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Multiresidue determination of persistent organochlorine and organophosphorus compounds in whale tissues using automated liquid chromatographic clean up and gas chromatographic–mass spectrometric detection

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Abstract

A multiresidue method based on normal-phase LC for the sample clean up of whale tissues extracts prior to GC–MS determination of residues of polychlorinated biphenyls, organochlorine pesticides and derivatives and lipophylic organophosphorus pesticides has been developed. Pesticides were extracted from blubber by fusing and dissolving the fat in *n*-hexane and from liver and kidney by reflux in *n*-hexane. Hexanic extracts were directly injected on the silicagel column of the automated LC clean up system, using *n*-hexane as mobile phase. Diode array detection allowed the on-line monitoring of lipids elution from the LC system. Purified extracts were analysed by GC using mass selective detection. The developed procedure was applied to different tissues from a whale specimen appeared in the Valencian coast, finding high concentrations of OCs (up to 7.3 $\mu g\ g^{-1}$ pp'-DDE, and 7.2 $\mu g\ g^{-1}$ PCBs). The method was validated by means of recovery tests for all the compounds detected in the whale and also for some other OCs and OPs studied in this paper. The method improves other current methods for the analysis of persistent organochlorines in marine mammals with regard to time of analysis, solvent expend and automation; solvent exchanges are not necessary before GC analysis, and it allows the simultaneous determination of organophosphorus pesticides.

Keywords: Organochlorine compounds; Organophosphorous compounds; Pesticides

1. Introduction

Persistent organochlorines such as polychlorinated biphenyls (PCBs) and diphenyl-dichloro-trichloroetane (DDT) and its derivatives (DDTs) are one of the most dangerous pollutants because of their high liposolubility and tend to bioaccumulate along the food chain involving a wide range of trophic levels. As a consequence, they are dispersed in the biotic compartment of the environment, world-wide.

Marine mammals are known to accumulate these contaminants at extremely high concentrations because they occupy a top trophic position in the ecosystem and have a low metabolic capacity to degrade toxic organochlorines. Even in marine mammals inhabiting pristine areas like the Arctic and the Antarctic regions, organochlorine compounds have been detected [1–4].

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Since the 1970s, the use of DDTs has been banned around the world, being replaced by other organochlorines (OC) and organophosphorus (OP) pesticides in the agricultural activities. As a consequence, OC pesticides such as lindane, chlordane, aldrin, dieldrin, heptachlor, hexachlorobenzene, and lipophylic OP pesticides – chlorpyrifos, fonofos, phorate, methyl-chlorpyrifos – have been detected in sea and surface waters and marine organisms [5–12].

Regulatory Agencies are increasingly interested in developing new screening protocols to detect residues of chemicals in aquatic species. Most of the existing analytical methods include laborious, and time and solvent consuming steps. Currently, procedures used for determination of OC compounds in fatty samples such as foods [13], fish oils [9,14], marine organisms [15] and marine mammals [3,4,16] require laborious clean up schemes including acid digestion or liquid–liquid partitioning followed by packed column chromatography with silicagel, Florisil or aluminium oxide, with various concentration steps and solvent exchanges.

The use of gel permeation chromatography (GPC) for the analysis of OCs in marine organisms [17–20] usually renders fractions of more than 50 ml and needs further clean up steps by means of Florisil or silicagel column chromatography to obtain fat-free extracts prior to the gas chromatographic (GC) determination.

Other approaches using modern techniques such as solid-phase extraction (SPE) [21] or supercritical fluid extraction (SFE) [22] try to minimise the sample treatment in the analysis of OC compounds in fatty samples.

The application of liquid chromatography (LC) as clean up step prior to the GC determination of OC and OP pesticides in fatty samples improves the efficiency of the purification, obtaining fat-free LC extracts of a few millilitres containing the analytes which are suitable for the subsequent GC analysis [12,23–27].

The aim of this work was to develop efficient and adequate analytical methodology for the simultaneous analysis of persistent OC compounds, as DDTs and PCBs, and some lipophylic OP pesticides in selected whale tissues. The appearance of a death specimen of common whale (*Balaenoptera physalus*, female) in the Valencian coast in March 1998, and our interest in investigating the causes of death [28] was the reason for this work. This paper describes an automated procedure based on normal-phase silicagel LC for the efficient clean up and fractionation of PCBs (IUPAC number 52, 118, 138, 153, 180), DDT and derivatives (DDTs), aldrin, dieldrin, lindane, chlordane, heptachlor, dicofol, Endosulfan A and B, and several lipophylic OPs (chlorpyrifos, methyl-chlorpyrifos, fonofos and phorate). The LC fractions are directly injected into the gas chromatograph without any further clean up steps or solvent exchanges. Analytical determination was carried out by mass selective detection in full scan mode for confirmatory purposes and single ion monitoring mode for quantitation.

2. Experimental

2.1. Reagents

Reference materials from Dr. S. Ehrenstrofer (Promochem, Wesel, Germany) with a purity >97–99% were used for preparation of standards of DDTs (op'DDE, pp'DDE, op'DDD, pp'DDD, op'DDT and pp'DDT), aldrin, dieldrin, chlordane, heptachlor, lindane, dicofol, endosulfan A+B, chlorpyrifos, methyl-chlorpyrifos, phorate and fonofos. PCB mix 3 from Dr. S. Ehrenstrofer (10 $\mu g \cdot ml^{-1}$ in isooctane) was used for single quantitation of PCBs congeners IUPAC number 52, 118, 138, 153 and 180.

The internal standard 2-chloro-octadecane from Fluka Chemie (Neu-Ulm, Switzerland) with a purity of 97% was used.

Working solutions were prepared by dilution in *n*-hexane. Ethyl acetate and *n*-hexane (pesticide residue analysis quality) were purchased from Scharlau (Barcelona, Spain). Anhydrous sodium sulphate was obtained from Baker (pesticide residue quality, Deventer, The Netherlands) and was purified for 18 h at 300°C before use. Celite was purchased from Merck (Darmstadt, Germany).

2.2. LC Instrumentation

A schematic representation of the LC system used in this work is shown in Fig. 1. It was constructed

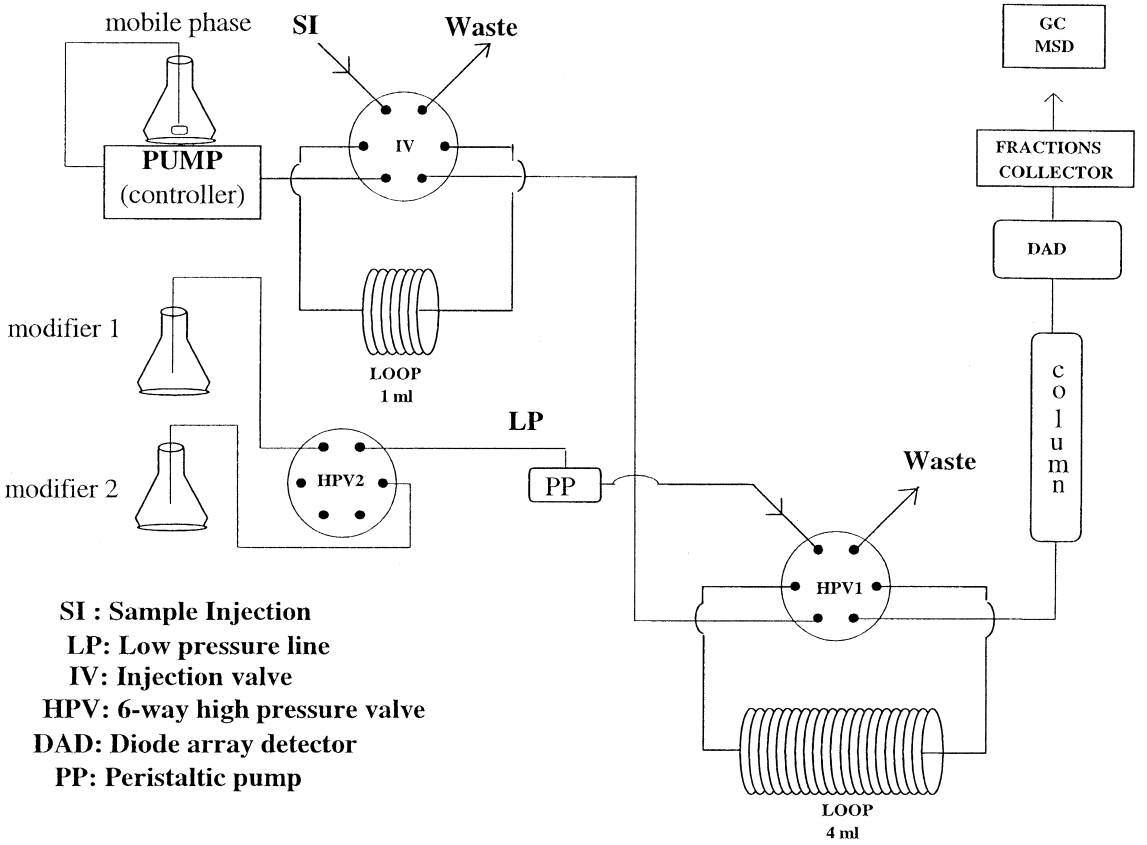

Fig. 1. Scheme of the automated LC system used for the development and optimisation of the analytical procedure.

from the following equipment: *HPLC Pump:* Master 305 piston pump, Gilson (Middleton, USA); *Peristaltic pump:* LKB (Bromma, Sweden). Solvent flex tubing (solvent resistant), ELKAY (Galway, Ireland); *Valves*: two six way high-pressure valves, VICI Valco, Europe Instruments (Schenkon, Switzerland), electronically controlled; *Sampler*: injection valve Rheodyne (Cotati, CA, USA) with 1.0 ml loop; *Column*: 150×3.9 mm ID packed with 4 μm silica Nova-pack (Waters, Milford, MA, USA); *Detector*: 2140 Rapid Spectral Detector LKB; *Fraction collector:* 2212 Helirac, LKB (Bromma, Sweden); *Mobile phases: n*-hexane, *n*-hexane–ethyl acetate mixtures and ethyl acetate; *Flow rate:* 1 ml min^{-1}.

2.3. GC instrumentation

GC–MSD analysis were performed with a Hewlett-Packard 5890 series II (Avondale, USA) which was equipped with a HP 7673 autosampler and a MSD 5971 mass selective detector. Splitless injections of 2 μl (purge off time: 0.75 min) were performed into a fused-silica Ultra 2 capillary column coated with crosslinked 5% phenyl methyl silicone with a length of 25 m×0.25 mm ID and a film thickness of 0.33 μm. Helium was applied as carrier gas at a flow of 0.7 ml min^{-1}. The oven temperature was programmed as follows: 90°C for 1 min, at 30°C min^{-1} to 180°C, at 4°C min^{-1} to 270°C with a final hold for 20 min.

Mass detector characteristics were as follows: electron impact (70 eV) mode with electron multiplier voltage of 1700; temperature of transfer line was 280°C. Analysis were carried out in selected ion monitoring mode using the single ions listed in Table 1.

Table 1
Selected ions for the single monitoring mode (MS–SIM)

Compound	Selected ions	Time (min)
op'DDE, pp'DDE	246, 318	14.50–16.50
op'DDD, pp'DDD	235, 165	16.50–27.50
op'DDT, pp'DDT	235, 165	16.50–27.50
PCB 52	292, 220	11.50–14.00
PCB 118	326, 254	14.80–18.00
PCB 138	360, 290	18.00–21.00
PCB 153	360, 290	18.00–21.00
PCB 180	394, 324	21.00–27.50
Lindane	183–219	9.00–10.50
Heptachlor	272–274	10.50–12.00
Aldrin	261, 263, 265	12.00–13.00
Dicofol (Dichlorobenzofenone)	139	13.00–14.00
Chlordane	373–375	14.80–15.00
Endosulfan A	195–339	15.00–15.50
Dieldrin	363–277	15.50–17.00
Endosulfan B	195–237	17.00–27.50
Phorate	97–121	7.50–9.00
Fonofos	109–246	9.00–10.50
Methyl–chlorpyrifos	125–286	10.50–11.50
Chlorpyrifos	197–314	12.45–13.50
2-chloro-octadecane (internal standard)	83–97	14.00–14.80

2.4. Procedure

2.4.1. Preparation of samples

A female common whale (*Balaenoptera physalus*) –weight: 20 Tm, size: 17 m– was found dead in the Valencian coast on 3 March 1998. Blubber, liver and kidney samples were collected in triplicate and frozen immediately at −20°C. After thawing, triplicate samples were cut into small cubes and mixed in order to obtain an homogeneous laboratory sample for each tissue.

2.4.2. Extraction procedure

Blubber: samples (15 g) were cut into cubes of approximately 10×10 mm and transferred to a glass funnel which was placed on top of an Erlenmeyer flask. Then, it was heated in a heating cabinet at 65°C for 8 h. The glass funnel and flask were rinsed with *n*-hexane and fused fat was diluted finally to 30 ml with *n*-hexane. Sodium sulphate was added to the hexanic extract before LC clean up. In this way, hexanic extracts of 500 mg of blubber per ml were obtained.

Liver and kidney: 30 g of sample cut into cubes of approximately 10×10 mm were mixed with 30 g sodium sulphate and 5 g Celite. The mixture was transferred to an Erlenmeyer flask and extracted under reflux in *n*-hexane (200 ml) for 1 h. Extract was filtered through filter paper and concentrated in a Kuderna Danish evaporator to 10 ml. Thus, hexanic extracts of 3 g sample per ml were obtained.

2.4.3. Automated Clean up Procedure

The mobile phase (*n*-hexane) was set at a flow-rate of 1 ml min^{-1} and a volume of 1 ml of the sample hexanic extract was injected into the LC column using the six-way injection valve (IV). Diode array detector (DAD) (280 nm) was used for monitoring lipid elution. 1 ml fat free fractions containing pesticides were collected by means of the fraction collector. Lipids started to elute at 15 min. Finally, 4 ml of ethyl acetate were injected using the valve HPV1 at 16 min, in order to elute the remaining lipids from the column. The peristaltic pump (PP) (2 ml min^{-1}) was used for loading the HPV1 loop (4 ml) with ethyl acetate through a low pressure line. The whole procedure was completely controlled from the HPLC pump (Fig. 1).

2.4.4. GC analysis

The LC fat-free fractions containing the analytes were injected directly into the GC system using MS detection (see Section 2.3) without any additional treatment. Quantitation was carried out using 2-chloro-octadecane as internal standard by adding 10 μl (100 μg ml^{-1}) at vials containing 1 ml of extract before injection.

2.4.5. Determination of the fat

The fat content in the extract and in the HPLC fractions was determined by a colorimetric method

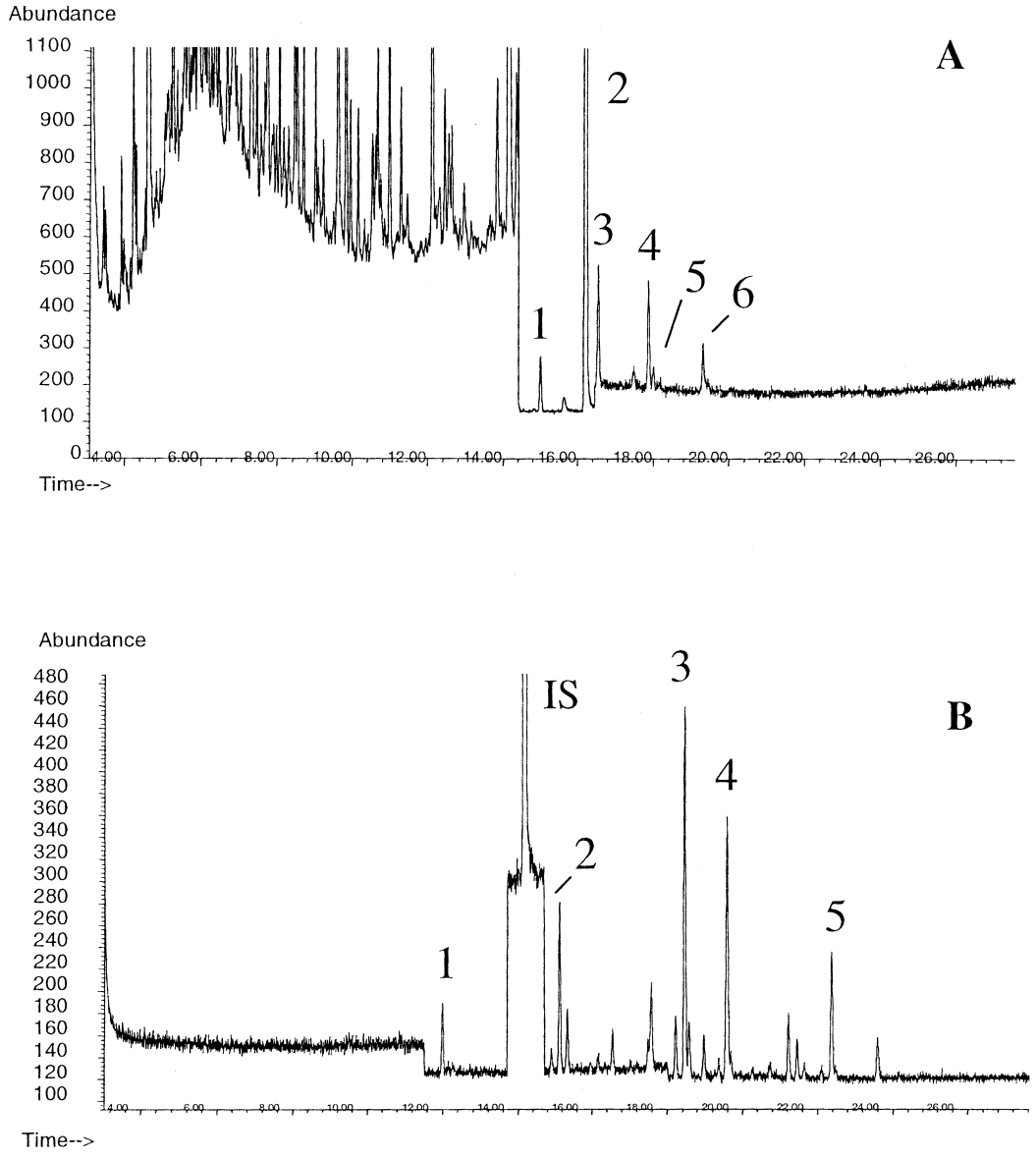

Fig. 2. GC–MSD (SIM)-chromatograms corresponding to the purified blubber whale sample, where several OCs and PCBs were detected. (A) (LC Fraction 3) 1–op'DDE, 2–pp'DDE, 3–op'DDD, 5–op'DDT, 6–pp'DDT; (B) (LC Fraction 3) 1–PCB 52, 2–PCB 118, 3–PCB 138, 4–PCB 153, 5–PCB 180, I.S.–internal standard.

for total lipids [29]. The colorimetric method used is based on the sulpho-phosphovainilline reactivity. Triacylglycerides calibrator solution (Sigma, St. Louis, MO, USA) was used as a standard in the range 125 to 1000 μg. The limit of detection was found to be 33 μg.

3. Results and discussion

The LC system shown in Fig. 1 was used for developing the clean up procedure. This system allows easy optimisation of LC clean up procedures for different fatty samples, as it allows on-line monitoring of the elution of fats using the DAD (λ=280 nm). In fact, this system has been successfully applied to such marine organisms as crustaceans, molluscs, fish and microalgae [12,25], which show different lipid elution patterns depending on the type of matrix.

The application of the optimised LC-clean up procedure to whale tissue extracts allowed us to obtain the analytes in 1 ml fat free fractions using *n*-hexane as the mobile phase: fraction 3 (DDTs, PCBs, heptachlor, aldrin, endosulfan A, endosulfan B, dicofol, dichlorobenzo-fenone), fraction 4 (chlordane), fraction 9 (lindane), fraction 12 (dieldrin), fraction 14 (phorate, fonofos, methyl-chlorpyrifos, chlorpyrifos). So, the more polar analytes (OP pesticides) eluted at minute 14, whereas fats started to elute at minute 15 in the blubber, liver and kidney extracts. The use of more polar mobile phases (by incorporating ethyl acetate as modifier) accelerated the elution of pesticides but unfortunately led to the coelution of the more polar pesticides together with the fat. Comparing the behaviour of whale fats to those observed by us, using the same LC approach, in other marine organisms (such as crustaceans, molluscs and fish) [12,25], it can be concluded that lipids of marine mammals are less polar than the above mentioned marine organisms.

The fat content in each fraction was determined by colorimetry, although in routine analysis the use of DAD is sufficient for obtaining on-line qualitative information about fat elution.

In order to validate the developed procedure, several experiments were carried out. Firstly, results obtained by injection of standard pesticide solutions (from 100 to 1000 ng ml^{-1}) directly into the GC system were compared with those obtained by

Table 2
Comparison of slopes and concentrations found in whale tissues obtained by direct calibration and by the standard addition method

	Slope (area units/ng)		Concentration (μg g^{-1})	
	Direct calibration	Standard additions	Direct calibration	Standard additions
Blubber				
o, p' DDE	936	833	0.09	0.09
p, p' DDE	349	331	7.30	7.92
o, p' DDD	497	402	0.43	0.35
p, p' DDD	409	365	0.64	0.61
o, p' DDT	60	63	0.71	0.61
p, p' DDT	66	28	0.59	0.63
PCB 52	2.9	2.2	1.86	1.79
PCB 118	19	14	4.28	4.11
PCB 138	42	30	7.11	7.07
PCB 153	26	19	7.23	7.25
PCB 180	21	16	3.78	3.55
Liver				
p, p' DDE	275	284	0.11	0.07
Kidney				
o, p' DDE	811	833	0.24	0.08
p, p' DDE	282	284	0.23	0.12
o, p' DDD	304	265	0.09	0.07

injection of standards into the LC system previously to the GC determination, showing that no losses took place during the LC clean up procedure (recoveries between 90 and 110%).

The injection of purified whale extracts in the GC–MSD system revealed the presence of several DDTs and PCBs in the real sample, whose chromatograms are presented in Fig. 2. The presence of a high number of peaks in the first 15 min of the gas chromatogram can be attributed to the selected ions for the internal standard detection (masses 83 and 97) by SIM mode, which present themselves in this part of the chromatogram. The quantitation was carried out by both direct calibration and addition of standards at three concentration levels (50, 100 and 150 ng ml^{-1}; or 500, 1000 and 1500 ng ml^{-1}, depending on the analyte concentration detected in whale tissues) to the hexanic extracts before LC clean up. Slopes of both calibration curves were similar, showing the lack of matrix influence (Table 2). Therefore, the concentrations of compounds detected in whale tissues obtained by direct calibration were in close agreement with those obtained by extrapolation of calibration graphs corresponding

Table 3
Recoveries ($n=4$)[a] after application of the overall analytical procedure for DDTs and PCBs in blubber, liver and kidney whale tissues spiked at three levels. Concentrations expressed in $\mu g\ g^{-1}$

Compound	Blubber			Liver			Kidney		
	blank	added	Rec%	blank	added	Rec%	blank	added	Rec%
op' DDE	0.09	0.1	85	nd[b]	0.05	96	0.24	0.1	88
		0.2	91		0.5	96		0.2	94
		0.3	90		1.0	116		0.3	104
pp' DDE	7.3	1.0	106	0.11	0.1	108	0.23	0.1	107
		2.0	91		0.2	106		0.2	105
		3.0	92		0.3	104		0.3	99
op' DDD	0.43	1.0	100	nd	0.05	88	0.09	0.1	89
		2.0	97		0.5	103		0.2	81
		3.0	90		1.0	81		0.3	83
pp' DDD	0.64	1.0	97	nd	0.05	88	nd	0.05	81
		2.0	90		0.5	86		0.5	83
		3.0	94		1.0	81		1.0	87
op' DDT	0.71	1.0	108	nd	0.05	83	nd	0.05	81
		2.0	108		0.5	103		0.5	88
		3.0	109		1.0	107		1.0	96
pp' DDT	0.59	1.0	108	nd	0.05	111	nd	0.05	92
		2.0	97		0.5	119		0.5	100
		3.0	99		1.0	112		1.0	92
PCB 52	1.86	1.0	108	nd	0.05	96	nd	0.05	92
		2.0	103		0.5	87		0.5	82
		3.0	100		1.0	97		1.0	103
PCB 118	4.28	1.0	103	nd	0.05	109	nd	0.05	108
		2.0	90		0.5	75		0.5	81
		3.0	83		1.0	93		1.0	103
PCB 138	7.11	1.0	105	nd	0.05	121	nd	0.05	83
		2.0	94		0.5	81		0.5	110
		3.0	104		1.0	93		1.0	100
PCB 153	7.23	1.0	95	nd	0.05	107	nd	0.05	80
		2.0	89		0.5	81		0.5	95
		3.0	89		1.0	87		1.0	105
PCB 180	3.78	1.0	99	nd	0.05	90	nd	0.05	92
		2.0	83		0.5	77		0.5	90
		3.0	88		1.0	94		1.0	106

[a] RSD for 4 replicates ranged between 3 and 13%.
[b] nd: Non detected.

Table 4
Recoveries (%) after application of the overall analytical procedure for other OCs y OPs in blubber, liver and kidney whale tissues spiked at three levels[a]

	Blubber			Liver			Kidney		
	μg g^{-1}			μg g^{-1}			μg g^{-1}		
	0.05	0.5	1.0	0.05	0.5	1.0	0.05	0.5	1.0
Aldrin	94	95	97	94	95	91	90	97	101
Chlordane	85	91	86	87	91	93	85	87	98
Dicofol	80	83	87	86	81	91	89	87	87
Dieldrin	97	102	100	91	94	98	95	91	104
Endosulfan A	91	86	89	94	91	99	93	96	96
Endosulfan B	79	87	85	76	89	88	85	79	81
Heptachlor	97	91	99	94	102	99	83	89	95
Lindane	91	97	100	87	90	105	93	99	102
Chlorpyrifos	85	88	87	88	89	87	86	89	90
Methyl-chlorpyrifos	85	85	87	83	80	87	91	96	92
Fonofos	98	100	105	98	89	101	90	91	99
Phorate	79	85	87	82	84	92	57	61	67

[a] RSD for 4 replicates ranged between 3 and 13%.

to the standard additions procedure used (Table 2), with DDTs in the kidney being the only exception.

High concentrations of DDTs –from 0.09 to 7.30 μg g^{-1}– and PCBs –from 1.86 to 7.23 μg g^{-1}– were found, which prove the exposure and bioaccumulation ability of these marine mammals to persistent organochlorine compounds. As expected, the metabolite pp'DDE was the predominant compound among all DDTs present in the blubber (7.3±0.58 μg g^{-1}). Besides, pp'DDE was also detected in liver and kidney (0.11±0.01 and 0.23±0.02 μg g^{-1}, respectively).

Recoveries for all the compounds studied in this work (those detected in the real-world sample and those not detected but also included in the optimizated LC procedure) were obtained by applying the overall analytical procedure (extraction, LC clean up and GC determination) to the selected whale tissues spiked at three concentration levels (Tables 3–4).

Results obtained were satisfactory at the three levels assayed for all compounds (recoveries between 79 and 119%), with relative standard deviations for four replicates between 3 and 13%, except for phorate, that showed lower recoveries in kidney. This compound also showed poor recoveries in other marine matrices, as the microalgae *Skeletonema*[12].

Figs. 3 and 4 show GC–MS (SIM) chromatograms corresponding to the blubber sample spiked at 50 ng g^{-1} level with the selected OCs and OPs, respectively. Due to the absence of fat in the extracts, after the proposed LC clean up, the GC chromatograms did not present interferences and a few ng·g^{-1} could be detected easily using GC–MS SIM mode. Limits of determination between 5 ng g^{-1} (fonofos) and 25 ng g^{-1} (phorate, methyl-chlorpyrifos, chlorpyrifos, dicofol, endosulfan) can be estimated from these chromatograms.

4. Conclusions

The method proposed in this paper allows the multiresidue determination of PCBs, OCPs and OPPs in blubber, kidney and liver of whale by means of normal-phase LC automated clean up of extracts prior to the GC–MSD determination. The application of this procedure presents several advantages: being more rapid and efficient than existing procedures for pesticide analysis in marine mammals and other marine organisms; the LC separation between fats and analytes can be monitored on line by DAD, thus rendering a fast and reliable optimisation of the system; and solvent exchanges are not necessary along the process, the extraction of sample included. The whole procedure, including extraction, LC clean up and GC–MS determination, allows the analysis of about 30 samples per day of blubber and 15–20 samples of liver or kidney depending of reflux sites.

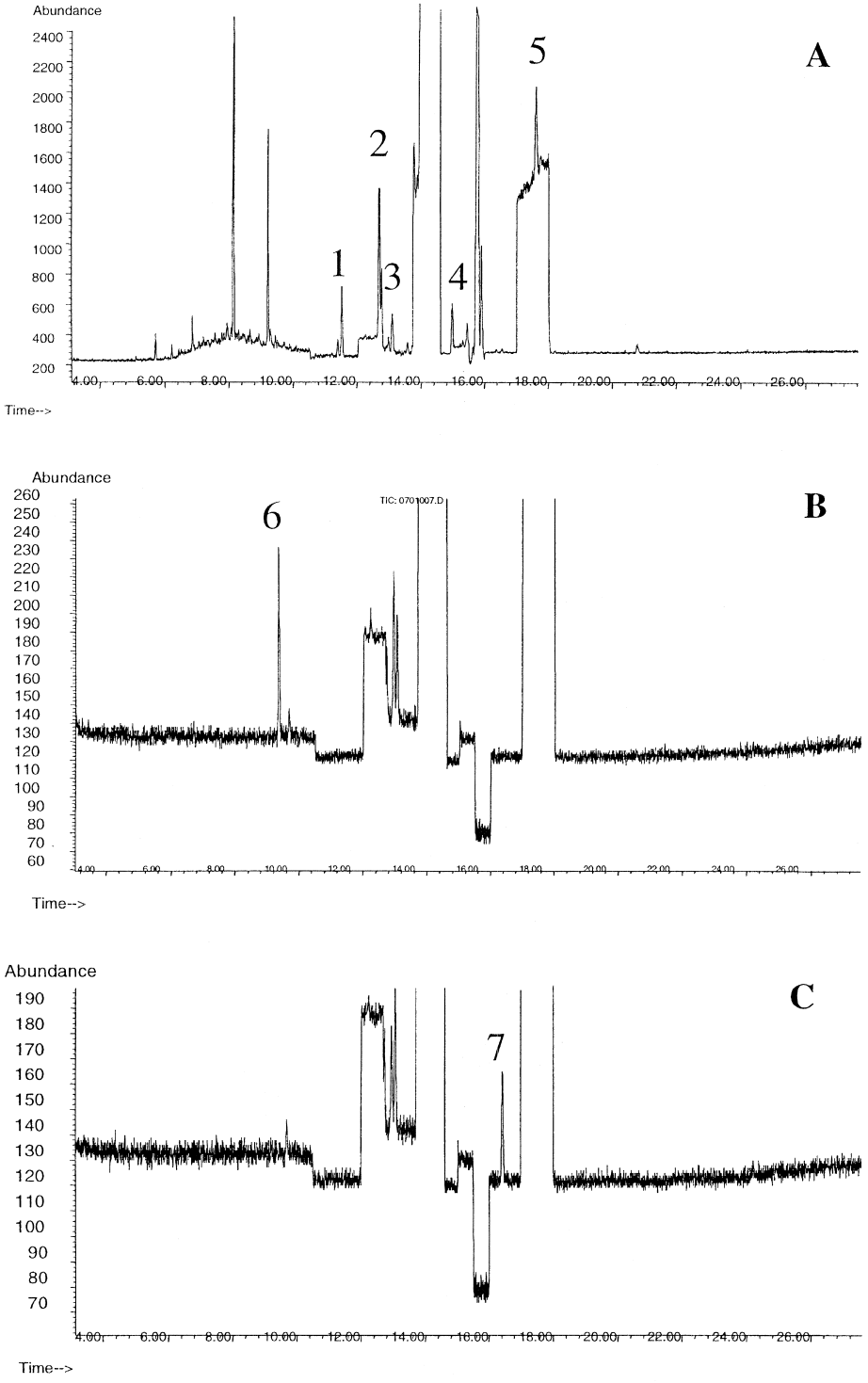

Fig. 3. GC–MSD (SIM)-chromatograms corresponding to the blubber whale sample spiked with 50 ng g^{-1} of selected OCs. (A) (LC Fraction 3) 1–heptachlor, 2–aldrin, 3–dicofol, 4–endosulfan A, 5–endosulfan B; (B) (LC Fraction 9) 6–lindane; (C) (LC Fraction 12) 7–dieldrin.

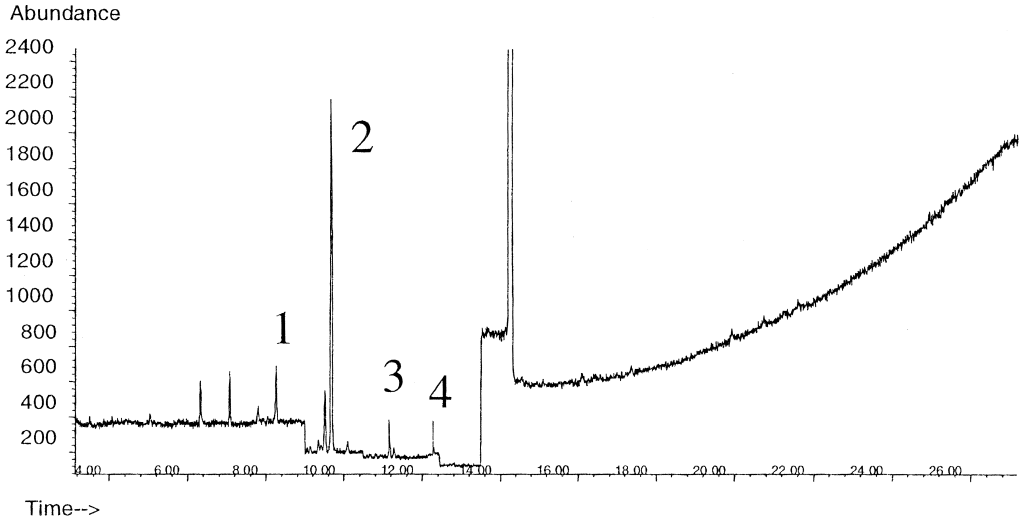

Fig. 4. GC–MSD (SIM)-chromatograms corresponding to the blubber whale sample spiked with 50 ng g^{-1} of selected OPs. (LC Fraction 14) 1–phorate, 2–fonofos, 3–methyl-chlorpyrifos, 4–chlorpyrifos.

The efficiency of the clean up step together with the selective determination by GC–MS led to unequivocal detection and confirmation of the analytes, making the procedure suitable for laboratory and environmental studies. LC fat-free fractions can be injected directly into the GC, which might finally lead to a fully automated LC–GC procedure in the near future. The use of the same LC silicagel column for two months did not affect the retention of pesticides and lipids.

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