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Rapid multiresidue extraction method of organochlorinated pesticides from fish feed☆

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Abstract

A rapid multiresidue extraction method for organochlorinated pesticides from fish feed was developed, which is based on the extracted fat treatment by *n*-hexane, concentrated sulphuric acid and ENVI-carb, a graphitized non-porous carbon material. The final residue, obtained in about 50 min, was dissolved in isooctane and analysed by gas chromatography with an electron capture detector (GC/ECD). The presence of the extracted pesticides was confirmed by gas chromatography–mass spectrometry (GC/MS). Concentration of sulphuric acid and amount of ENVI-carb were optimized in order to improve analytes recovery, accuracy and detection limits. This simple and relatively fast method allowed a high recovery of the HCB, Lindane, HEPO, *p,p′*-DDE, *p,p′*-DDD, *p,p′*-DDT residues, with mean values in the range 68–124% at four fortification levels (12.5, 25.0, 50.0, 100.0 ng/g), and coefficients of variation between 1.9 and 20.2%. Detection limit were equal to 3.0 ng/g, related to fat, for all pesticides, and calibration curves were linear ($r > 0.999$) in the range of explored concentrations from the detection limit to 100 ng/g. For all pesticides a good repeatability was obtained (CV% values in the range 0.23–4.16%) when a sequence of six injections of the isooctane extraction solution was performed. The usefulness of the proposed method has been tested by the analysis of fish feed samples.

Keywords: Multiresidue extraction; Fish feed; Cleanup methods; Organochlorine compounds; Pesticides

1. Introduction

The increasing interest for the sea culture farms has boosted remarkably modern techniques of intensive breeding, with a particular focus on the rationalization of feeding systems. Nevertheless, the use of highly nutritious feed requires accurate sanitary controls of public health. In fact, in such matrixes it is frequent to find highly toxic chemical products (heavy metals, PCBs, dioxins and pesticides) or substances not permitted, added for a preservative purpose or as growth promoters.

A particular attention should be addressed to organochlorinated pesticides that, in some cases, are still used in the worldwide or they are present as persistent residues of previous uses. In fact, fish feed, contaminated by such substances, represent a potential way of direct introduction into fishes, where they accumulate in various organs especially in adipose tissue [1], and then into human beings. Therefore, to prevent the human health risk, a monitoring of these pesticides in fish feed, also to identify their origin, is absolutely required.

Note that fish feed are very complex matrixes for the presence, among the other components, of animal origin products too. Consequently, in the analysis of such matrixes a difficult task is represented by the sample cleanup that should be efficient enough, in terms of analyte recovery, elimination of interferences and rapidity, to allow a reliable screening of contaminated samples.

Actually, a number of methods are currently used to extract organochlorinated pesticides from fatty samples. The most commons involve adsorption chromatography on

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Florisil, [2,3] Alumina, [4,5] and Silica gel [6,7]. These procedures are expensive, owing to the relatively high cost of adsorbents, and have a low potential for automation. Alternatively, gel permeation chromatography (GPC) [8,9] is used, which offers a high degree of automation but it is a relatively slow sequential sample cleanup, with a large volume solvent use. Moreover, this technique is time consuming and the relevant analyses of the extracts by gas chromatography with electron capture detector (GC/ECD) reveal many interfering peaks [10]. Furthermore, previous papers, report either direct treatment with concentrated sulphuric acid [11] or cleanup on Extrelut column, soaked with sulphuric acid [12], before the analysis by GC/ECD. Syhre et al. [10] developed a different cleanup approach for the monitoring of chlorinated compounds in animal feed, based on a chromatographic step with ENVI-carb column.

On the best of our knowledge, there are not papers in literature reporting the extraction and analysis of organochlorinated pesticides in fish feed.

In the present paper, a rapid multiresidue extraction method of organochlorinated pesticides in fish feed has been developed, which involves only the fat extraction from feed samples by Soxhlet with petroleum ether, and the following cleanup, with a simple procedure based on the use of *n*-hexane, concentrated sulphuric acid and ENVI-carb, a graphitized non-porous carbon material.

The cleanup has been optimized in terms of ENVI-carb amount and concentration of sulphuric acid, in order to improve recovery of analytes, accuracy and detection limits.

The proposed method has been employed to detect the presence of organochlorinated pesticides in fish feed samples collected from local fish farms.

2. Experimental

2.1. Reagent and materials

All solvents were ultra-Resi-Analyzed grade from Merck (Darmstadt, Germany). Organochlorinated pesticides (Lindane, HEPO, HCB, *p,p′*-DDT, *p,p′*-DDE, *p,p′*-DDD) (Supelco Inc., Bellefonte, PA, USA) (reference standards) were from the collection in our Laboratory. All of them were neat compounds (purity >96%). Standard solutions of pesticides were prepared in isooctane and stored in freezer at −20 °C. The stock standard solution of each pesticide was of 2 mg/l. Intermediate standard solutions were prepared by dilution in isooctane to give working concentrations of 3.0–6.0–12.5–25.0–50.0–100.0 ng/ml.

ENVI-carb (120/140 mesh, 100 m^2/g) was purchased from Supelco Inc. (Bellefonte, PA, USA). To remove all impurities, it was plentifully washed with different solvents, in the following order: *n*-hexane, cyclohexane and toluene, and finally again with *n*-hexane.

All glassware were treated at first with sulphochromic mixture (Carlo Erba-Milano) and, then, washed with different solvents in the order of HPLC grade water, acetone and *n*-hexane.

2.2. GC/ECD and gas chromatography–mass spectrometry (GC/MS) conditions

2.2.1. GC/ECD

A Perkin-Elmer (Monza, Italy) Gas chromatograph Mod. 8500 with electron capture ^{63}Ni detector, equipped with Perkin-Elmer Software TC4, was used for GC/ECD determination. Chromatographic separations were performed using an Alltech AT-5 (Deerfield, IL, USA) fused-silica capillary column (30 m × 0.25 mm i.d.) with 5% diphenyl-95% dimethylsiloxane liquid phase, (0.25 μm film thickness) with a relative deactivated retention gap.

The oven temperature has been programmed as follows: 80 °C for 2 min, ramped at 10 °C/min to 160 °C and then ramped at 5 °C/min to 260 °C. The final isotherm has been of 260 °C for 20 min, with a total run of 50 min. The carrier gas was helium, with a flow of 0.8 ml/min (160 °C) and a column head pressure of 1.02 atm.

A split/splitless injector with 2 mm i.d. glass-liner has been used in the splitless mode for 1 min. Injector temperature was 240 °C and split flow was of 24 ml/min until the end of analysis; the injection volume was of 1 μl. The electron capture detector (ECD) temperature was of 300 °C and nitrogen was the auxiliary gas with a flow of 55 ml/min.

2.2.2. GC/MS

A Perkin-Elmer (Monza, Italy) Gas chromatograph Mod. AutoSystem XL with Turbomass Gold Mass Spectrometer, equipped with Perkin-Elmer Software Turbomass Ver. 4.4.0, was used for GC/MS confirmation.

Chromatographic separations were performed using a PE-5 MS (Monza, Italy) fused-silica capillary column (30 m × 0.25 mm i.d.) with 5% diphenyl–95% dimethylsiloxane liquid phase, (0.25 μm film thickness). The oven temperature has been programmed as follows: 80 °C for 2 min, ramped at 10 °C/min to 160 °C and then ramped at 5 °C/min to 260 °C. The final isotherm was of 260 °C for 20 min, with a total run of 50 min. The carrier gas was helium with a constant flow of 1 ml/min. A PSS injector with 1 mm i.d. quartz liner was used in the following splitless mode: time 1 = −0.50 value 0; time 2 = 1.50 value 100; time 3 = 1.80 value 10. The injector temperature was 250 °C, and the injection volume was of 1 μl. GC/MS detection functions are summarized in Table 1.

2.3. Method

2.3.1. Sample preparation

The fish feed samples were ground in a mill (Foss Tecator Cemotec Mod. 1090, Hoganas, Sweden) and kept dry until the fat extraction. The fat was extracted from fish feed by Soxhlet (Soxtec System: Foss Tecator Mod. 1046 Servit Unit/Mod. System HT2 1045, Hoganas, Sweden) under the

Table 1
GC/MS detection functions

Solvent delay (min)	5
MS pressure (Torr)	7.69e−6
Ionization mode	Electron impact (EI+)
Function 1	
Scan duration (s)	0.10
Interscan delay (s)	0.020
Retention window (min)	Always on
Function type	Scan
Mass range	45–450
Function 2	
Inter channel delay (s)	0.001
Retention window (min)	0.000–50.000
Function type	SIR
Chan mass	282.00–284.00–286.00–181.00 183.00–246.00–248.00–235.00–237.00
Dwell (s)	0.05

following conditions: the thimble was loaded with about 10 g (W_1) of the mixed sample and covered with a thin layer of cotton, previously scoured by petroleum ether, then it was inserted into the Soxtec HT; the extracted fat collection cup was dried and pre-weighed (W_2), 80 ml of petroleum ether were added into the cup and then it was inserted into the Soxtec HT. The time required for this extraction was 1 h at 103 °C. After solvent evaporation, the cup was released, dried at 100 °C for 3 h, cooled in a glass dryer for 30 min, and then weighed (W_3). Percentage of fat was calculated according to the formula: $\text{fat}\,(\%) = (W_3 - W_2)/W_1 \times 100$. The fat residue was transferred to a glass vial and preserved in freezer at −20 °C until the cleanup.

2.3.2. Cleanup

The extracted fat was purified in less than 50 min by a simple procedure based on the use of *n*-hexane, concentrated sulphuric acid and ENVI-carb, a graphitized non-porous carbon material.

Cleanup procedure was carried out as follows: 2 ml of *n*-hexane were added to about 0.5 g of fat in a Pyrex tube, and mixed by vortex for 1 min; 0.1 g of ENVI-carb, previously washed with different solvents (in the order *n*-hexane, cyclohexane, toluene and again *n*-hexane), were added and mixed by vortex for 1 min; 2 ml of concentrated sulphuric acid (90%) were pipetted slowly into the Pyrex tube, avoiding to touch the inner walls. The Pyrex tube was cooled and then the solution was mixed by vortex for 1 min. The mixture was centrifuged for 30 min at 5000 rpm (Tehtnica-Centric 322A-Mod. TEH 464000, Zelezniki, Slovenia), and the upper clear organic phase solution was pipetted by a Gilson Microman Pipette (Gilson-Mod. M250 S/N-Villiers-le-Bel, France) and the volume was accurately measured.

The solution was carefully evaporated by Bain Marie at 45 °C under a nitrogen flow, the residue was dissolved in 1 ml of isooctane, and the obtained solution was analysed by GC/ECD and GC/MS.

3. Results and discussion

3.1. Cleanup optimization

Accurate analyses of fish feed samples, which are contaminated by organochlorinated pesticides, require an efficient method of extraction and cleanup.

Among all the reagents, typically employed on the pesticides extraction cleanup from fat samples, the potential of combined use of ENVI-carb, a graphitized non-porous material with adsorbent properties and of the concentrated sulphuric acid, have been studied.

In a preliminary screening the cleanup efficiency was evaluated by the treatment of pesticide-free fish feed fat samples (in the following referred to blank samples), and the relevant GC/ECD analysis was performed only when the extract solution was colourless and clear.

The choice of an appropriate ENVI-carb amount was essential to obtain a good cleanup in terms of interferences elimination. Five different amounts of a such graphitized non-porous material, in the range 0.05–0.4 g, were used in the cleanup procedure of blank samples with a 90% concentrated sulphuric acid solution; for each amount of ENVI-carb the experiment was performed twice. The best results were obtained using 0.1 g of ENVI-carb, in fact higher amounts gave pink coloured solutions that clearly indicated an inefficient elimination of interferences. Moreover, by using 0.05 g of ENVI-carb, a faint coloured solution was obtained, which, as expected, showed a number of negative and positive interfering peaks in the GC analysis and a high background noise.

In the extraction cleanup of pesticides from fat samples, the concentration of sulphuric acid plays an important role in the fat digestion and degradation of organic substances.

By using the optimized amount of ENVI-carb, the effect of sulphuric acid concentration in the range 70–95% has been studied. Best results in terms of fat digestion efficiency, colourless solutions, background noise and absence of negative or positive interfering peaks have been obtained at concentrations of 90 and 95%.

In fact, using such two concentrations, the results obtained by the analysis of spiked blank samples at 100 ng/g fortification level of a standard solution have shown that for *p*,*p*′-DDT and its congeners the mean peak areas are not significantly different. On the contrary, HEPO, Lindane and HCB have shown higher values when a concentration of 90% has been employed. Under these latter cleanup conditions, lower noise and higher signal to noise ratios have been obtained.

These results can be explained considering a stronger action of the sulphuric acid at 95%, which probably determines a partial degradation either of the less resistant pesticides or of ENVI-carb, with a consequent release of interfering substances instead of their elimination.

In the light of these results, all the following experiments have been carried out using 0.1 g of ENVI-carb and sulphuric acid at 90%.

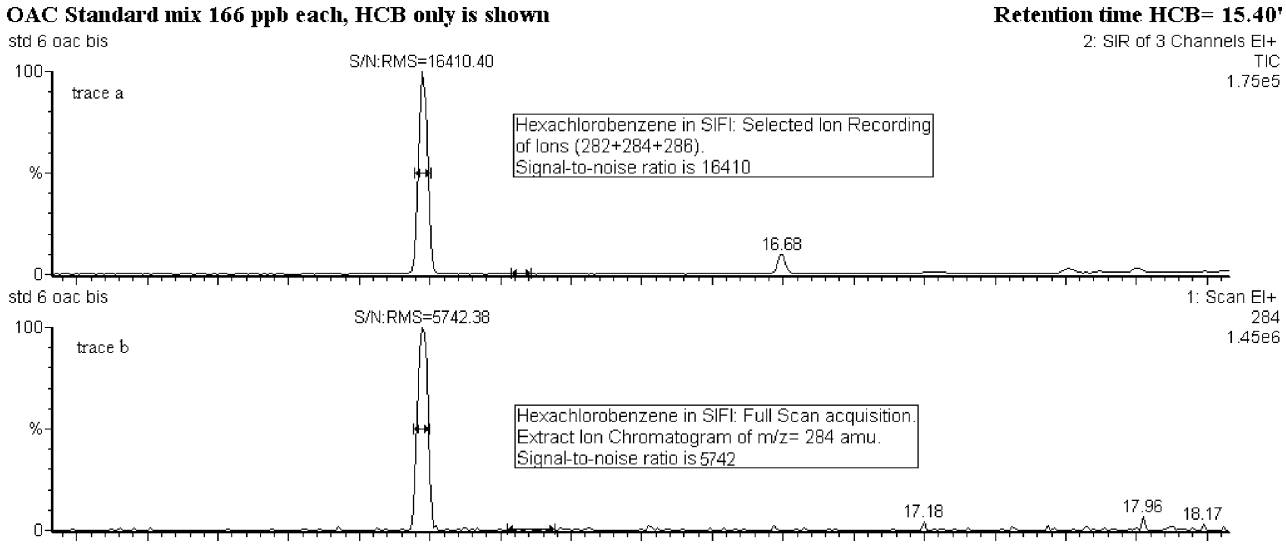

Fig. 1. GC/MS analysis in SIFI of HCB at a 166 ng/g concentration.

The high efficiency of the cleanup of the method was also assessed by the analysis of standard solutions and spiked blank samples in GC/MS, which is the most useful technique in the confirmation of the chromatographic peaks assignment.

A 166 ng/g standard solution of organochlorinated pesticides has been analysed by GC/MS in selected ion and full ion (SIFI) mode acquisition, and in Fig. 1 are shown the results for HCB. In particular, "trace a" shows the selected ion recording (SIR) acquisition of the three characteristic ion ($282 + 284 + 286$) for HCB, while "trace b" shows the extract ion chromatogram ($m/z = 284$) from the full scan acquisition.

Although SIR mode increases efficiency and sensitivity by a selective scan of individual m/z ratio, for the best identification of each compound the full scan mode has been used to allow the acquisition of library-searchable spectra. The full mass spectra of each pesticide present in the extract of the spiked blank sample fitted well with those obtained from the standard solution. In Fig. 2 is reported an example of

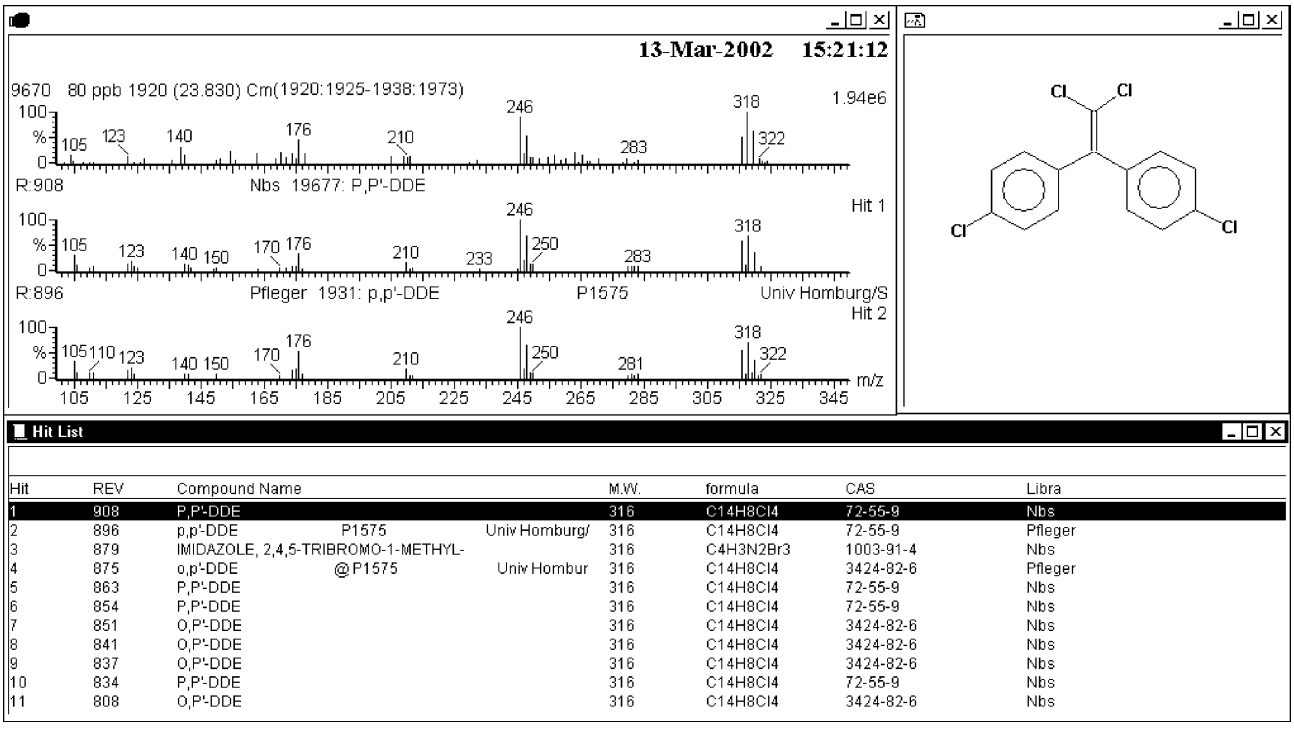

Fig. 2. Library search result for *p,p′*-DDE.

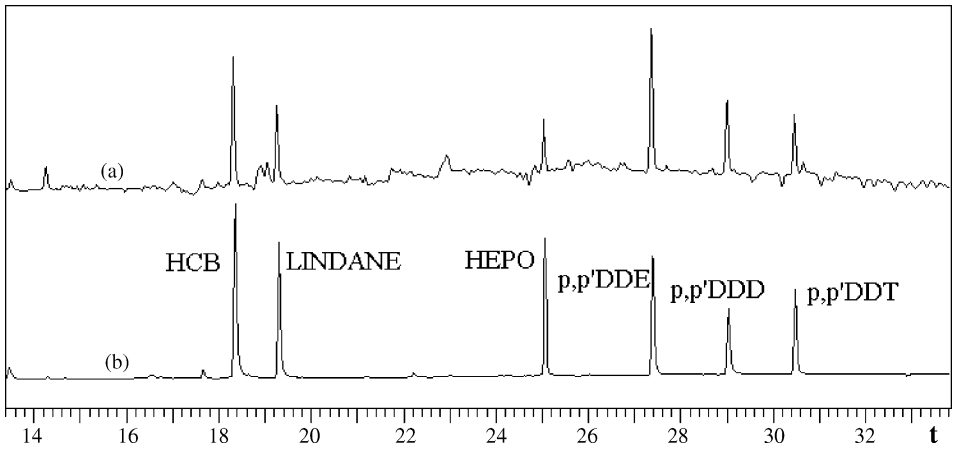

Fig. 3. Overlay of a spiked blank sample (a) and a standard solution (b) of organochlorinated pesticides at a 100 ng/g concentration.

library search result, relevant to *p,p′*-DDE, where the experimental mass spectra is compared with the two best hits of the Hit List. As it can be seen from the high value of reverse fits (90%) a good confirmation was achieved for *p,p′*-DDE, notwithstanding the real blank sample was spiked with only 80.0 ng/g of *p,p′*-DDE. Same results were obtained for the other pesticides.

3.2. GC/ECD determination

The GC experimental conditions were optimized in terms of temperature program that allowed an improvement of the time and the chromatographic run resolution. Moreover, to avoid the cross contamination between high and low spiked blank samples, the sequence of injections was in the following order: solvent, blank sample, spiked blank sample, and finally standard solutions.

Typical GC/ECD chromatograms are shown in Fig. 3 for a spiked blank sample and a standard solution of organochlorinated pesticides. The gas chromatogram of the spiked blank sample seems to be free of interfering peaks and it shows a satisfactory overlay, in terms of retention times, with that of the standard solution. Quantification has been carried out through peak area comparison with the external standard technique and a six-level calibration (3.0–6.0–12.5–25.0–50.0–100.0 ng/g). The calibration curves of the analysed pesticides present a good regression line ($r > 0.999$) in the range of explored concentrations, from the detection limit to 100 ng/g. The detection limit of each persistent pesticide was of 3.0 ng/g, related to fat, calculated automatically by Turbochrom–Perkin-Elmer Software as a signal to noise ratio of five. This low detection limit was achieved because of the efficient cleanup step that allows the elimination of possible interfering substances, obtaining then a low noise value.

The repeatability of GC/ECD determinations was assessed in 5 h period by a series of six replicate injections of the final isooctane extraction solution from a spiked blank sample, fortified at a 100 ng/g level. A good precision was obtained for all investigated pesticides, as evidenced from the coefficient variation values in the range 0.23–4.16%. The highest CV% obtained for *p,p′*-DDT can be ascribed to the random breakdown of this pesticide in the injector (vide infra). Even if the HEPO CV% was very low (1.03%), a slight natural degradation was observed by the decrease of peak areas during the sequence of injections.

3.3. Recovery study

The recovery study, performed on blank feed fat samples spiked with known levels of the six organochlorinated pesticides are summarized in Table 2. Six replicates for each sample have been carried out at four fortification levels of 12.5–25.0–50.0–100.0 ng/g. and the relevant recovery results, given as mean values, were in the range of 68–124%. In our opinion, higher recoveries observed for *p,p′*-DDE (124%), *p,p′*-DDD (122%), and *p,p′*-DDT (116%) are ascribed to matrix effect. Besides, a lower recovery for *p,p′*-DDT, respect to *p,p′*-DDE and *p,p′*-DDD, is probably due to its thermal breakdown in the injector ($T = 240\,^{\circ}C$). This degradation produces *p,p′*-DDE and *p,p′*-DDD as evidenced in the chromatogram of Fig. 4. The recoveries are satisfactory for all compounds apart for HEPO (not greater than 76%); this low value can be ascribed to the acidic treatment that probably converts such epoxide into the correspondent

Table 2
Results for the recovery experiments of the six organochlorinated pesticides from spiked blank feed fat samples

Pesticide	Average recovery (%) ± S.D.[a]				
	12.50	25.00	50.00	100.00	Mean
HCB	91 ± 16	103 ± 7	107 ± 4	113 ± 10	103 ± 9
Lindane	104 ± 13	102 ± 6	103 ± 2	110 ± 9	105 ± 7
HEPO	76 ± 2	61 ± 5	62 ± 4	72 ± 12	68 ± 6
p,p′-DDE	134 ± 10	121 ± 17	122 ± 10	121 ± 8	124 ± 11
p,p′-DDD	124 ± 20	129 ± 14	115 ± 9	120 ± 10	122 ± 13
p,p′-DDT	119 ± 24	116 ± 11	111 ± 14	118 ± 14	116 ± 16

[a] S.D.: standard deviation ($n = 6$).

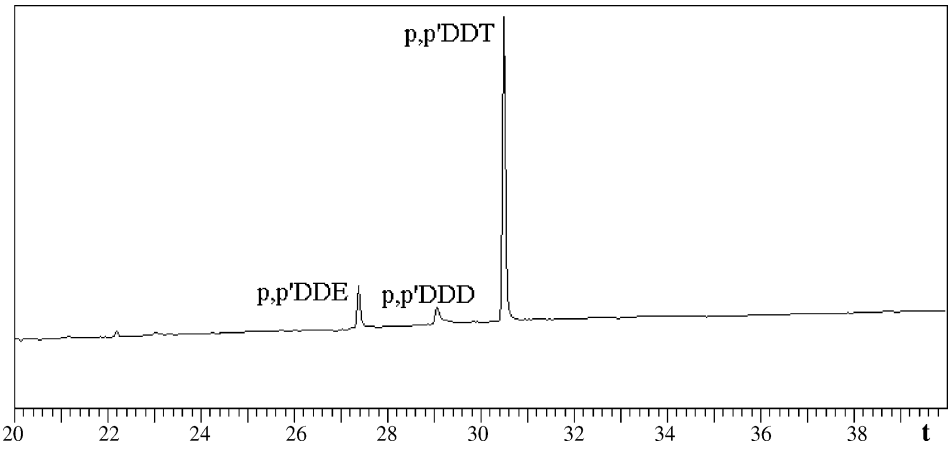

Fig. 4. Example of thermal breakdown of *p,p′*-DDT, obtained by the injection of 100 ng/g standard solution.

diol or sulphate, which are much more soluble in the water phase and then not extractable by *n*-hexane. In fact, as it can be observed in Fig. 5, the recoveries for HEPO became acceptable (85%) when the sulphuric acid was replaced with the HPLC grade water, during the cleanup procedure. However, during the cleanup, an occurring natural degradation of this less resistant pesticide, giving water-soluble products, can not be ruled out (vide ante).

The combined use of sulphuric acid and ENVI-carb powder represents a valid alternative cleanup procedure over the existing methods suggested in the European Committee for Standardization (CEN) guide-line [13], as well as other methods proposed in the literature [10–12].

In addition to the high cost of adsorbent materials, the sample cleanup based on Florisil columns requires the use of a large volume of different eluting solvent mixtures to recovery all pesticide species. GPC based methods are time consuming procedure and they require expensive instrumentation, without assuring an efficient elimination of interfering substances when used in the extraction of pesticides from vegetable based animal feed [10].

On the other hand, the use of sulphuric acid is a simple, fast and efficient cleanup method when applied to foods [11,12], but in the case of feedingstuffs for poultry [11] a number of coeluting peaks in the chromatogram affect the determination of PCBs.

In the same way, even if vegetable based feed are processed, the use of cartridges home-packed with ENVI-carb [10] gives chromatograms showing a high background noise and a number of interfering peaks that could affect the determination of pesticides when real-life samples are analysed. Moreover, the fortification of reference matrix is carried out on the extract from accelerated solvent extraction (ASE) system, which has not yet been validated [14]. Finally, the cartridges present overloading problems, so that the amount of the ASE extract to be purified should be optimized dependently on the particular real matrix processed.

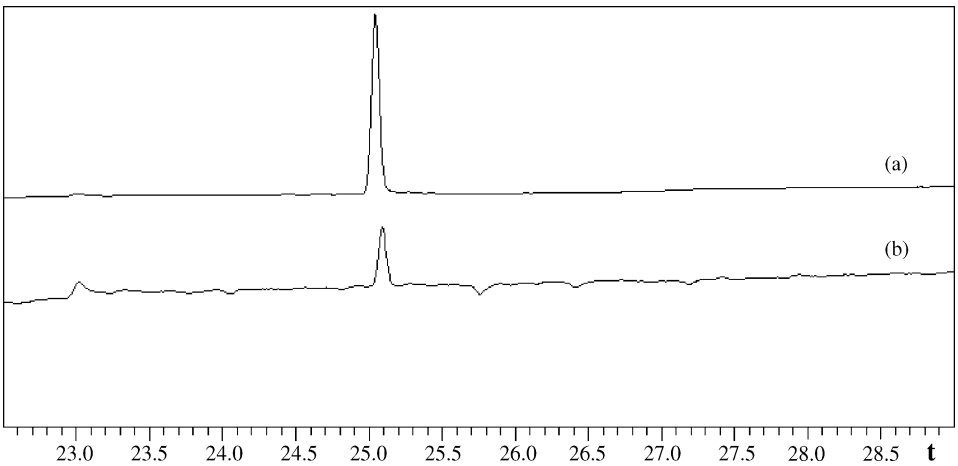

Fig. 5. Overlay of two procedural blanks: (a) procedural blank of HPLC grade water, spiked with HEPO; (b) procedural blank of concentrated sulphuric acid, spiked with HEPO.

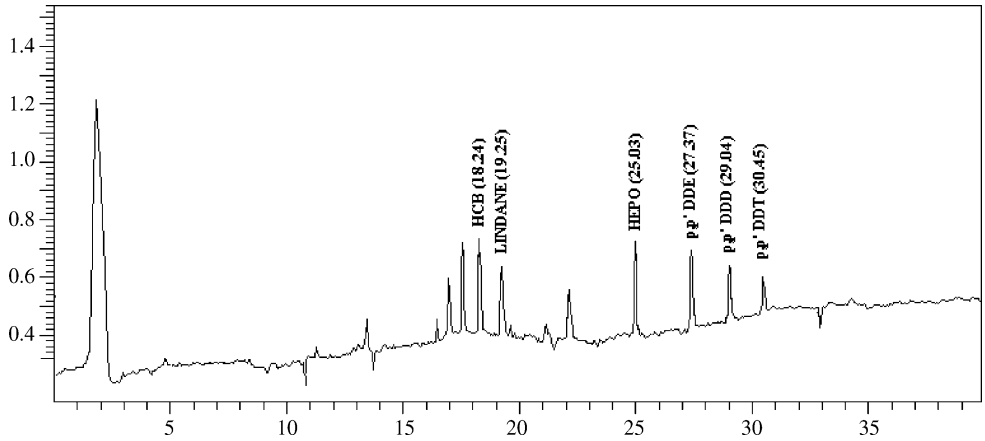

Fig. 6. Chromatogram of a naturally contaminated fish feed sample after fat extraction and cleanup.

Table 3
Content of organochlorinated pesticides in contaminated fish feed samples

Sample	Fish feed fat (%)	Average concentration (ng/g fat) ± S.D. ($n = 3$)					
		HCB	Lindane	HEPO	*p,p′*-DDE	*p,p′*-DDD	*p,p′*-DDT
1	20.3	7.56 ± 0.19	13.29 ± 0.39	10.78 ± 0.38	6.32 ± 0.40	7.81 ± 0.33	7.02 ± 0.27
2	22.6	8.10 ± 0.18	14.26 ± 0.25	<LOD[a]	5.74 ± 0.39	6.75 ± 0.46	7.37 ± 0.34
3	23.2	7.92 ± 0.37	15.10 ± 0.31	10.18 ± 0.49	6.25 ± 0.28	6.79 ± 0.40	7.68 ± 0.41

[a] LOD: limit of detection (signal-to-noise ratio of 5).

On the contrary, in the analysis of more complex matrices as fish feed, by the synergic effect of ENVI-carb and sulphuric acid chromatograms with low noise levels and without interfering peaks have been obtained. In spite of the simplicity of operations and the low-cost of materials and instrumentations, pesticide recovery values, detection limits and reproducibility compared-well with those obtained by the other methods.

3.4. Analyses of real samples

Recently, the European Parliament and the Council have published [15] a new directive, where have been fixed the limits of several residues in feedingstuffs including organochlorinated pesticides. To assess the potential of the proposed sample cleanup method 20 fish feed samples, collected from local fish farms, have been analysed. The results have evidenced the presence of pesticides in three of the samples but at concentrations below the legal limits; Fig. 6 shows a typical chromatogram obtained for a naturally contaminated fish feed. Quantification of pesticides has been carried out through peak area comparison with the external standard technique and the correspondent concentration values are given in Table 3 together with the percent of the fat content. The elimination of interfering substances and the low background noise, obtained by the efficient cleanup step, have permitted a high precision (CV% in the range 1.7–6.8%) and accuracy in the determination of each compound; in fact the results compared-well, according to a *t*-test at 95% confidence level, with those obtained by GC/MS.

4. Conclusions

The developed rapid multiresidue extraction method is suitable for monitoring of organochlorinated pesticides in fish feed. The combined use of concentrated sulphuric acid and ENVI-carb allows a quantitative cleanup extraction of the analytes. No complicated apparatus are required and other advantages are the need of a low organic solvent volume and a non-intensive manual labour requirement. By this method good results are obtained over a wide range of analyte concentrations in terms of precision, linearity, accuracy, detection limits and recovery.

The usefulness of the proposed method has been demonstrated by the determination of organochlorinated pesticides in feed fish samples collected from local fish farms.

This low-cost and simple procedure, based on rapid and safe operations, may be a useful tool in routine analysis of the organochlorinated pesticides, in place of the currently used conventional techniques.

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