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Rapid analytical method for the determination of pesticide residues in sunflower seeds based on focused microwave-assisted Soxhlet extraction prior to gas chromatography–tandem mass spectrometry

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Abstract

A rapid analytical method for determination of organochlorine pesticide residues in sunflower seeds based on focused microwave-assisted Soxhlet extraction has been developed. The main factors affecting the extraction efficiency—namely microwave power, irradiation time, volume of extractant and number of cycles—were optimized by a two-level factorial fractional design. After extraction, a liquid–liquid extraction and a clean-up step including the use of Florisil macrocolumns were required prior to injection of the extracts into the chromatograph in order to isolate the pesticide residues from the lipid fraction of the original extract. The MS–MS ion preparation mode was selected due to the high sensitivity and selectivity it provides. Seed samples were used collected near a crop subjected to aerial pesticide application. Residues of hexachlorocyclohexane isomers and endosulfan were found in the seeds although they were not subjected to pesticide application, thus showing the spray-drift contamination. The validation of the proposed approach was carried out by comparison with the ISO 659-1988 reference extraction method obtaining similar, or even better efficiencies by the proposed approach.

Keywords: Focused microwave-assisted Soxhlet extraction; Extraction methods; Plant materials; Factorial design; Pesticides; Organochlorine compounds; Endosulfan; Hexachlorocyclohexanes

1. Introduction

Spray drift contamination is one of the most serious problems derived from aerial application of pesticides to crops [1]. The study of spray drift of endosulfan and other organochlorine pesticides (OCPs) has been carried out after aerial application to cotton growing in Australia showing an off-target deposition 500 m downwind of 2% of the field applied rate [2]; both water and other crops were affected. This problem is aggravated when the affected non-target crops are oil-seed type as the pesticides are absorbed in the lipid fraction, remaining in it after oil extraction and thus passing to the oil consumers. This, added to the large amounts of different pesticides applied to crops make mandatory the control of pesticide residues in crops in general and, more especially, in oil seeds type crops.

Previous extraction steps are required for the determination of pesticide residues in seeds. The extraction of the lipid fraction from seeds is usually

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carried out by classical methods (especially Soxhlet extraction) and there are few references in the literature about new extraction methods—namely, supercritical fluid extraction (SFE), pressurised liquid extraction or microwave-assisted extraction (MAE)—used for this purpose [3–5]. There are some reasons for the scarce development of these methods, namely: seeds are very difficult matrices where some characteristics such as particle size, moisture, etc., are decisive on extraction efficiency. Most lipids (85–95%) are easily extractable by the sole use of an appropriate solvent (hexane or ether) but the rest of the lipid material, which is strongly bound to the matrix, requires exhaustive treatment in order to be isolated. This fact makes the reference extraction methods adopted very tedious and time-consuming. The use of focused microwave-assisted Soxhlet extraction (FMASE) has proved to be a good alternative for the isolation of lipids and fat content from different matrices [6,7]. The key aspect of FMASE is that it maintains the advantages of conventional Soxhlet extraction (namely: sample–fresh solvent contact during the whole extraction step, no filtration required after extraction, easy manipulation, well-known procedures and large experience in the extraction field for more than a century) and circumvents the shortcomings of conventional Soxhlet by accelerating the process and minimizing environmental pollution due to the small amount of solvent release to the atmosphere. However, the focused microwave-assisted Soxhlet extractors used so far did not allow automation of the total extraction process. Recently, a new prototype of focused microwave-assisted Soxhlet extractor has been designed and constructed [8] providing automation of the whole extraction process. This system has been selected in the present research to carry out the extraction of OCPs from sunflower seeds.

Interference from a lipid matrix may cause problems in the analytical signal of the target analytes [9]; so, subsequent steps for isolation of the OCPs from the lipid fraction are needed. Several techniques are in use including separatory-funnel partition between immiscible solvents, size-exclusion chromatography either with macro or minicolumns or with sweep co-distillation. Adsorption column chromatography on a Florisil macrocolumn or Florisil minicolumn, alumina or silica gel have been used for further clean-up steps before determination by gas chromatography (GC) with electron-capture detection (ECD) [10]. The absence of these clean-up steps would involve potential peak overlapping and frequent cleaning of the instrument [11]. The necessity to confirm the results makes mass spectrometry (MS) an indispensable tool in the laboratory. Nevertheless the usefulness of a typical bench top MS for screening pesticides in complex matrices such as oil seeds is making the use of GC combined with tandem mass spectrometry (GC–MS–MS) more popular [12]. Recently, the joint use of a Carbofrit in the glass liner injector and a pre-column have reduced the pretreatment steps of the extract before its introduction into the chromatograph [12].

The present contribution has focused on the development of a rapid analytical method for the determination of OCP residues in sunflower seeds. The proposed method consisted of: (1) an extraction step based on FMASE using a new automated, extractor; (2) the isolation of the OCPs from the lipid fraction by a two-step liquid–liquid extraction procedure; (3) a clean-up step using Florisil macrocolumns and a (4) separation–identification–quantification step using GC–MS–MS equipped with a precolumn and a plug of Carbofrit in the glass liner injector.

2. Experimental

2.1. Chemicals and materials

A mixture of OCPs, the US Environmental Protection Agency (EPA) 608 pesticide mixture (Table 1), was obtained from Supelco (Bellefonte, PA, USA) and used for preparing the standard solutions in pesticide residue-grade cyclohexane (Panreac, Barcelona, Spain). This mixture was selected as it contains a wide number of OCPs, including, hexachlorocyclohexane (HCH) isomers and endosulfan isomers, which are the analytes most likely to be found in the samples under study.

All the solvents used [dichloromethane, acetonitrile, *n*-hexane, cyclohexane, diethyl ether and light petroleum (b.p.=40–55 °C)] were of pesticide residue-grade and were obtained from Panreac; 20-ml

Table 1
Analytes present in the EPA 608 pesticide mixture

Aldrin	4,4′-DDD	**Endosulfan sulfate**
α-**HCH**	4,4′-DDE	Endrin
β-**HCH**	4,4′-DDT	Endrin aldehyde
γ-**HCH** (**lindane**)	Dieldrin	Heptachlor
δ-**HCH**	**Endosulfan I**	Heptachlor epoxide (isomer B)
	Endosulfan II	

Residues found in the seed samples are shown in bold.

columns packed with 5 g of LC-Florisil (Supelco, Bellefonte, PA, USA) were used in the clean-up step.

2.2. Sample collection

Sunflower seeds were collected from a sunflower crop located 300 m from a corn crop subjected to aerial applications of OCPs. They were collected at the end of July according to the normal collection time of sunflower seeds at this latitude. The sample collection was carried out following the 79/700/CEE directive for preservation of seeds until extraction [13]. After collection of the sunflowers, the seeds were extracted from the sunflower crown and crushed and sieved to different sizes (0.2, 0.8, 2.0 mm) in order to study the optimum particle size for the extraction process. All samples were stored at 4 °C in the dark until extraction.

2.3. Instruments and apparatus

A focused microwave-assisted Soxhlet extractor (MIC V, SEV, Puebla, Mexico) was used to perform the extraction step (Fig. 1). Some improvements with respect to that previously developed by the authors enable total automation of the extraction process. The system consists of a single unit where the glassware pathway enables reception of the solvent vapour from the distillation flask on a refrigerant connected to the top of the sample-cartridge vessel, subsequently condensing and dropping onto the solid sample. Two extraction units allow the simultaneous processing of two samples with good reproducibility [8]. It also includes an optical sensor that is positioned at the desired siphon height so that the magnetron starts irradiation of the sample when the solvent reaches a preset level. This sensor can be placed along the siphon, which has a length of 18 cm, in order to control the volume of extractant in contact with the sample at the irradiation moment. A solenoid valve is placed in the bottom of the siphon for unloading the extract from the vessel to the distillation flask after the irradiation step. A microprocessor programmer controls the parameters involved in the microwave-assisted extraction, namely: microwave power, irradiation time, number of extraction cycles and volume of extractant, as well as the functioning of the optical sensor and the solenoid valve.

A 50-ml Soxhlet extractor (Probus, Barcelona, Spain) was used to carry out the conventional oil extraction procedure ISO 659-1988 [14] and a rotary evaporator (R-200, Büchi, Switzerland) was used to release the solvent after each conventional Soxhlet extraction.

The extracts were analysed using a Varian CP 3800 gas chromatograph coupled to a Saturn 2200 ion trap mass spectrometer (Varian, Sugar Land, TX, USA). Separations were conducted on a Varian CP-SIL 8 CB-MS WCOT fused-silica capillary column, 30 m×0.25 mm I.D., 0.25 μm. For preservation of the column, an uncoated and deactivated fused-silica pre-column, 2.5 m×0.25 mm I.D., 0.25 μm was used.

All the injections were carried out using a Varian 8200CX autosampler and a plug of Carbofrit (Restek, Bellefonte, PA, USA) was placed into the glass liner injector reducing the amount of sample matrix entering the column and detector.

2.4. Reference extraction procedure (ISO 659-1988)

Ten grams of seeds were weighed to the nearest 1 mg and placed in a cellulose extraction cartridge. The cartridge was plugged with cotton wool and

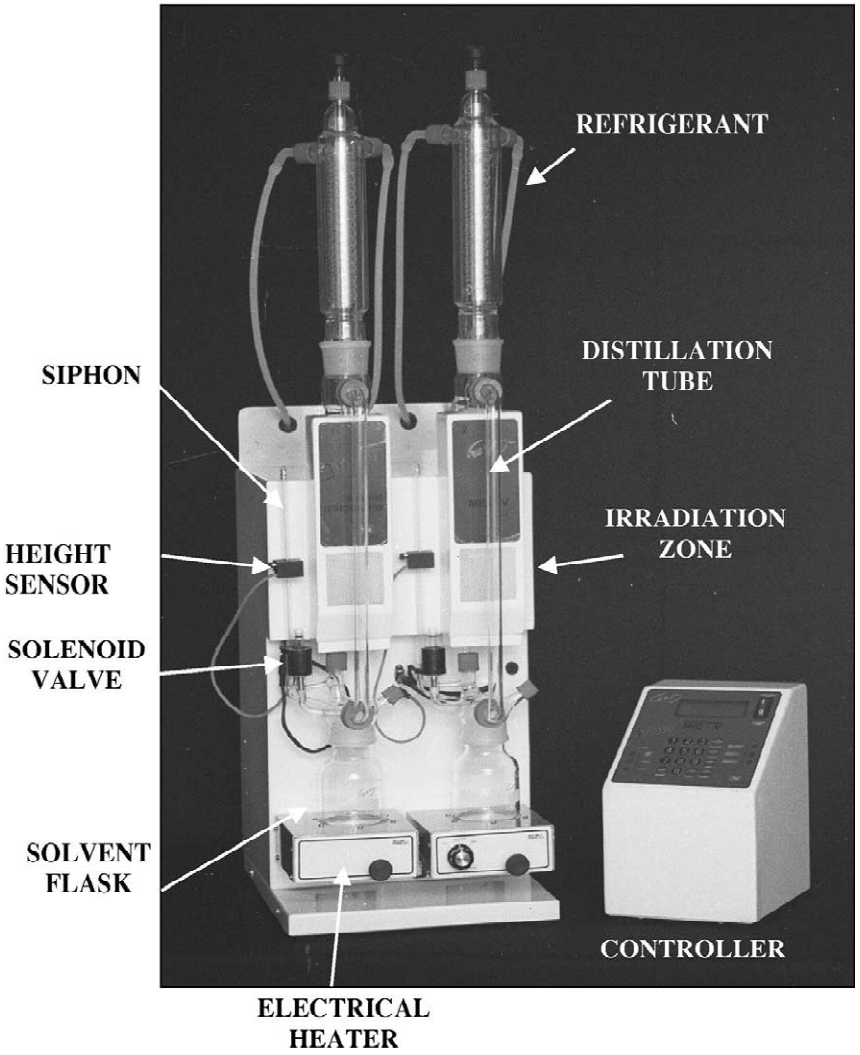

Fig. 1. Photograph of the automated focused microwave-assisted Soxhlet extractor used.

placed in a 50-ml Soxhlet extractor; 100 ml of dichloromethane was added to the distillation flask and 2–3 boiling glass regulators were added. After extraction for 4 h, the cartridge was allowed to cool and unloaded in a mortar. An amount of 10 g of sand previously washed with hydrochloric acid and calcined was then added, and the mixture was ground as finely as possible. The mixture was placed back into the cartridge and then into the Soxhlet chamber (that was fitted in a new pre-tared distillation flask) for back-extraction for 2 h (this step was repeated until the amount weighed was less than 2 mg). Separation of the OCPs from the lipid fraction and a clean-up step were required after each extraction.

2.5. Focused microwave-assisted Soxhlet extraction procedure

Five grams of seeds were weighed into a cellulose extraction cartridge and put into the vessel located in

the zone of microwave irradiation. Then 160 ml of dichloromethane was poured into the distillation flask. The isomantle rheostat was set at 100% in order to reach a continuous flow of extractant from the distillation flask to the sample cartridge vessel. The extraction program consisted of a number of cycles that depend on the extraction kinetics of the target analytes. Each cycle involved three steps:

1. Filling the sample cartridge by the extractant evaporated from the distillation flask, condensed in the refrigerant and dropped onto the sample. The position of the optical sensor determined the volume of the extractant put into contact with the sample.
2. Microwave irradiation of the sample for 90 s at 300 W after the solvent reached the optical sensor position.
3. Unloading of the extractant from the vessel to the distillation flask by automatic switching of the solenoid valve.

After the last cycle, the optical sensor was disconnected and the remaining solid was removed from the extraction vessel. Then, only step (1) was carried out again in order to reduce the volume of the extract contained in the distillation flask and to recycle the extractant. After complete drying of the extract with an N_2 stream, the OCPs were isolated from the lipid fraction as discussed in Section 2.6.

It is worth emphasising that the ISO method pays special attention to moisture. FMASE does not need previous sample drying due to its ability to remove moisture during the initial cycles.

2.6. Isolation of the OCPs from the lipid fraction and clean-up procedures

Two millilitres of *n*-hexane, 1 mg of Na_2SO_4 and 10 ml of acetonitrile saturated with *n*-hexane (90:10, v/v) were added to the oil extract and the mixture was shaken for 30 min. After this, the acetonitrile phase was removed (after complete phase separation) and the liquid–liquid extraction step was repeated with another 10-ml portion of acetonitrile saturated with *n*-hexane. Both extracts were mixed and the acetonitrile was released by a rotary evaporator at 55 °C and the residue obtained was passed through a Florisil column for clean-up. Previously, the column was conditioned by passing 20 ml of light petroleum. The analytes were eluted with 40 ml of a diethyl ether–light petroleum (6:94, v/v) mixture. The eluate was dried by a rotary evaporator at 55 °C. Finally, the extract was recomposed to 1 ml with pesticide residue-grade cyclohexane and stored in the dark in a sealed vial at −4 °C until analysis. The flow-rate of each clean-up step was strictly controlled in order to obtain good reproducibility of the results.

2.7. GC–MS–MS procedure

The analysis of the extracts was carried out using helium as a carrier gas at a constant flow-rate of 1 ml/min. The column temperature program was 70 °C, held for 3.5 min, then increased at 25 °C/min to 180 °C, held for 10 min and, finally, increased at a rate of 10 °C/min to 300 °C. The temperature program of the injection (10-μl injection volume) started at 70 °C, held for 0.5 min and then increased at 100 °C/min to 300 °C, then kept there for 15 min. The injection was in split–splitless mode. The splitter was opened (50:1) for 0.5 min, closed for 3 min and then opened at 100:1 split ratio for 2 min.

For the MS detector, automatic gain control (AGC) was used to optimize the sensitivity by completely filling the trap with the target ions. The value was optimized to 5000 for the electron impact ionization (EI) mode. The parent ion mass selected was of the highest intensity. A non-resonant wave form (collision induced dissociation, CID) was selected for all compounds. The instrumental parameters were set at the following values: a filament emission current of 80 μA, an electron multiplier voltage offset of +200 V, and a modulation amplitude of 4.0 V, using perfluorotributylamine (FC-43) as a reference gas. For analysis, the ion preparation was with MS–MS and the conditions established for the different analytes are shown in Table 2.

The pesticides detected in the samples are shown in bold in Table 1. As can be seen, all the analytes present in the sunflower seeds were also contained in the EPA 608 mixture used for preparing the standards, thus making possible quantification of all pesticides in the samples. It can also be concluded from the MS data that no further pesticides were present in the sample.

Table 2
Instrumental variables for MS–MS detection

Compound	$t_{R\ (min)}$	ESL[a] (m/z)	EA[b] (V)	Parent ion mass	Quantification ion
α-HCH	12.762±0.12[c]	100	75	219	183
β-HCH	13.479±0.10	100	75	219	183
γ-HCH (lindane)	14.185±0.21	100	85	219	183
δ-HCH	15.263±0.17	100	70	219	183
Heptachlor	18.351±0.11	75	60	272	231
Aldrin	20.731±0.11	90	100	263	191
Heptachlor epoxide (isomer B)	23.308±0.16	75	54	353	263
Endosulfan I	25.171±0.15	125	51	339	267
4,4′-DDE	26.607±0.19	100	94	318	246
Dieldrin	27.005±0.23	100	93	277	204
Endrin	27.873±0.18	75	59	281	243
Endosulfan II	28.509±0.17	125	57	339	267
4,4′-DDD	28.937±0.18	80	69	235	165
Endrin aldehyde	29.324±0.15	75	62	281	243
4,4′-DDT	30.566±0.11	71	44	387	289
Endosulfan sulfate	30.772±0.10	75	76	235	165

[a] ESL, excitation storage level.
[b] EA, excitation amplitude.
[c] Standard deviation ($n=5$).

3. Results and discussion

3.1. Study of the particle size

Different particle sizes (namely: 0.2, 0.8 and 2.0 mm) were studied. This variable is of paramount important in the extraction procedure due to: (1) the heterogeneity of the crushed raw samples (a mixture of seed and shell is obtained in the crushing process with different proportions of seed and shell depending on the particle size—richer in seed as the size decreases) and (2) the interaction between the OCPs and the lipid fraction of the seeds, that is located in the seed fraction. For these reasons, better results are foreseen for lower particle size because of the highest content in lipids of the seed compared with the shell. However, sample compactness was observed when the smallest particle size (0.2 mm) was used. Better results were obtained for medium particle size (0.8 mm) compared with the results from the other sizes, so it was selected as the optimum size.

3.2. Optimization of focused microwave-assisted Soxhlet extraction

The variables requiring to be optimized in FMASE were irradiation power, irradiation time, extractant volume in the sample chamber at the moment of starting irradiation and number of cycles needed for total extraction of the target compounds. A half-fractioned 2^{4-1} type IV resolution design involving eight randomized runs plus three centered points was developed as a first screening study of the behaviour of the main variables affecting the extraction efficiency, using the Statgraphics Plus for Windows software package (v.2.1, 1992; Statgraphics, Rockville, MD, USA). The power of irradiation was tested between 40 and 100% of the power provided by the microwave device. The irradiation time was studied in the range 30–90 s, the number of cycles between 1 and 5 and the extractant volume in the sample chamber between 25 and 35 ml. The upper and lower values were selected from the available data and experience gathered in previous research with the focused microwave-assisted Soxhlet extractor. Portions of 5 g of sunflower seeds crushed and sieved at 0.8 mm were used through the optimization.

The conclusion of this first design was that the irradiation time was the main significant variable for the overall compounds. This variable was fixed at 90 s as this is the highest value allowed by the device. The power of irradiation showed a positive effect over the response and it was also fixed at the

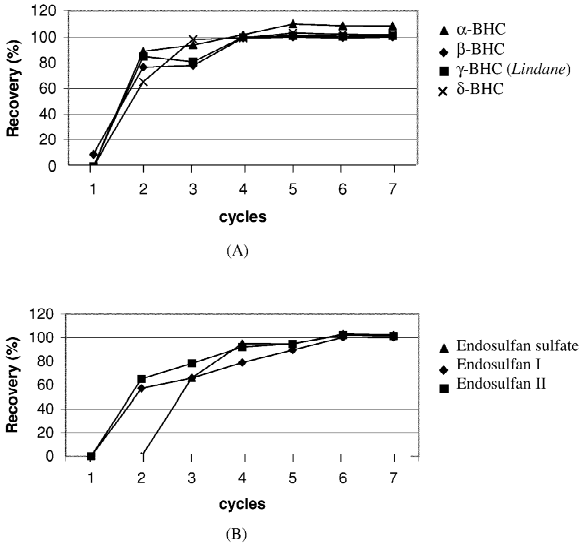

Fig. 2. Kinetics of the focused microwave-assisted extraction. (A) HCH isomers, (B) endosulfan isomers.

highest value tested: 100% of the maximum power provided by the device. The extractant volume in the sample chamber was not significant so the lowest value tested, 25 ml, was selected for further experiments in order to make the extraction time as short as possible. Finally, the number of cycles was a significant variable for some of the target analytes. For this reason, and in order to optimize the time needed for quantitative extraction of all the analytes, a study of the extraction kinetics was carried out.

3.3. Study of the extraction kinetics

As can be seen in Fig. 2(A,B), the extraction kinetics of the target analytes is very different: α-HCH, β-HCH, γ-HCH (lindane) and δ-HCH were extracted after four cycles but more retained compounds such as endosulfan I, endosulfan II and endosulfan sulfate needed six cycles for quantitative removal. More than six cycles did not provide higher extraction efficiencies for any of the analytes so six cycles were selected as the optimum value for the FMASE procedure.

3.4. Method validation

To confirm that the method is suitable for its intended use, a validation process was carried out by establishing the basic analytical requirements of the performance to be appropriate for quantitative determination of OCPs in sunflower seeds. Precision, linear dynamic range and both instrumental and method detection limits were evaluated for the analytical approach developed.

3.4.1. Precision

In order to evaluate not only the extraction efficiency of the proposed method but also the precision, seven extractions were carried out on seven consecutive days. The precision for the different analytes, expressed as relative standard deviation, is shown in Table 3. It ranged between 4.4% for endosulfan I, and 11.35% for β-HCH.

3.4.2. Linear dynamic ranges and instrumental limits of detection

The calibration curves were run using standard solutions of the EPA 608 pesticide mixture in chromatographic grade cyclohexane. The concentrations of the standards fitted within the linear portion of the calibration curve were between 40 and 300 ng/ml. The calibration graph for each analyte as well as the correlation coefficients are shown in

Table 3
Precision (RSD), limits of detection (LODs) and method detection limits (MDLs) for the residues of OCPs encountered

Compound	RSD (%)	LOD (ng)	MDL (ng/g)	Calibration graph	r^2
α-HCH	8.01	0.0234	0.468	$y=4842.2x-849$	0.9812
β-HCH	11.35	0.0401	0.802	$y=5735.7x-3950.1$	0.9867
γ-HCH (lindane)	6.40	0.1082	2.164	$y=1710.1x-121.1$	0.9877
δ-HCH	10.81	0.0469	0.938	$y=3597.4x-741.2$	0.9943
Endosulfan I	4.40	0.1862	3.724	$y=809.6x-671.6$	0.9879
Endosulfan II	10.23	0.8507	17.014	$y=530.5x-579.7$	0.9875
Endosulfan sulfate	6.23	0.2245	4.491	$y=312.3x-214.9$	0.9978

Table 3. The instrumental limit of detection (LOD), expressed in nanograms entering on-column and reaching the detector, which gives a signal that is 3σ above the mean blank signal (where σ is the standard deviation of the blank signal), ranged between 0.0234 ng for α-HCH and 0.8507 ng for endosulfan II (Table 3).

3.4.3. Method detection limit

The method detection limit (MDL) was estimated from the statistical information derived from the standard calibration curves used to determine the instrumental LODs [15]. The MDL was estimated from the LOD multiplied by the final volume and divided by sample mass and injected volume [MDL=(LOD×final volume)/(sample mass× injected volume)] [16]. Table 3 shows the MDL values obtained for each compound.

3.5. Comparison of the proposed method with the reference ISO method

The proposed FMASE method and the reference ISO extraction were applied to sunflower seeds in order to evaluate if both methods provide similar results. Table 4 shows the analytes extracted using both methods as well as the factor *f* defined as the amount of analyte extracted by FMASE/amount of analyte extracted by the ISO method. As can be seen, the efficiency provided by FMASE is similar or even better than that obtained by the reference method, thus showing the suitability of microwaves to break the analyte–matrix bonds of strongly retained analytes and also the lack of a reference method for providing quantitative extractions of these analytes.

Table 4
Comparison of the extraction efficiencies (ng) obtained by FMASE and the ISO 659-1988 extraction procedure

Compound	FMASE	ISO 659	*f* [a]
α-HCH	1.39	1.02	1.36
β-HCH	2.41	2.13	1.13
γ-HCH (lindane)	3.51	3.48	1.01
δ-HCH	1.65	1.54	1.07
Endosulfan I	2.53	2.52	1.00
Endosulfan II	2.01	1.95	1.03
Endosulfan sulfate	2.65	2.50	1.06

[a] *f*, amount extracted by FMASE/amount extracted by the ISO extraction procedure.

4. Conclusions

A rapid and fully automated method has been proposed for the extraction of OCP residues in sunflower seeds prior to GC–MS–MS determination. The use of FMASE provides the following advantages:

(a) Shortening of the extraction time: 45 min (six cycles) versus at least 7 h for the reference extraction procedure. In addition, the use of FMASE does not require manipulation of the sample prior to or during the extraction procedure.

(b) Higher recoveries than those provided by the reference method are obtained showing the efficacy of microwaves for the extraction of strongly retained compounds.

(c) Use of samples as received without the moisture adjustment usually required in conventional Soxhlet-based methods.

Additional advantages derived from the use of Carbofrit in the GC injector combined with the MS–MS detection system are: (a) the high selectivity achieved; thus avoiding interference problems, (b) high sensitivity, allowing detection limits of these pesticide residues in sunflower seeds at low ng/ml levels.

Another conclusion from this research is related to the fact that residues of OCPs have been found in sunflower seeds although this crop was not treated with these pesticides. This fact is due to the proximity of the sunflower crop to a corn crop which was subjected to aerial application of these OCPs, thus demonstrating the problem of spray-drift contamination and the risk to human health as the residues pass directly to the oil and, then, to the oil consumers.

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