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Simultaneous Extraction and Cleanup Method Based on Pressurized Solvent Extraction for Multiresidue Analysis of Pesticides in Complex Feed Samples

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The development of a multiresidue method based on pressurized solvent extraction (PSE) to determine a large number of pesticides (mostly pyrethroids and organochlorines) in cattle feed is described. As far as we know, this is the first work dedicated to the PSE of many of the target pesticides from animal feed. A mixed level fraction design was performed to investigate the influence of several operational variables on the PSE procedure; integrated cleanup strategies were also assessed allowing the direct chromatographic analysis of the extracts. Method accuracy was evaluated by the analysis of a certified reference material (BCR-115) and different fortified cattle feed samples. Most analytes were recovered in the range of 70−110%, with relative standard deviations generally lower than 15%. Limits of detection (LODs) were below the maximum residue levels (MRLs) set by the European Union for animal feed and main crops used in the preparation of feedingstuffs. The applicability of the proposed method was demonstrated by the analysis of real cattle feed samples collected from 23 dairy farms located in Galicia (NW Spain).

KEYWORDS: Pressurized solvent extraction; feed analysis; cattle feed; pyrethroids; organochlorine pesticides; pesticides; experimental design

INTRODUCTION

The yield of agricultural and horticultural crops can be severely decreased as a result of infestation by pests and diseases. The widespread use of pesticides to control pests may result in the contamination of products intended for animal feed, which can endanger animal health or, because of their presence in livestock products, human health and the environment (*1*, *2*). Therefore, Maximum residue levels (MRLs) have been set to protect consumers from exposure to unacceptable levels of pesticides in feedingstuffs and in main crops used for their preparation. According to the European directive 2002/32/EC on undesirable substances in animal feed, MRLs between 5 and 2000 ng g^{-1} have been established for the most common organochlorine pesticides (*2*). From September 2008 onward, a new regulation that covers not only products for human food but also those intended for animal feed (*3*, *4*) came into force in the European Union (EU). This Regulation includes pesticides used in agriculture at present or in earlier times inside or outside the EU and raises the question on how the current directive 2002/32/EC, which does not mention any modern pesticide, will be affected. The current EU and US legislations for the pesticides investigated in the present work with respect to their residues in the most common cattle feed components (maize, barley, soya bean, and wheat) are outlined in **Table 1** (*1*, *3*−*5*).

Feedingstuffs contain additional substances (especially fats) that make extraction of pesticides much more difficult than from a feed component (maize, wheat, etc.). Therefore, the analysis of pesticides in feed samples is a very difficult task, not only because of the low detection levels required by the legislations but also because of the complexity of the matrix. For these reasons, selective, sensible, and, in short, reliable analytical methods are needed. Regardless of the progress in the development of highly efficient analytical instrumentation for final determination, sample preparation remains a very important part of obtaining accurate quantitative results. Methods based on classical Soxhlet or solvent extraction have been employed in the extraction of pesticides from animal feed (*6*, *7*). Alternatively, faster and more automated extraction techniques such as ultrasonic extraction (UE), fluidized-bed extraction (FBE), and microwave-assisted extraction (MAE) have been applied to the analysis of chlorinated pesticides in pig feed (*8*, *9*). The QuEChERS (quick, easy, cheap, effective, rugged, and safe) method has been also used in order to prepare samples of cereal grain and some dry feedingstuffs for the determination of pesticide residues (*10*). In a previous paper, the authors optimized and validated a matrix solid-phase

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Table 1. Current European (EU) and American (US) Legislation Regarding the Maximum Residue Levels of the Target Pesticides in the Most Common Ingredients Used in the Preparation of Cattle Feedingstuffs (*4*, *5*)

		MRL (ng g^{-1})							
		barley		soya bean		maize		wheat	
class	pesticide	EU	US	EU	US	EU	US	EU	US
organochlorine	γ-lindane	10		10		10		10	
	heptachlor (sum of heptachlor and heptachlor epoxide expressed as heptachlor)	10		10		10		10	
	aldrin and dieldrin (aldrin and dieldrin combined expressed as dieldrin)	10		20		10		10	
	chlordane (sum of α- and γ-chlordane)			20					
	endosulfan (sum of isomers I, II, and endosulfan sulfate expressed as endosulfan)	50	300	500		50		50	300
	DDT (sum of *p*,*p*′-DDT, *o*,*p*′-DDT, *p*,*p*′-DDE and *p*,*p*′-TDE (DDD) expressed as DDT)	50		50		50		50	
	endrin	10		10		10		10	
	methoxychlor	10		10		10		10	
chloroacetanilide	acetochlor	10	50	10	100	100	50	10	
	alachlor	50	50	200	1000	200	200	50	50
organophosphorus	chlorpyrifos	200		50	300	50	50	50	500
	fenitrotion	500[a]		10		500[a]		500[a]	
pyrethroid	tefluthrin	50		50		50	60	50	
	λ-cyhalothrin	50	50	50	10	20	50	20	50
	permethrin (sum of isomers)	50		50	50	50	50	50	
	cyfluthrin (cyfluthrin including other mixtures of constituent isomers; sum of isomers)	20	150	20	30	50	50	20	150
	cypermethrin (cypermethrin including other mixtures of constituent isomers; sum of isomers)	2000		50	50[b]	50	50[b]	2000	200[b]
	flucythrinate	50		50		50		50	
	fenvalerate and esfenvalerate (sum of RR and SS isomers)	200		50	50[c]	20	20[c]	50	
	fenvalerate and esfenvalerate (sum of RS and SR isomers)	50		50		20		20	
	deltamethrin	2000[d]	1000	50[d]	100	2000[d]	1000	2000[d]	1000

[a] Temporary MRL until 1 June 2009. [b] MRL for *zeta*-cypermethrin. [c] MRL for fenvalerate. [d] MRL for *cis*-deltamethrin.

dispersion (MSPD) method for the simultaneous extraction of a high number of common pesticides and breakdown products in cattle feed (*11*).

Popularity of pressurized solvent extraction (PSE) has increased since its acceptance as an official US Environmental Protection Agency (EPA) method for the determination of persistent organic pollutants (POPs) in a variety of environmental solid samples (*12*, *13*). It has been successfully applied for the extraction of pesticide residues from various matrices, such as fruits and vegetables (*14*), cereals (*15*), soya bean (*16*), or food (*17*). Nevertheless, only very few data are available in the literature about the PSE of pesticides from feedingstuffs, and they are limited to organochlorine compounds (*8*, *18*). In PSE, pressure is applied to allow the use of liquids as extraction solvents at temperatures greater than their normal boiling point (*19*). Nevertheless, the extraction selectivity also decreases under these conditions because only the target analytes are solubilized. Most common postcleanup approaches for fatty samples include adsorption columns using alumina (*20*), silica gel (*18*), graphitized nonporous carbon (ENVI-Carb) (*21*), florisil (*22*), or sulfuric acid-impregnated silica gel (*23*), and gel-permeation chromatography (GPC) (*18*, *24*). Frequently, more than one step is required (*18*). In order to avoid laborious cleanup of extracts prior to GC analysis and to increase the automation possibilities, several reports have focused on the development of in situ cleanup methods. In these cases, the elimination of lipids and other coextractable materials was achieved by adding fat retaining sorbents to the PSE cell, such as Florisil (*25*), alumina (*26*), or sulfuric acid-impregnated silica gel (*23*).

In the present work, a pressurized solvent extraction (PSE) cleanup procedure is proposed as a simple, rapid, and reliable alternative for the multiresidue analysis of pesticides in feedingstuffs. The complete list of pesticides comprises 36 compounds, mostly organochlorines (including some metabolites) and pyrethroids, although several common organophosphorus and chloroacetanilides were also investigated. The effect of the solvent type and sample size on the pesticide recovery, as well as some PSE operational variables such as temperature and static time, was evaluated by means of an experimental design. Several cleanup strategies were also assessed in order to obtain suitable chromatographic extracts. Finally, the optimized extraction−cleanup methodology was validated and applied to real cattle feed samples.

MATERIALS AND METHODS

Chemicals. Tefluthrin, transfluthrin, allethrin (mixture of stereoisomers), tetramethrin, λ-cyhalothrin, cyphenothrin (mixture of cis and trans isomers), permethrin (mixture of cis and trans isomers), cyfluthrin (mixture of isomers), flucythrinate, fenvalerate, acetochlor, and chlorpyrifos were of Pestanal grade and were purchased from Riedel-de-Häen (Seelze, Germany). A standard mix solution containing organochlorinated pesticides and some metabolites (α-chlordane, methoxychlor, γ-chlordane, endrin ketone, endrin aldehyde, aldrin, α-lindane, β-lindane, γ-lindane, δ-lindane, *p*,*p*′-DDD, *p*,*p*′-DDE, *p*,*p*′-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, heptachlor, and heptachlor epoxide isomer B) with a concentration of 2000 μg mL^{-1} of each compound in toluene/hexane (50:50), cypermethrin (mixture of isomers), and deltamethrin were supplied by Supelco (Bellefonte, PA, USA). Fenitrotion and alachlor were acquired from Dr. Ehrenstorfer (Augsburg, Germany). PCB-166 and PCB-195 (employed as internal standard and surrogate, respectively) were obtained as 10 μg mL^{-1} solutions in isooctane also from Dr. Ehrenstorfer.

Acetone (pesticide grade) was acquired from Prolabo (VWR, Fontenay-sous-Bois, France). *N*-Hexane (GC grade), ethyl acetate (HPLC grade), isooctane (for organic trace analysis), methanol (gradient grade), and toluene (HPLC grade) were obtained from Merck (Mollet del Vallés, Barcelona, Spain).

Sand (white quartz, 50−70 mesh, suitable for chromatography) was supplied by Sigma-Aldrich (Madrid, Spain). Anhydrous Na_2SO_4 was provided by Panreac (Barcelona, Spain). Neutral alumina (150 mesh), Florisil (60−100 mesh) and C18 (70−230 mesh) were achieved from Aldrich (Milwaukee, WI, USA), and silica (230−400 mesh) from Merck. Supelclean PSA SPE (primary secondary amine, solid phase extraction) bulk packing (38−35 μm) and graphitized nonporous carbon (Supelclean ENVI-Carb SPE Bulk Packing, 100−400 mesh) were obtained from Supelco. Before being used, Florisil, alumina, and silica were activated at 130 °C for 12 h and then allowed to cool down in a desiccator, while graphitized nonporous carbon was prewashed with acetone, hexane, and ethyl acetate and then thoroughly vacuum-dried and also kept in a desiccator. Alumina N, Florisil, Silica, and C18 Sep-Pak cartridges were acquired from Waters (Milford, MA, USA).

Preparation of Solutions. Individual standard stock solutions of 1.000−10.000 μg mL^{-1} of pyrethroid, chloroacetanilide, and organophosphorus pesticides were prepared by accurate weighing and dissolution in the appropriate solvent (acetone, methanol, isooctane, or ethyl acetate). By dilution of stock solutions and commercial organochlorinated pesticide solution, intermediate mixture solutions of 100 μg mL^{-1} in acetone were also prepared. Working solutions in acetone (to spike cattle feed samples) or ethyl acetate (to direct injection into the GC) containing the target pesticides were obtained by convenient dilutions of the intermediate solutions. Working solutions of PCB-195 in isooctane were also prepared. Stock, intermediate, and working solutions were stored in a freezer at −20 °C protected from light.

Feed Samples. The feedingstuffs are very complex samples elaborated by mixing several products (more than 10 in most cases) containing a high percentage of various cereal meals, vegetal oils, oxides, and salts.

The cattle feed samples included in the present study were collected from 23 dairy farms located in NW Spain. They were ground, and the residual moisture content was calculated. Then, they were stored in their original containers at −20 °C until their analysis.

A feed sample spiked at 100 ng g^{-1} was employed for method optimization. None of the target compounds was detected in this sample. Fortification of the sample was performed by weighing 60 g in a glass vessel and pouring 12 mL of a 500 ng mL^{-1} solution of the target pesticides in acetone. Then, an extra volume of acetone (about 30 mL) was added all over the sample so that it got completely coated with organic solvent. The resulting slurry was allowed to stand (36 h at room temperature, in a switched off hood) and stirred occasionally until the acetone was completely evaporated. Then, 1 and 3 g fractions were collected and kept at −20 °C until 5−10 min before the analysis. For the analytical performance evaluation, aliquots of the same cattle feed sample spiked at concentration levels ranging from 5 to 100 ng g^{-1} were analyzed. Other feed samples were spiked at 100 ng g^{-1} for recovery studies. In addition to these samples, nonspiked feedingstuffs were analyzed for the monitoring of the target pesticides.

The certified reference material (BCR-115) employed for method accuracy evaluation is an animal feed product certified for the content of 10 organochlorine pesticides: hexachlorobenzene (HCB) (19.4 ± 1.4 ng g^{-1}), β-lindane (23 ± 3 ng g^{-1}), γ-lindane (21.8 ± 1.9 ng g^{-1}), heptachlor (19.0 ± 1.5 ng g^{-1}), γ-chlordane (48 ± 5 ng g^{-1}), endosulfan I (46 ± 4 ng g^{-1}), dieldrin (18 ± 3 ng g^{-1}), endrin (46 ± 6 ng g^{-1}), *o,p′*-DDT (46 ± 5 ng g^{-1}), and *p,p′*-DDE (47 ± 4 ng g^{-1}). It was prepared by mixing different ingredients such as wheat, corn, soya bean, oil meal, tapioca, and others to mimic a mixture of pig and poultry feeds. This material was provided by the EC Community Bureau of Reference (Brussels, Belgium).

PSE and Cleanup Procedures. Extractions were performed on an ASE 200 system (Dionex, Co., Sunyvale, CA, USA) equipped with a 24-sample carousel, 11-mL stainless steel cells, and 40-mL collection vials. To avoid the collection of suspended powders in the extraction, filters (Dionex) were placed at each end of the PSE cell. When in situ cleanup was performed, the corresponding sorbents (see Results and Discussion) were introduced into the cell, followed by the mixture of the sample (1 or 3 g) and 1 g of drying agent (anhydrous Na_2SO_4). In all experiments, 10 μL of PCB-195 surrogate solution (1 μg mL^{-1}) was added to each sample before extraction. Finally, the dead volume of the cell was filled up with clean sand. The packing of the extraction cell is outlined in **Figure 1**. The cell was tightly closed and placed into the carousel of the ASE system. Extractions were performed by preheating the cell before filling with solvent

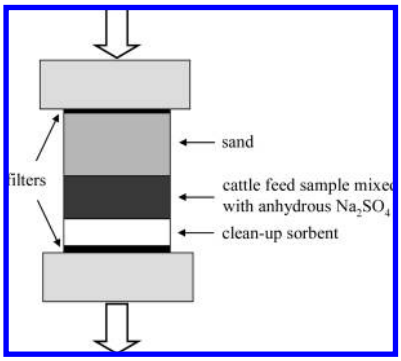

Figure 1. Packing of the extraction cell.

(preheat method). The extraction pressure was set to 1500 psi, the flush volume was 60%, and the purge time was set to 90 s. Hexane/acetone (1:1, v/v) or ethyl acetate were employed as extraction solvents, depending on the experiment. The remaining variables (extraction temperature, extraction time, and number of cycles) also varied during optimization of the method. The PSE extract (of approximately 15 mL) was concentrated to 5 mL under a nitrogen stream. At this point, a cleanup stage might be required depending on the sample (see Results and Discussion). Then, the extract was filtered through a PVDF 22 μm filter (Millex CV). One milliliter of the eluate was evaporated to dryness and rediluted in 200 μL of ethyl acetate. Finally, 1 μL of PCB-166 internal standard solution was added, and 1 μL of the final extract was injected.

For purification based on adsorption chromatography applying classical solid-phase extraction (SPE) procedures, Silica, C18 Sep-Pak, Alumina N, and Florisil cartridges were conditioned with approximately 5 mL of organic solvent. Then, the extract (5 mL) was added to the cartridge and eluted under gravity flow. In dispersive-solid-phase extraction (dSPE) experiments, an amount of 500 mg of sorbent (alumina, Florisil, a mixture of both sorbents, PSA, or silica) was weighed and mixed with the eluate (5 mL). This mixture was shaken twice for 2 min.

Chromatographic Conditions. Gas chromatographic analysis was carried out in a Hewlett-Packard 6890 GC system equipped with a ^{63}Ni microelectron capture detection, a 7683B autosampler and a split/splitless injector. Data were acquired and processed by GC Chemstation software. A 30 m × 0.32 mm i.d. HP-5MS capillary column with a stationary phase thickness of 0.25 μm was used for the chromatographic separation of the target compounds. The GC oven temperature program was as follows: initial temperature, 80 °C (held for 2 min); increased at 15 °C min^{-1} to 200 °C; increased at 3 °C min^{-1} to 235 °C (held for 1 min); and finally increased at 20 °C min^{-1} to 300 °C and held at this temperature for 10 min, with a total acquisition program of 35.92 min. Samples were injected in the splitless mode (split opened after 2 min) at an injector temperature of 280 °C. Helium was employed as carrier gas at a constant flow rate of 1 mL min^{-1}, while nitrogen was used as makeup gas at 30 mL min^{-1}. Detector temperature was 300 °C. For confirmation of positive results, some of the real feed samples were analyzed using a Varian 3800 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) coupled to an ion trap mass detector Varian Saturn 2000 (Varian Chromatography Systems), operated in the electron impact ionization (EI) positive mode (+70 eV). The mass range was scanned in full scan mode from 80 to 500 m/z at 0.6 s scan^{-1}. The system was operated by Saturn GC/MS Workstation v5.4 software.

Statistical analysis. Basic and descriptive statistics and experimental design analysis were performed using the Statgraphics XV Centurion (Rockville, MD) software package. The experimental design was applied in the optimization of the extraction method to analyze the simultaneous effect of the main parameters affecting PSE.

RESULTS AND DISCUSSION

Optimization. Numerous parameters can potentially influence the efficiency of the pressurized solvent extraction: on the one hand, some specific PSE variables such as the temperature and pressure of the extraction, flush volume, extraction and purge time, or the number of cycles and on the other hand, as in

Table 2. Factors and Levels Considered in the Experimental Design

factor	code	low level (−)	intermediate level	high level (+)	continuous
temperature (°C)	A	80	100	120	yes
solvent	B	acetone/hexane (1:1, v/v)		ethyl acetate	no
time (min)	C	5		15	yes
sample size (g)	D	1		3	yes

conventional solid−liquid extractions, solvent nature, sample size, and cleanup stages must be also investigated.

Pressure generally has a negligible effect on the extraction yield (*27*), and therefore, all experiments were conducted at 1500 psi, which is the standard operating pressure in PSE extractions (*28*). Flush volume and purge time were set at 60% and 90 s, respectively. The influence of the remaining variables was studied as described below. Method optimization was performed on fortified cattle feed samples. Since drying of the sample is essential, in all experiments 1 g of anhydrous sodium sulfate was added to the extraction cell. Sand was employed to avoid the dead volume (see the Materials and Methods section).

In-depth cleanup of eluates prior to chromatographic analysis can be avoided (or, at least, simplified) by performing an in situ cleanup step by adding certain sorbents to the PSE cells. In this way, lipids and other coextractable materials are prevented from coming out to the extract. Experiments with and without 1 g of sorbent (Florisil) placed under the sample were performed. A deep yellow extract was indeed obtained when the cell was only filled with the sand and the mixture between the sample and the anhydrous Na_2SO_4, leading to a chromatogram with an obvious increase in the baseline and in the chromatographic artifacts compared to the one obtained when Florisil was added to the cell. Then, different sorbents (Florisil, alumina, a mixture of both sorbents, and silica) were evaluated. Silica led to the worst chromatographic profile, while the cleanest chromatograms were obtained after using alumina. The simultaneous use of Florisil and alumina did not mean any improvement with regard to alumina. Thus, in the subsequent trials, 1 g of alumina was packed in the PSE cells as the cleanup sorbent.

The use of several static cycles that introduce fresh solvent during the extraction process assists in keeping a favorable extraction equilibrium. Static cycles have proven to be useful for samples with very high analyte concentration or for samples in which the matrix hampers the solvent diffusion (*28*). In our case, extraction efficiency was not improved, while the background increased when two static cycles were conducted instead of one. This result is in agreement with the official pressurized fluid extraction (PFE) method 3545 (*12*), which recommends the use of only one static cycle as the optimal extraction conditions for semivolatiles including organochlorine pesticides and herbicides. The use of extra cycles could favor the coextraction of interfering compounds, as was observed in our case.

The optimization of the remaining variables was accomplished using an experimental design in order to detect the most influential factors and their optimum levels, evaluating also possible interactions between variables, and minimizing the number of trials needed. The studied factors were extraction temperature (A), solvent nature (B), extraction time (C), and sample size (D) (see factors and levels in **Table 2**).

Temperature is an important parameter that favors PSE extractions, although the use of high temperatures can reduce selectivity (*29*) and cause compound degradation as well, as has been described for DDT (*30*). This factor (A) was evaluated at three levels: 80, 100, and 120 °C. The choice of an appropriate solvent (B) is another essential aspect in the development of extraction methods. For an efficient extraction, the solvent must solubilize the target analytes while leaving the sample matrix as intact as possible (*28*). Two solvents were investigated: acetone/hexane (1:1, v/v) and ethyl acetate. The use of mixtures of solvents of different polarities is usual when a broad range of compound classes has to be extracted, and acetone/hexane has proved to be suitable to extract pesticides from animal feed (*8*, *18*) and other environmental matrixes (*31*). In other studies, ethyl acetate has been also used in the PSE extraction of several pesticides from meat and food samples (*17*, *24*). PSE extraction times are very short compared to those required in conventional solid−liquid extraction techniques. A few minutes are often enough, although higher static times are sometimes needed to extract the analytes strongly retained in pores or other structures of certain samples (*28*). In the present work, extraction time (C) was assessed at 5 and 15 min. Finally, the effect of sample size (D) was evaluated at two levels: 1 and 3 g.

Table 3. Experimental Conditions of the $3 \cdot 2^{3-1}$ Experimental Design[a]

experiment	temperature (°C)	solvent	time (min)	sample size (g)
1*	100	acetone/hexane	10	2
2	80	acetone/hexane	5	1
3	80	acetone/hexane	15	3
4	80	ethyl acetate	5	3
5	80	ethyl acetate	15	1
6	100	acetone/hexane	5	3
7	100	acetone/hexane	15	1
8	100	ethyl acetate	5	1
9	100	ethyl acetate	15	3
10	120	acetone/hexane	5	1
11	120	acetone/hexane	15	3
12	120	ethyl acetate	5	3
13	120	ethyl acetate	15	1
14*	100	ethyl acetate	10	2

[a] Central points are marked with an asterisk.

A $3 \cdot 2^{3-1}$ mixed level fraction design was proposed (Statgraphics XV Centurion). In contrast to other screening designs, this one allows running one quantitative factor (A) at 3 levels rather than 2. The resolution of the design is V, enabling an estimation of all main effects and all two-factor interactions. Two center points were added to increase the degrees of freedom to evaluate the experimental error. Thus, 14 experiments were run under the conditions specified in **Table 3**. In all experiments, the extraction cell was loaded with 1 g of alumina followed by the mixture of the fortified sample (at 100 ng g^{-1}) and anhydrous Na_2SO_4. Sand was also added at the top, and one cellulose filter was placed at each end of the cell. The instrumental settings were those mentioned above (60% flush volume, 90 s of purge time, 1500 psi, and one static cycle).

The outcomes of the experimental design can be simply interpreted by visualizing several intuitive software tools provided by Statgraphics. For practical reasons, only some representative examples are illustrated in **Figures 2** to **4**. In the Pareto charts (**Figure 2**), the standardized effects are plotted in decreasing

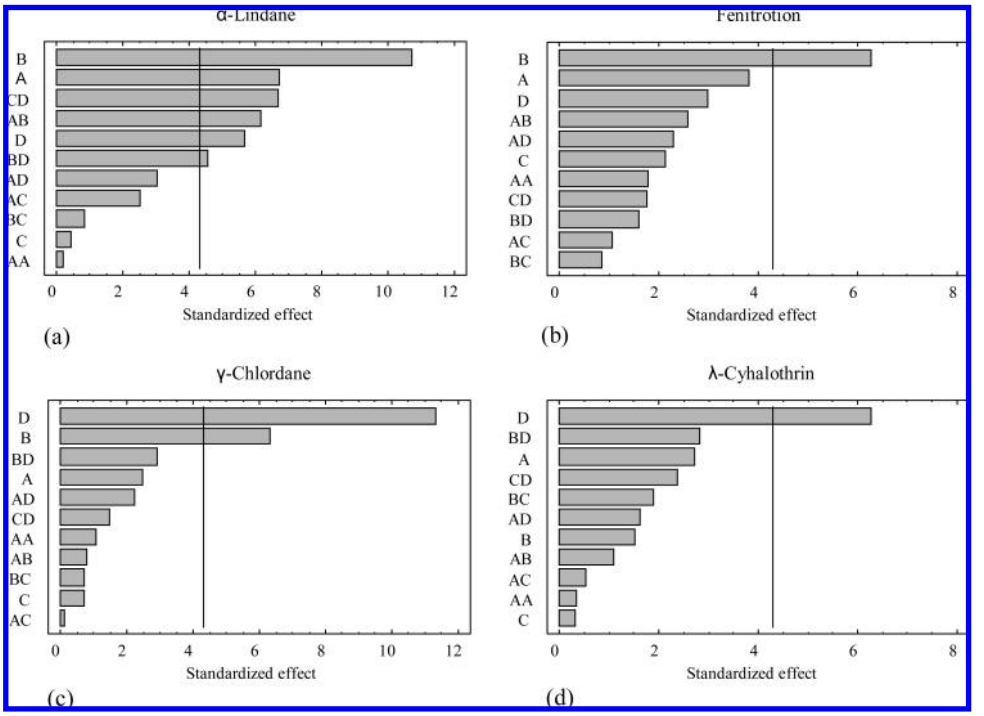

Figure 2. Pareto charts for α-lindane, fenitrotion, γ-chlordane, and λ-cyhalothrin.

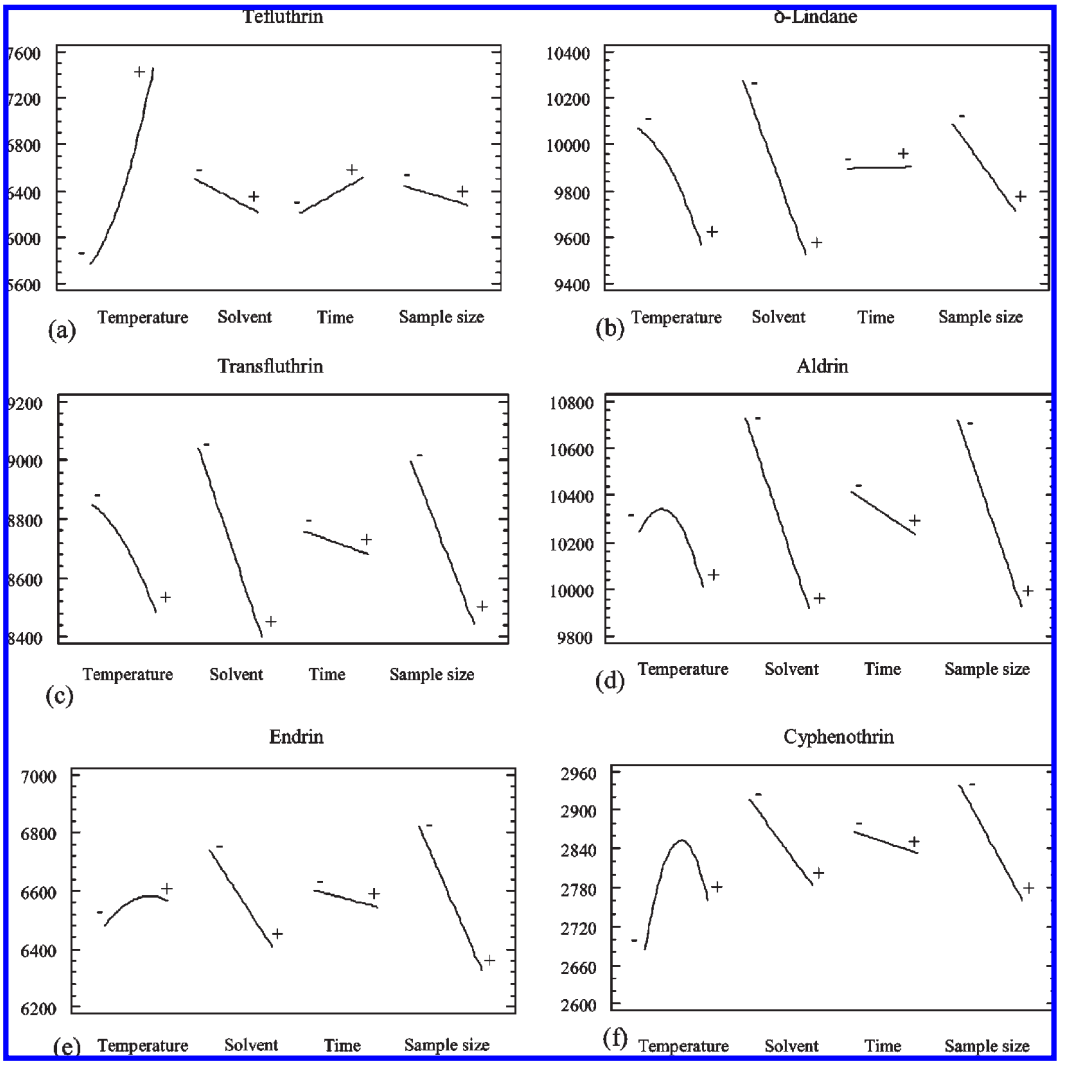

Figure 3. Main effect plots for tefluthrin, δ-lindane, transfluthrin, aldrin, endrin, and cyphenothrin.

order of absolute magnitude, thus making it easier to see which ones are the most important factors and interactions. In addition, the line drawn on the chart indicates whether an effect is statistically significant at a specified significance level (in this case, 95%). Main effect plots (**Figure 3**) show how the response varies when each factor is changed from its low level to its high level, while all other factors are held at the center of the experimental domain. Finally, in the interaction plots (**Figure 4**), the predicted response for each combination of the low and high levels of two factors is displayed at the end of each line segment.

Analyzing the Pareto charts (**Figure 2**), it was observed that solvent (B) and sample size (D) were the most important parameters for the extraction efficiency. For almost all of the pesticides for which factor B was significant, acetone/hexane led to higher recoveries (see **Figure 3**). Therefore, acetone/hexane was selected as the most efficient solvent for the extraction of the target compounds from cattle feed. Regarding sample size, in general, its effect was negative, meaning that higher responses and, thus, more efficient extractions were obtained working with 1 g of sample (see **Figure 3**). Influence of extraction temperature (A) was not so important in the studied range. In fact, this parameter was statistically significant only for four of the 36 compounds: α- and δ-lindane, tefluthrin, and endrin aldehyde. In the case of lindanes (see **Figure 3** for δ-lindane), 80 °C was the optimal temperature, while 120 and 107 °C were the most favorable temperatures for tefluthrin (see **Figure 3**) and endrin aldehyde, respectively. A compromise solution was taken by selecting 100 °C as the most satisfactory extraction temperature. Finally, extraction time (D) was not a significant variable for any of the studied pesticides. With the aim of making experiments as short as possible, increasing sampling throughput, 5 min was chosen as the most adequate extraction time.

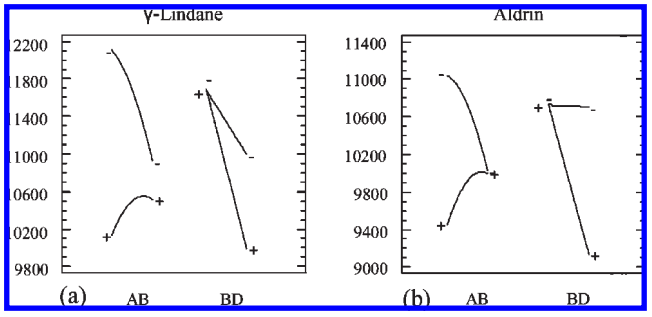

Figure 4. Temperature-solvent (AB) and solvent-sample size (BD) interaction plots for γ-lindane and aldrin.

Table 4 summarizes the optimal conditions for the extraction of the target pesticides. The last columns also include the significant interaction effects. As can be deduced from this Table, only eight analytes showed significant second-order effects; moreover, these interactions did not change the optimal conditions selected after main effects analysis. As an example, **Figure 4** shows the temperature−solvent (AB) and solvent−sample size (BD) interactions for γ-lindane and aldrin. AB interaction was

Table 4. Optimal Experimental Conditions Given for Each Compound by the $3 \cdot 2^{3-1}$ Mixed Level Fraction Design[a]

	factors				interactions					
	temperature (°C)	solvent	time (min)	sample size (g)	AB	AC	AD	BC	BD	CD
α-lindane	**80**	**acetone/hexane**	5	**1**	*				*	*
β-lindane	80	**acetone/hexane**	5	1						
γ-lindane	80	**acetone/hexane**	5	**3**	*	*	*		*	*
tefluthrin	**120**	ethyl acetate	15	1						
δ-lindane	**80**	**acetone/hexane**	5	**3**	*	*	*		*	*
acetochlor	80	ethyl acetate	5	1						
transfluthrin	80	**acetone/hexane**	5	**1**						*
alachlor	80	ethyl acetate	15	1						
heptachlor	80	**acetone/hexane**	15	1						
fenitrotion	80	**acetone/hexane**	15	1						
aldrin	80	**acetone/hexane**	5	**1**	*				*	*
chlorpyrifos	80	acetone/hexane	15	1						
heptachlor epoxide	80	acetone/hexane	15	1						
allethrin	115	acetone/hexane	15	1						
γ-chlordane	80	**acetone/hexane**	5	**1**						
p,p′-DDE	80	acetone/hexane	15	**1**						
dieldrin	80	acetone/hexane	15	1						
endrin	120	**acetone/hexane**	15	**1**		*	*	*	*	*
endosulfan II	101	acetone/hexane	5	**1**						
p,p′-DDD	104	**acetone/hexane**	5	**1**						
endrin aldehyde	**107**	**ethyl acetate**	15	**1**	*			*	*	*
endosulfan sulfate	111	acetone/hexane	5	**1**						
p,p′-DDT	120	acetone/hexane	5	1						
endrin ketone	80	**acetone/hexane**	15	**1**	*					
tetramethrin	80	acetone/hexane	5	1						
methoxychlor	93	**acetone/hexane**	5	**1**						
λ-cyhalothrin	120	ethyl acetate	15	**1**						
cyphenothrin	108	acetone/hexane	5	1						
permethrin	109	ethyl acetate	15	1						
cyfluthrin	80	acetone/hexane	15	1						
cypermethrin	80	ethyl acetate	15	1						
fenvalerate	120	acetone/hexane	15	1						
deltamethrin	85	ethyl acetate	5	1						

[a] Significant factors are in bold. *, refers to a significant interaction.

significant for α-, γ- and δ-lindane, aldrin, endrin aldehyde, and endrin ketone. In these cases, slight differences between the types of solvent were observed when working at 120 °C; however, at 80 °C, these differences were important, and better results were obtained when using acetone/hexane as solvent. Regarding the BD interaction, in general the extraction of 1 g of feed with both solvents led to comparable responses, while better efficacies were obtained with acetone/hexane when the sample size was 3 g.

After optimization of the investigated factors, the recommended procedure for the simultaneous extraction of the target pesticides in cattle feed was established as follows: temperature of 100 °C, acetone/hexane (1:1, v/v) as solvent, 5 min of extraction time, and 1 g of sample.

Several additional experiments regarding the number and type of filters placed at the bottom of the PSE cell were performed. The use of two filters instead of one led to less muddy extracts. Cellulose and glass fiber filters were compared, and no differences between them were noticed; however, because of economic reasons, the former filters were selected, and thus, in the subsequent experiments one and two cellulose filters were placed at the top and at the bottom of the cell, respectively.

Concentration and Cleanup Strategies. An important improvement in the sensibility of the developed method was achieved by means of extract concentration. Aliquots of 1 mL of PSE extracts were evaporated to dryness under nitrogen and rediluted with 200 μL of ethyl acetate. A 5-fold increase in the peak areas of the target pesticides compared to those attained after the chromatographic analysis of the corresponding extracts without concentration was observed. In this way, the absence of losses by volatilization during solvent evaporation was demonstrated.

Because of the complexity of the studied matrix, even though a layer of alumina was included in the PSE extraction cell, interfering compounds present in the eluate had to be removed in order to minimize adverse effects for the target compound detection. Thus, several further cleanup procedures were investigated. First, 0.5 g of graphitized nonporous carbon was placed in the PSE cell on the top of alumina. In most cases, this procedure delivered colorless and lipid-free eluates leading to better chromatographic profiles. In general, the extracts obtained in this way were adequately clean for their direct analysis, even after concentration.

Nevertheless, in the case of extremely complex feed samples, this extra in situ cleanup may not be sufficient, and other cleanup assays were performed in order to find the best protocol for further purification. In this way, classical SPE using Silica, Alumina N, Florisil, and C18 Sep-Pak cartridges was tried. Alternatively, dispersive-SPE (dSPE) was evaluated with alumina, Florisil, a mixture of both, PSA, and silica. In both procedures

Table 5. Linearity, Precision, and Limits of Detection (LODs) and Quantification (LOQs) of the Proposed Method

			precision (RSD, %)				
		linearity	within-a-day		among days		
code	pesticide	R	20 ng/g (n = 3)	100 ng/g (n = 3)	20 ng/g (n = 5)	LOD (ng g^{-1})	LOQ (ng g^{-1})
1	α-lindane	0.9996	9	6	14	0.05	0.17
2	β-lindane	0.9998	3	5	12	0.20	0.67
3	γ-lindane	0.9997	8	6	7	0.09	0.30
4	tefluthrin	0.9994	7	10	12	0.12	0.40
5	δ-lindane	0.9993	6	7	12	0.10	0.33
6	acetochlor	0.9999	4	8	4	1.8	6.0
7	transfluthrin	0.9996	13	4	11	0.10	0.33
8	alachlor	0.9988	12	9	12	1.8	6.0
9	heptachlor	0.9998	10	5	14	0.09	0.30
10	fenitrotion	1.0000	7	6	6	0.09	0.30
11	chlorpyrifos	1.0000	6	7	7	0.10	0.33
12	aldrin	0.9997	6	6	9	0.12	0.40
13	allethrin	0.9998	6	6	5	0.09	0.30
14	heptachlor epoxide	0.9999	4	6	11	0.30	1.0
15	γ-chlordane	0.9998	10	6	14	0.09	0.30
16	endosulfan I	0.9998	11	6	8	0.10	0.33
17	α-chlordane	0.9999	8	6	9	0.12	0.40
18	4,4′-DDE	0.9992	7	6	11	0.11	0.37
19	dieldrin	0.9999	8	6	14	0.11	0.37
20	endrin	0.9997	5	7	9	0.10	0.33
21	endosulfan II	0.9998	10	7	10	0.09	0.30
22	4,4′-DDD	0.9994	7	7	10	0.09	0.30
23	endrin aldehyde	0.9999	11	11	20	0.25	0.83
24	endosulfan sulfate	0.9998	10	7	8	0.13	0.43
25	4,4′-DDT	0.9993	14	6	20	0.30	1.0
26	endrin ketone	0.9999	10	7	11	0.12	0.40
27	tetramethrin	0.9996	11	9	9	0.80	2.7
28	methoxychlor	0.9999	11	6	20	0.70	2.3
29	λ-cyhalothrin	0.9993	7	7	9	0.24	0.80
30	cyphenothrin	0.9995	8	7	12	0.48	1.6
31	permethrin	1.0000	12	6	21	1.5	5.0
32	cyfluthrin	0.9983	3	8	4	1.3	4.3
33	cypermethrin	0.9990	10	8	10	0.60	2.0
34	flucythrinate	0.9993	2	8	3	0.90	3.0
35	fenvalerate	0.9996	14	10	11	1.3	4.4
36	deltamethrin	0.9993	14	5	11	1.5	5.0

(for further details see the Materials and Methods section), alumina led to good and similar results, although the background was slightly lower in the case of dSPE. Interfering chromatographic peaks still appeared in some samples, although most of them did not hamper the detection and quantitation of the analytes.

Thus, when additional removal of the coextracted matrix components was required, eluates were cleaned by dSPE using alumina as the sorbent.

Method Validation: Application to Real Samples. With the aim of verifying that the PSE/GC-μECD developed method was suitable for the quantitative determination of pesticides in cattle feed, method quality parameters were estimated (**Table 5**).

The instrumental linearity was evaluated at a concentration range between 1 and 100 ng mL^{-1} (including six concentration levels) using standard solutions prepared in ethyl acetate. Each concentration level was injected in triplicate, and the response function was found to be linear with correlation coefficients (R) higher than 0.9983.

Precision and limits of detection and quantification were assessed by analyzing spiked cattle feedingstuff samples containing known concentrations of the investigated pesticides. Previous analysis of this sample did not show detectable concentrations of the target analytes. Method precision was studied within a day at two fortification levels (20 and 100 ng g^{-1}) and among days at 20 ng g^{-1}. RSDs for the intraday precision ranged from 2 to 14% with an average value of 7.6%, while the RSDs for the interday precision ranged from 3 to 21% with an average value of 10.8% (Table). The limits of detection (LOD) and limits of quantification (LOQ) of the overall method were calculated as the

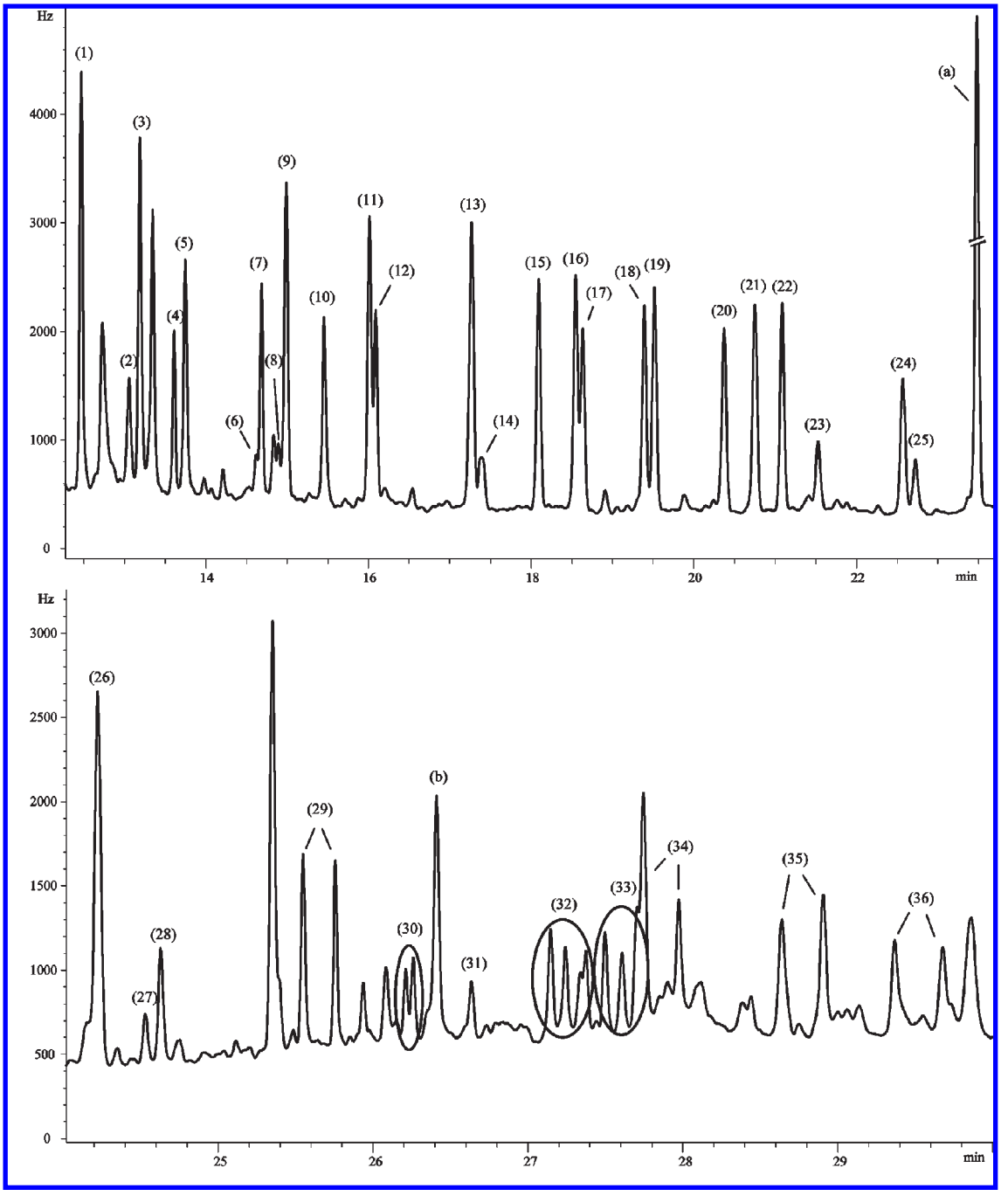

Figure 5. PSE/GC-μECD chromatogram of a spiked cattle feed sample (20 ng g^{-1}). See number code equivalence (1)−(36) in **Table 5**; (a) PCB-166, (b) PCB-195.

concentration giving a signal-to-noise ratio of 3 (S/N = 3) and 10 (S/N = 10), respectively. These limits were estimated using the PSE extract of a feedingstuff spiked at 5 ng g^{-1}. LODs and LOQs values were between the sub-ng g^{-1} and low-ng g^{-1} levels, and both were lower than the MRLs for the target pesticides in cereals and animal feeds set by the European (EU) and American (US) legislations (see **Tables 1** and **5**). It must be also highlighted that these LODs were lower than those achieved in the same matrix by other authors (*7*−*10*, *18*) and very close to those we have obtained using MSPD (*11*).

Table 6. Recovery of Pesticides (%) from Several Cattle Feed Samples

	sample					
	A		B	C	D	E
pesticide	20 ng g^{-1}	100 ng g^{-1}		100 ng g^{-1}		
α-lindane	94	98	93	91	99	90
β-lindane	86	95	85	90	90	85
γ-lindane	80	93	91	84	85	90
tefluthrin	73	87	94	70	77	82
δ-lindane	78	103	79	101	94	88
acetochlor	67	91	109	110	91	114
transfluthrin	74	94	91	85	80	82
alachlor	78	101	110	89	96	98
heptachlor	71	89	88	75	79	76
fenitrotion	74	101	87	92	103	82
aldrin	78	90	101	86	86	84
chlorpyrifos	78	85	84	80	77	76
heptachlor epoxide	84	90	88	89	87	75
allethrin	79	86		74	78	84
γ-chlordane	77	86	83	71	73	76
endosulfan I	60	80	85	72	77	72
α-chlordane	60	74	78	72	73	70
p,p′-DDE	86	92	91	80	81	83
dieldrin	73	83	80	71	72	73
endrin	88	91	85	79	81	81
endosulfan II	86	81	75	72	72	73
p,p′-DDD	74	80	85	81	81	87
endrin aldehyde	25	31	30	25	44	21
endosulfan sulfate	68	72	72	72	78	65
p,p′-DDT	114	101	86	81	88	58
endrin ketone	77	82	83	76	82	66
tetramethrin	79	80	100	80	100	81
methoxychlor	116	113	100	85	96	67
λ-cyhalothrin	90	93	99	90	94	85
cyphenothrin	94	102	107	98	99	100
permethrin	120	107	103	101	100	81
cyfluthrin	95	102	108	103	104	94
cypermethrin	92	95	107	91	99	101
flucythrinate	98	101	110	100	105	116
fenvalerate	119	109	100	107	114	120
deltamethrin	97	106	105	102	109	98

Table 7. Validation of the Method: Analysis of an Animal Feed Certified Reference Material (BCR-115)

pesticide	estimated concentration (ng g^{-1})	recoveries (%)
α-lindane[a]	18.7 ± 1.0	
β-lindane	24.9 ± 1.0	108
γ-lindane	21.3 ± 1.8	98
heptachlor	22.0 ± 3.4	116
aldrin[a]	15.1 ± 4.1	
heptachlor epoxide[a]	22.5 ± 5.4	
γ-chlordane	38.6 ± 5.6	80
endosulfan I	30.3 ± 3.3	66
4,4′-DDE	39.9 ± 4.2	85
dieldrin	11.9 ± 0.8	66
endrin	42.5 ± 7.9	92
p,p′-DDD[a]	43.8 ± 8.6	
p,p′-DDT[a]	35.6 ± 5.4	

[a] Not certified.

Recovery studies were carried out by applying the optimized PSE method to the extraction of five cattle feed samples (A−E) spiked at 100 ng g^{-1} with the target pesticides. Sample A was also fortified at 20 ng g^{-1}. Previous analyses of some of these samples showed the presence of some of the target analytes at low concentration levels (< 10 ng g^{-1}), and these initial concentrations were taken into account to calculate the recoveries. As can be seen in **Table 6**, recoveries were between 70 and 110% for most compounds in all samples. The lower recovery for endrin aldehyde may be attributed to its strong retention on the solid adsorbents. In fact, a similar behavior has also been reported for the extraction of this endrin metabolite from animal feed and vegetables (*9*, *32*). The observed variability (RSD) between the feedingstuff samples can be attributed to experimental error. These results demonstrate that the developed PSE/GC-μECD method allows the quantification of the target compounds in cattle feed samples of different composition.

Method accuracy was also evaluated by analyzing a certified reference material (BCR-115) containing some of the target pesticides. **Table 7** shows the estimated concentrations and the recovery values for the studied organochlorine pesticides. For γ-chlordane, β-lindane, γ-lindane, *p,p′*-DDE, dieldrin, endosulfan I, endrin, and heptachlor, the obtained values were in good agreement with the certified ones. Other target analytes (α-lindane, aldrin, heptachlor epoxide, *p,p′*-DDD, and *p,p′*-DDT) were also found in this sample. Although certified concentrations were not provided for them, values about (or below) the maximum content possible for each of these pesticides in the final material were obtained (*33*). Thus, the suitability of the optimized method for the analysis of pesticides at trace levels in cattle feed samples is demonstrated.

The validated method could then be employed for the analysis of the target pesticides in cattle feed samples collected from 23 dairy farms located in Galicia (NW Spain). No pesticide residues at levels exceeding the MRLs were found in any of the analyzed samples with the exception of one, in which the insecticide chlorpyrifos was detected at a concentration of 79 ng g^{-1} (above the legislated values in Europe for soya bean, maize, and wheat, and in US for maize; see **Table 1**).

The GC-MS analysis of this sample confirmed the presence of chlorpyriphos, and the corresponding ion chromatogram, as well as the full mass spectra, is shown in **Figure 6**. This insecticide was found in the other two samples at lower concentrations (8 ng g^{-1}). Other target analytes were found at low levels: endrin aldehyde (3.1−4 ng g^{-1}), β-lindane (5.6 ng g^{-1}), fenitrotion (1.6−9.0 ng g^{-1}), endosulfan I (1.1−5.3 ng g^{-1}), endosulfan II (1.5−3.9 ng g^{-1}), and endosulfan sulfate (1.7−3.4 ng g^{-1}).

Several samples were analyzed as well by the MSPD method previously developed by the authors (*11*). **Table 8** includes the comparative results obtained for one of these samples in which an organophosphorus pesticide (fenitrotion), two organochlorine pesticides (endosulfan I and II), and one degradation product (endosulfan sulfate) were detected (see chromatogram in **Figure 7**). As can be seen in **Table 8**, equivalent values were obtained using the MSPD and PSE based procedures.

Therefore, pressurized solvent extraction coupled to GC-μECD detection has been successfully applied to the

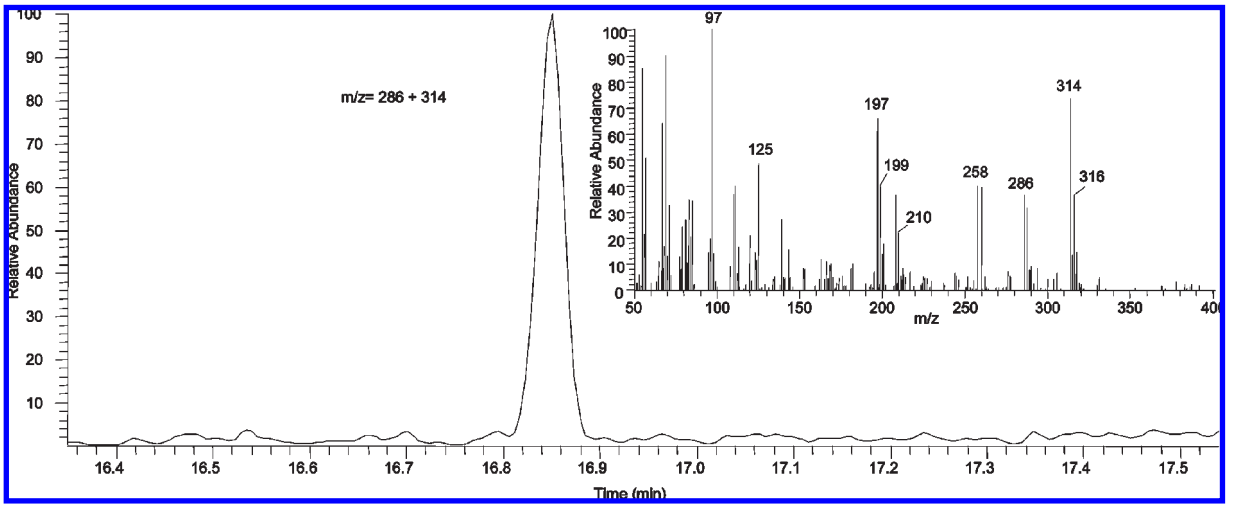

Figure 6. PSE/GC-MS reconstructed ion chromatogram (*m/z* 314 + 286) of a real cattle feed sample contaminated with chlorpyriphos. The obtained mass spectrum is also displayed.

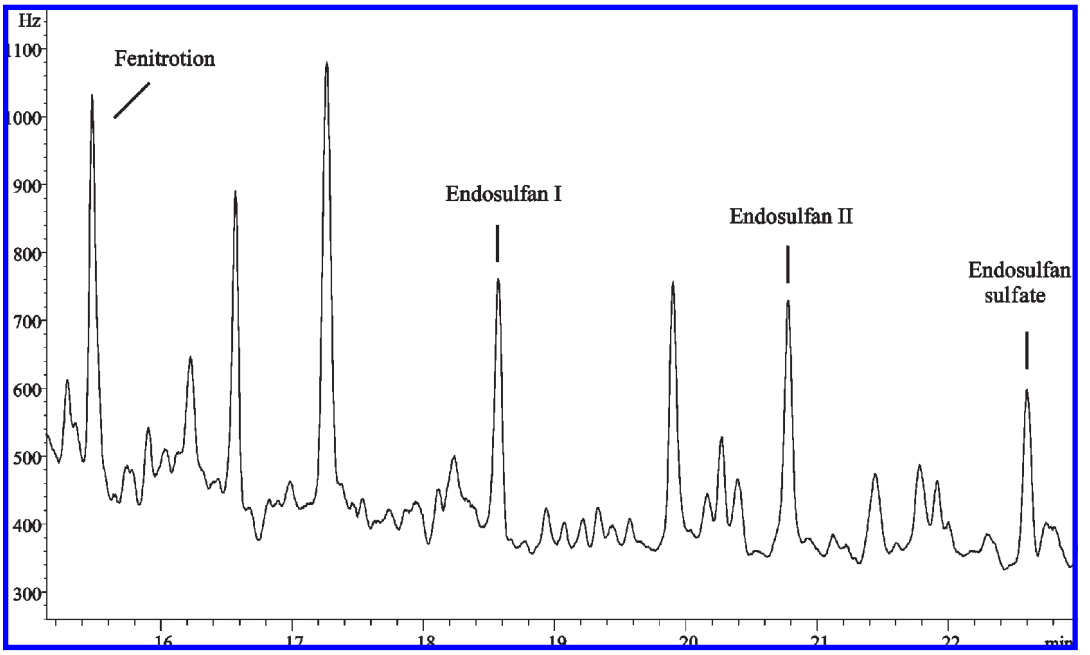

Figure 7. PSE/GC-μECD chromatogram of a cattle feed sample contaminated with several pesticides.

Table 8. Comparative analysis of a real cattle feed sample by PSE and MSPD

	concentration (ng g^{-1})	
pesticide	PSE	MSPD
fenitrotion	8.7 ± 1.5	9.3 ± 2.0
endosulfan I	3.4 ± 0.6	3.7 ± 0.4
endosulfan II	3.9 ± 0.7	3.8 ± 0.1
endosulfan sulfate	3.3 ± 0.2	3.8 ± 0.7

determination of multiclass pesticides in cattle feedingstuffs. With the developed method, the most relevant criteria required for an extraction procedure, such as low solvent consumption, short process times, and possibility of automation, were fulfilled. Integrated cleanup strategies have been employed in order to simplify the sample preparation step as much as possible, and only in the case of extremely complex samples, a postcleanup step based on dSPE using alumina as adsorbent was needed.

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