standard calibration curves and the matrix-matched standard calibration curves. Moreover, experimental matrix effect values with GC-MS/MS(MRM) were compared with those previously obtained with GC-MS(SIM), as *Figure 4* shows.

Obtained values show a great decrease on matrix effect when GC-MS/MS system is used. As a first approach, this fact can be explained by the use or a more selective method, avoiding most of the interferences and background noise present when GC-MS was used. On the other hand, the injection of a smaller amount of extract decreases the introduction of matrix and co-extractives which block the active sites of the analytical system and reduces matrix effects (enhancement or suppression).

5.2. Method validation

Sanco document [37] distinguishes between qualitative screening methods and quantitative ones. For the first ones, the Limit Of Detection (LOD) is defined as the lowest concentration for which has been demonstrated that a certain analyte can be detected in at least, 95% of the samples (i.e. a false negative rate of 5% is accepted). In the case of selectivity, the presence of false detects should be verified using unspiked samples (verifying the absence of interfering peaks whose response > 30% LOD). For each commodity group, a basic validation of a qualitative method should involve analysis of at least 20 samples spiked at the anticipated LOD. The selected samples should cover multiple matrices from the commodity group, with a minimum of two samples for matrix, and being representative for the matrix scope of the laboratory. Its application in routine analysis, on-going QC data should be acquired and the performance of the method should be periodically reassessed.

Quantitative methods must be tested to assess for sensitivity, mean recovery, precision, linearity, and Limit Of Quantitation (LOQ). This means that spiked recovery experiments to check the accuracy of the method, should be undertaken. A minimum of 5 replicates is required (to check the precision and accuracy) at the LOQ, and at least another higher level (HL). The LOQ is defined as the lowest validated spiked level meeting the method performance acceptability criteria (mean recoveries for each representative commodity in the range 70-120%, with an RSD ≤ 20%).

Experimental obtained values for the validation of the screening (qualitative) method are resumed on *Table 5*.

In fat and poultry meat samples, all the studied pesticides have been validated at initially proposed LODs. In the case of eggs, *Cyfluthin, Permethrin*, and *Methidation* could not be validated the proposed levels, 0.05, 0.05 and 0.02 mg/kg respectively. *Cyfluthrin* because a lack of sensibility on the confirmation transition; *Permethrin* because one of its isomers has an important interference; and *Methidathion* because of the inconsistent values on

confirmation transition. In shellfish, *Deltamethrin* and *Permethrin* had selectivity problems, and *Malathion* sensibility ones, so the proposed method is not valid for proposed LODs (0.01, 0.005 and 0.02 respectively). In liver matrices, *Malathion* and *Endosulfan-β* show interferences at LOD levels (0.002 and 0.005 mg/kg respectively). Finally, in milk products the screening method had not sensibility enough to detect *Alachlor* and *Parathion-m* at LOD levels (0.0004 and 0.0008 mg/kg respectively), as well as *Deltamethrin* and *Malathion* had some interferences at LODs (0.004 and 0.0008 mg/kg respectively).

Experimental obtained values for the validation of the quantitative method are resumed on *Table 6*.

Most pesticides gave 70 – 120 % acceptable recoveries with associated RSD < 20 %. Some organophosphorous pesticides show values out of these ranges: *Parathion*, *Parathion-m*, *Malathion*, and *Methidation* in both levels and *Triazophos* in high level. For those positive detections, alternative methods are necessary. *HCB* at high level gave low recovery (64%), but very reasonably consistent RSD (5%). The reason is a physicochemical partitioning factor between lipids and acetonitrile phases and the retention in C_{18} sorbent. Accurate quantification, of *HCB* will require either the use of internal standard that are matched more closely to the lipophilic analytes or the use of an extracted matrix matched calibration or to correct the results for the recovery factor. Improvements in the accuracy are feasible using deuterinated pesticides or ^{13}C labelled standard.

5.3. Quality control

Method verification is necessary during routine analysis (analytical quality control and on-going method validation).

For qualitative screening sequences, blank and a duplicate sample spiked at LOD level are introduced. Blank sample must agree with the blank criteria (no peaks with response higher than 30 % of the response at LOD level) and fortified samples classified as positive.

In quantitative sequences, introduced positives and blank controls and single recovery values must be in the range 60 – 140 %. Recoveries outside the mentioned range require re-analysis of the batch but can be acceptable in certain justified cases. Where the individual recovery is unacceptably high and no residues are detected, it is not necessary to reanalyse the samples to prove the absence of residues. However, consistently high recoveries should be investigated. If a significant trend occurs in recovery, or potentially unacceptable (RSD beyond ± 20 %) results are obtained, the cause(s) must be investigated. Linearity is checked for selected analytes by calculated the residuals, which must be within ± 20%.

The laboratory must participate regularly in relevant proficiency tests. When a low number of compounds (e.g.: < 90%) are analysed with respect to the pesticides present in the test sample, false positive(s) or negative(s) are reported or the accuracy achieved in any of the tests is questionable or unacceptable, the problem(s) should be investigated. Particularly for false positive(s), negative(s) and, or unacceptable performance, have to be rectified before proceeding with further determinations of the involved analyte/matrices combinations.

Matrix	Fat	Eggs	Milk	Shellfish	Chicken Meat	Liver
Compound name	LOD (mg/kg fat)	LOD (mg/kg fat)	LOD (mg/kg product)	LOD (mg/kg product)	LOD (mg/kg product)	LOD (mg/kg product)
Alachlor	0.01	0.01	NV	0.001	0.001	0.001
Aldrin	0.05	0.05	0.002	0.005	0.005	0.005
Bifenthrin	0.05	0.05	0.002	0.005	0.005	0.005
Chlordane-cis	0.05	0.05	0.002	0.005	0.005	0.005
Chlordane-trans	0.05	0.05	0.002	0.005	0.005	0.005
Chlorpyrifos-m	0.05	0.05	0.002	0.005	0.005	0.005
Chlorpyrifos	0.05	0.05	0.002	0.005	0.005	0.005
Cyfluthrin	NV	0.05	0.002	0.005	0.005	0.005
Cyhalothrin-λ	0.05	0.05	0.002	0.005	0.005	0.005
Cypermethrin	0.1	0.1	0.004	0.01	0.01	0.01
DDD	0.02	0.02	0.0008	0.002	0.002	0.002
DDE	0.02	0.02	0.0008	0.002	0.002	0.002
DDT-op	0.02	0.02	0.0008	0.002	0.002	0.002
DDT-pp	0.02	0.02	0.0008	0.002	0.002	0.002
Deltamethrin	0.1	0.1	NV	NV	0.01	0.01
Diazinon	0.05	0.05	0.002	0.005	0.005	0.005
Dieldrin	0.05	0.05	0.002	0.005	0.005	0.005
Endosulfan sulphate	0.05	0.05	0.002	0.005	0.005	0.005
Endosulfan-α	0.05	0.05	0.002	0.005	0.005	0.005
Endosulfan-β	0.05	0.05	0.002	0.005	0.005	NV
Endrin	0.04	0.04	0.0008	0.004	0.004	0.004
Fenthion	0.05	0.05	0.002	0.005	0.005	0.005
HCB	0.02	0.02	NV	0.002	0.002	0.002
HCE-endo	0.05	0.05	0.002	0.005	0.005	0.005
HCE-exo	0.05	0.05	0.002	0.005	0.005	0.005
HCH-α	0.05	0.05	0.002	0.005	0.005	0.005
HCH-β	0.05	0.05	0.002	0.005	0.005	0.005
Heptachlor	0.05	0.05	0.002	0.005	0.005	0.005
Lindane	0.015	0.015	0.0006	0.0015	0.0015	0.0015
Malathion	0.02	0.02	NV	NV	0.002	NV
Methidathion	NV	0.02	0.0008	0.002	0.002	0.002
Parathion	0.05	0.05	0.0008	0.005	0.005	0.005
Parathion-m	0.02	0.02	NV	0.002	0.002	0.002
Permethrin	NV	0.05	0.002	NV	0.005	0.005
Pirimiphos-m	0.05	0.05	0.0008	0.005	0.005	0.005
Trifluralin	0.01	0.01	0.0004	0.001	0.001	0.001

Table 5. Validation results for screening procedure applied to several animal origin matrices
NV, not validated compound

| Compound | LOQ Level | | | | Higher level | | | |
Name	mg/kg	n	R%	RSD%	mg/kg	n	R%	RSD%
Alachlor	0.01	8	106	10	0.10	6	105	8
Aldrin	0.05	8	81	16	0.50	6	76	6
Endosulfan-α	0.05	8	97	8	0.50	6	93	5
HCH-α	0.05	8	99	11	0.50	6	96	7
Endosulfan-β	0.05	8	108	11	0.50	6	97	5
HCH-β	0.05	8	117	15	0.50	6	107	19
Bifenthrin	0.05	8	100	14	0.50	6	96	8
Cyfluthrin	0.05	8	106	20	0.50	6	99	20
Cypermethrin	0.1	8	103	13	1.00	6	94	16
Chlordane-cis	0.05	8	96	13	0.50	6	91	7
DDD	0.02	8	95	18	0.20	6	90	6
DDE	0.02	8	78	17	0.20	6	84	6
Deltamethrin	0.1	8	109	15	1.00	6	99	18
Diazinon	0.05	8	103	13	0.50	6	105	6
Dieldrin	0.05	8	98	11	0.50	6	95	3
Chlorpyrifos	0.05	8	104	11	0.50	6	103	5
Parathion	0.05	8	123	14	0.50	6	110	21
Endosulfan sulphate	0.05	8	119	15	0.50	6	113	20
Endrin	0.04	8	98	14	0.40	6	93	4
Fenthion	0.05	8	115	14	0.50	6	107	11
HCB	0.02	8	71	18	0.20	6	64	5
HCE-endo	0.05	8	101	10	0.50	6	98	5
HCE-exo	0.05	8	100	10	0.50	6	99	5
Heptachlor	0.05	8	89	11	0.50	6	88	8
Cyhalothrin-λ	0.05	8	106	16	0.50	6	96	16
Lindane	0.015	8	103	8	0.15	6	98	10
Chlorpyrifos-m	0.05	8	104	13	0.50	6	102	4
Parathion-m	0.02	8	128	23	0.20	6	120	28
Pirimiphos-m	0.05	8	106	14	0.50	6	105	4
Malathion	0.02	8	126	20	0.20	6	124	26
Methidathion	0.02	8	123	15	0.20	6	116	25
DDT-op	0.02	8	101	10	0.20	6	96	8
Permethrin	0.05	8	92	16	0.50	6	90	9
Pyrazophos	0.02	8	109	13	0.20	6	102	15
DDT-pp	0.02	8	96	16	0.20	6	91	5
Chlordane-trans	0.05	8	94	14	0.50	6	92	4
Triazophos	0.01	8	111	36	0.10	6	130	42
Trifuralin	0.01	8	104	13	0.10	6	100	7

Table 6. Validation results obtained in fat sample at two levels studied (LOQ and HL).
N = nº of spiked samples; R = recovery (%) and RSD = relative Standard deviation (%)

6. Application to the analysis of real samples

The method was applied for routine analysis of lipophilic pesticides. Samples of different animal origin were collected during 2010 and 2011 from several farms from Comunidad Valenciana (Spain) and analyzed. 167 samples of animal fat (19% from swine, 19% from bovine, 29% from sheep, 25% from chicken, 5% from rabbit and 3% from horse) were analyzed. Sixteen samples were contaminated by *DDE* with values above the LOD (0.02 mg/kg): fourteen of them were sheep fat, with *DDE* values between 0.021 and 0.175 mg/kg; and two of them were bovine fat, with 0.210 and 0.221 mg/kg of pesticide. Only one sample showed the presence of *HCB* in a higher concentration than the LOD (0.02 mg/kg); it was a porcine fat with 0.036 mg/kg of pesticide. The rest of the samples presented concentrations below the LOD values.

Pesticides from other matrices were analyzed too: eggs (20), milk (4), fish (2), shellfish (4), liver (10), and chicken meat (10). All of them showed absence of pesticides above LOD level.

7. Future research

Our working team intends to continue its research on the determination of pesticides in animal origin food following two ways:

a. The extension of the laboratory scope in both, analytes and matrices; by the increase of analyzed pesticides and to extend and complete the list of the included substances on the definition of residue. In the case of matrices, the main objective is to increase the number of covered matrices.
b. Instrumental development on GC-MS/MS by the comparison of negative chemical ionization and EI ionization modes. The improvement of selectivity and sensibility by the use of back-flush technology. The complementation of GC chromatography with the use of UHPLC-MS/MS system, to extend the list of analyzed pesticides with other families as triazines, carbamates, etc., also included on the definition previously mentioned.

8. Conclusions

Official control laboratories are required to handle a large number of analytes in different matrices with validated methods. That is the reason that quick, and easy methods are required.

In this chapter, extraction and clean-up efficiency have been evaluated (due to the success of the analysis of lipophilic pesticides in fatty matrices critically relies on the efficiency of the clean-up) in order to: a) reduce instrumental maintenance, b) reduce solvents consumption, and c) minimize time and effort consumption in sample treatment. This evaluation, lead us to choose modified QuEChERS procedure as the best one.

Previously observed matrix interferences in GC-MS for some pyrethroids, as well as sensibility limitations, have been reduced with the use of a more selective instrument, as GC-MS/MS is. Matrix-matched standard calibration is still necessary for an adequate quantification although an important decrease on matrix effects have been observed when using GC-MS/MS equipment.

Furthermore, an efficient data processing system has been developed; as a first approach by the use of screening sequences, discriminating all the samples without detected analytes; and secondly, applying a quantitative method to the previously ones detected as positive samples. This working methodology allows faster data processing, concentrating our efforts mainly in those samples which need to be carefully evaluated because of their potential damage on human health.

All these incorporations have become a powerful tool to improve laboratory efficiency, developing a method that allow the multi-residue determination of 38 representative pesticides including organochlorine, organophosphate, and pyrethroid pesticides in animal origin food samples. Thereby, this method can be used in routine analysis laboratories for national and community monitoring programs of different families of pesticides in animal origin food with equipments that allows screening as a first approach, quantitation and confirmation when necessary.

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