Analyses of lindane, vinclozolin, aldrin, p,p'-DDE, o,p'-DDT and p,p'-DDT in human serum using gas chromatography with electron capture detection and tandem mass spectrometry[☆]

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Abstract

A sensitive, selective and reliable procedure was developed and validated to determine organochlorinated compounds, which present endocrine-disrupting effects, lindane, vinclozolin, aldrin, p,p'-DDT, o,p'-DDT and p,p'-DDE, in human serum. The analytical methodology combines serum extraction with organic solvents, clean-up of the organic extract using acid treatment with H_2SO_4, elution of the cleaned-up extract through liquid column chromatography system and analysis of the fraction eluted by gas chromatography (GC) with electron capture detection (ECD) and tandem mass spectrometry (MS–MS) detection mode. Performance characteristics, such as linearity, sensitivity, precision, accuracy or recovery, of both chromatographic methods were studied. The proposed analytical methodology was applied to analyse the target compounds in serum samples from women living in agricultural areas of Almería (Spain). Comparison of the MS–MS and the ECD results was made and the advantage of the MS–MS operation mode to determine endocrine disrupting compounds in complex matrices is also presented. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Evidence has been accumulating which indicates that humans, domestic and wildlife species have suffered adverse health consequences from exposure to environmental chemicals that interact with the endocrine system [1–5] and so they are more accurately named "endocrine disrupting" [6]. There are many factors which influence whether a compound will affect humans, depending on how the compound enters the body, how it is distributed, what hormonal or other processes it interacts with and what other endocrine disrupting or complex mixture of them is present. Nowadays, pesticide blood levels are considered a good indicator of

pesticide body burden as close correlations between the concentrations of pesticides in blood and fat have been established even in the case of non-occupationally exposed individuals [7]. As a consequence the determination of such compounds in biological samples, specifically blood, from exposed populations is considered of high interest.

Effects of some types of these compounds, mainly organochlorinated pesticides, have been already studied. Lindane may produce kidney and liver changes in some experimental animals and, more importantly, there have been several cases of aplastic anaemia with a clear association between their recent or, less frequently, remote exposure [8]. Occupational overexposure to aldrin produces excitation of the nervous system. Ethane, 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl) or *o,p*′-DDT and ethane, 1,1,1-trichloro-2, 2-bis(*p*-chlorophenyl) or *p,p*′-DDT, the primary isomer used extensively worldwide since the late 1940s, have been banned in the U.S. and Europe for nearly 25 years on account of their toxic effect on wildlife and their potential human carcinogenicity, however, they are still widely used in other regions of the world, primarily for vector control where malaria is endemic. A few recent reports [9] have linked human exposure to *p,p*′-DDT, or its main persistent metabolite 1,1-bis(*p*-chlorophenyl)-2,2-dichloroethylene (*p,p*′-DDE), with an increased risk of breast cancer in women, possibly as a result of *p,p*′-DDT's known estrogenic properties [10,11] although these findings, are inconsistent [12].

Perhaps one of the most pertinent issues is risk assessment of the effects of long-term exposure to trace levels of these chemical pollutants. The low concentrations of these products in biological fluids led us to carefully address the analytical determination and quality assurance to obtain accurate measurements of biological exposure.

The most frequently used detection method for organochlorinated pesticides (OCs) is gas chromatography (GC) with electron capture detector (ECD) [13–17]. Its application requires, on one hand, an adequate extraction and isolation technique, by which compound enrichment, since these compounds are present at trace levels, and on the other hand a clean-up step, to obtain clean solutions and eliminat-

ing interfering compounds that limit performance of capillary columns and the certainty of the analysis.

Nowadays, the tandem of GC with Mass Spectrometry (MS) has become the ideal analytical tool for confirmatory analysis [18]. The introduction of MS–MS capabilities to benchtop instruments, the improvement of chemical ionization or the introduction of the ion trap mass spectrometers [19] have been important evolutions in benchtop GC–MS. They offer tandem MS that improves selectivity in the analysis of mixtures and, therefore, can lower detection limits by eliminating or minimizing chemical interferences [20–24]. Ion traps perform tandem in time MS, i.e. analyte-specific ions of a selected mass/charge ratio are first isolated and then fragmented. Following fragmentation, the fragment ions are scanned. Both MS stages occur in a single ion trap. Tandem MS usually results in higher analytical sensitivity than single stage MS analyses [23–25].

On the other hand, MS–MS provides information for structural elucidation, which can be helpful in the case of unknowns. So, if an unknown compound is detected and the electron ionization (EI) spectrum is not informative enough to tell the structure, the ion trap gives the possibility of doing MS–MS and learning more about the structure of that compound.

Several groups of compounds [2–4] have been reported to exhibit hormone-disrupting effects such as OCs, PCBs, alkylphenols, bisphenol A or phthalates. It is likely other hormone-disrupting chemicals will be discovered as experimental procedures are developed. So, there is an urgent need to establish validated assays to test the activity of endocrine-disrupting chemicals, as well as validated analytical methods to determine trace levels of these compounds.

In this paper a procedure is described for the simultaneous determination of trace levels of lindane, vinclozolin, aldrin, *p,p*′-DDE, *o,p*′-DDT and *p,p*′-DDT in human blood-serum samples. The procedure consists of a solvent extraction step followed by a sample clean up. Determination was carried by capillary GC–ECD and GC–MS–MS. Both chromatographic methods were developed taking into account criteria established for validation of routine quantitative analytical compounds [26–28]. The limitations and advantages of using GC–ECD or

M. Moreno Frías et al. / J. Chromatogr. B 760 (2001) 1–15

3

GC–MS–MS techniques for quantitative and qualitative determinations in serum samples are compared and discussed.

The procedure was applied to the simultaneous determination of the target compounds in blood serum samples from people living in an agricultural area (Almería, Spain). The target pesticides studied here reported to have endocrine-disrupting effects [16].

2. Experimental

2.1. Chemicals and reagents

Standards of the pesticides were obtained from Riedel-de-Haën (Seelze-Hannover, Germany) always with purity higher than 99%. The internal standard (ISTD), heptachlor (99% of purity) was supplied by Aldrich (St. Louis, MO, USA). Stock standard solutions, 200 μg ml^{-1}, were prepared by exact weighting and dissolving them in n-hexane. Working standard solutions were prepared by appropriate dilutions and stored in a refrigerator (4°C). Pesticide quality solvents: n-hexane, MeOH, diethyl ether and 2-propanol were supplied by Panreac (Barcelona, Spain). Reagent grade concentrated sulphuric acid was also supplied by Panreac. Organic free water was prepared by distillation and then by Milli-Q SP treatment (Millipore Corporation, USA). Sex steroids-free serum was obtained according to the method described by Brotons et al. [29].

2.2. Sampling and sample storage

Six serum samples were obtained from women breast cancer patients from Almeria's province. Around 10 ml of venous blood were collected into vacutainer tubes (Venoyect, Terumo Europe N.V. 3001, Leuven, Belgium) without preservatives. The samples were left to coagulate naturally for 3–7 h and then, they were centrifuged to separate the serum that was transferred into glass vials capped with teflon-faced silicone rubber septa. These vials were stored at −20°C until analysis.

All instruments and vials used were made of glass; sample contact to plastic materials was reduced to a minimum in order to avoid adsorptive sample losses. Instruments and vials were cleaned with hexane and acetone.

2.3. Extraction procedure of pesticides from the serum

Four ml aliquots of blank serum matrix were spiked and equilibrated for 3 h at room temperature in a test tube. To the samples were sequentially added 2 ml of MeOH, mixed in a rotatory mixer for 1 min, and 5 ml of n-hexane:ethyl ether (1:1) v/v, agitating on a rotary mixer for 2 min and then centrifuging at 2500 g for 5 min. The organic phase was collected, and the aqueous phase was extracted twice more with 5 ml of n-hexane:ethyl ether (1:1, v/v). The organic phases were evaporated and concentrated to approximately 1 ml under a gentle stream of nitrogen.

2.4. Sample clean-up

Chemical clean-up: 0.5 ml of H_2SO_4 was added to the concentrated 1 ml sample of organic phase, mixed for 1 min and centrifuged for 5 min at 2500 g. The organic phase was collected and the aqueous phase was extracted twice more with 1 ml of n-hexane; the organic phases were evaporated and dried completely under a stream of nitrogen; the samples were dissolved in 1 ml of n-hexane and then injected into the HPLC system.

2.5. Chromatographic techniques

Clean-up procedure using high-performance liquid chromatography with photodiode-array detector (HPLC–DAD): the ml of the previous extract in n-hexane was injected into the HPLC–DAD and the fraction eluted for the first 15 min, where the OC compounds were present, was collected, dried under nitrogen stream and eluted with 1 ml of n-hexane. One μl of this extract was injected into the GC–ECD system and 2 μl into the GC–MS–MS instrument.

A Waters (Milford, MA, US) model 990 liquid chromatographic system was used, equipped with a Model 600 E constant-flow pump, a Rheodyne six-port injection valve with a 1 ml sample loop, a

Lichrospher Si 250×4 mm (5 μm particle size) column from E. Merck (Darmstadt, Germany), a Model 990 UV-visible photodiode-array detector (DAD), a printer/plotter and a microcomputer using the Waters 991 software package. The mobile phase, under gradient conditions, was as follows: initially 2 min isocratic with 100 phase A (*n*-hexane), 15 min linear gradient to 60% phase A, 40% phase B (*n*-hexane:MeOH:2-propanol, (40:45:15), v/v), 20 min linear gradient to 100% phase B and 30 min linear gradient to 100% phase A. An additional time of 5 min with this composition of mobile phase was enough to return the system to the initial conditions for subsequent analysis. Samples of 1 ml were injected with the solvent flow-rate maintained at 1 ml min^{-1}. Photometric detection was performed in the range 200–280 nm, with a spectral resolution of 1.4 nm.

2.5.1. GC–ECD analysis

A gas chromatograph Hewlett-Packard (Palo Alto, CA, USA) model 5890 equipped with a ^{63}Ni ECD, a split/splitless injector operated in the splitless mode, a fused-silica capillary HP-1 chromatographic column (60 m×0.25 mm id); film thickness 0.25 μm and an HP 7673 autosampler. A HP 3365 Chemstation software was used for instrument control and data treatment. Operating conditions were as follows: initial column temperature 130°C (1 min), increased at 10°C min^{-1} to 200°C (11 min); injector temperature 250°C; detector temperature 300°C. Carrier gas N$_2$ at a flow-rate 0.85 ml min^{-1}; make-up gas N$_2$ at a flow-rate 60 ml min^{-1}; operated in the splitless mode; purge off time 2 min; injection volume 1 μl.

2.5.2. GC–MS analysis

A Saturn 2000 ion trap mass spectrometer from Varian Instruments (Sunnyvale, CA, USA) equipped with an autosampler 8200, a split/splitless programmed temperature injector SPI/1078 operated in the splitless mode and a DB5-MS (30 m×0.25 mm id.), film thickness 0.25 μm chromatographic column was employed. The ion trap mass spectrometer was operated in the electron ionisation (EI) mode and the MS–MS option was used. The computer, which controlled the system, had an EI–MS–MS library specially created for the target analytes in our

experimental conditions. In addition, another EI–MS library was available.

GC conditions were as follows: initial column temperature 80°C (2.5 min), increased from 50°C min^{-1} to 140°C (1 min) and finally increased from 5°C min^{-1} to 260°C (held for 3 min); initial injector temperature 90°C (0.1 min) and increased from 200°C min^{-1} to 280°C (held 31 min); carrier gas He (99.999%) at a flow rate of 1 ml min^{-1} at 150°C oven temperature; manifold, transfer-line and trap temperatures were 45, 280 and 200°C, respectively; operated in the splitless mode; injection volume 2 μl.

GC–MS conditions were: solvent delay 11 min; 70 eV of electron impact energy; scan rate 0.6 scans s^{-1}; scanned-mass range 85–450 *m/z*. The Automatic Gain Control (AGC) was switched on with a target fixed at 5000 counts. Helium (99.999%) at a flow rate of 1 ml min^{-1} was used as the carrier and collision gas. The mass spectrometer was calibrated weekly. The MS–MS parameters are shown in Table 1.

2.6. Assay validation

All validation samples were prepared using sex steroids-free serum [29]. Calibration standards were prepared by adding appropriate working standard solutions to the final (and dried) extract collected during the first 15 min. The validation of the methods included the following studies:

2.6.1. Linearity

Linear calibration graphs in human serum were constructed by least squares regression of concentration versus height or area ratio (analyte/internal standard) of the calibration samples.

2.6.2. Sensitivity

In GC–ECD, detection (LOD) and quantitation (LOQ) limits were calculated on the values of the blank at the retention times of the analytes (10 injections). In GC–MS–MS, LOD was determined as the lowest concentration giving a response of three-times the average of the baseline noise and LOQ as the lowest concentration of a pesticide giving a response of ten-times the average of the baseline.

M. Moreno Frías et al. / J. Chromatogr. B 760 (2001) 1–15

5

Table 1
MS–MS parameters

Pesticide	Activation		Parent ion (m/z)	Excitation amplitude (V)	Excitation storage level (m/z)
	time (min)	m/z Range			
Lindane	11.0–13.5	85–230	219	57	75
Vinclozolin	15.0–16.5	85–290	212	66	71
ISTD	15.0–16.5	85–290	272	80	100
Aldrin	16.5–17.9	85–310	263	96	90
p,p'-DDE	20.5–22.0	85–380	318	88	110
o,p'-DDT	20.5–22.0	85–380	235	64	80
p,p'-DDT	22.0–24.5	85–290	235	65	80

2.6.3. Specificity

Specificity or the existence of potential interferences in the chromatograms from the biological samples was monitored by running control blank samples in each calibration. The absence of any chromatographic components at the same retention time as target pesticides suggested that no chemical interferences were occurring. Using MS–MS if a coeluted interferent has the same identification ion as the analyte, the interference can be avoided using selected experimental conditions for the collision-induced dissociation (CID).

2.6.4. Precision

The intra- and inter-day precision observed in one analysis were assessed using quality control (QC) samples at three concentration levels, 20, 100 and 200 μg l^{-1} in GC–ECD. The samples were all run in pentuplicate ($n=5$) on three different days and the relative standard deviation (RSD) and the relative error (RE) were calculated for each. The intra- and inter-day precision was found by performing a one-way analysis of variance (ANOVA). The intra-assay precision can be interpreted as the intra-run variability and the inter-assay precision as the inter-run variability. Acceptable precision for biological samples was considered to be an RSD of ≤15% at the concentration limits and 10% at higher concentration levels [30].

2.6.5. Stability

The stability stock solutions of the target compounds, as well as the stability of human serum extract samples was studied. The stability of serum extracts was evaluated at room temperature, after 24 h in an autosampler and after three freeze–thaw cycles. In addition, the stability of unreconstituted dry extracts after thirty days of storage in a $-20°C$ freezer was also tested. QC samples of the medium-level concentration, 160 μg l^{-1}, were used in this study.

2.6.6. Recovery

The extraction recovery of the compounds at three QC levels (20, 100 and 200 μg l^{-1}) was determined by comparing the height or area ratio of the compound signals to the internal standard in the extracted samples with the corresponding calibration curves prepared in blank sample extracts. The internal standard was added to both sets of samples after the extraction and clean-up procedures.

3. Results and discussion

3.1. GC–ECD analysis

The separation of the target compounds was optimised by GC–ECD automatically injecting 1 μl of a standard solution containing 200 μg l^{-1} of each compound in n-hexane. Fig. 1a shows the GC–ECD chromatogram of a mixture containing the target compounds and the internal standard. A good separation between them was reached under the final chromatographic conditions selected, that was suitable for obtaining accurate calibrations. This situation was also adequate for the analysis of real samples, where there is always the possibility of poor resolution between the analysed analytes and other compounds frequently used in the area, or even
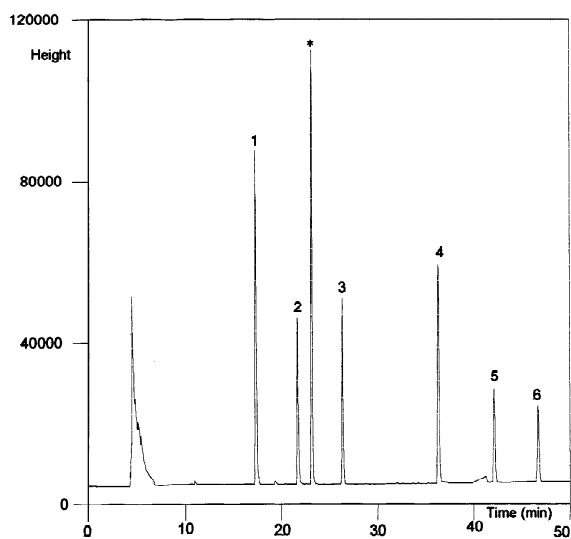
Fig. 1. GC–ECD chromatogram of: (a) a standard solution of the pesticides in *n*-hexane at 200 μg l^{-1}: 1, lindane; 2, vinclozolin; *, ISTD; 3, aldrin; 4, *p,p'*-DDE; 5, *o,p'*-DDT; 6, *p,p'*-DDT.

co-elution of analytes with matrix components. In Table 2 the %RSD for retention times and retention time windows (RTW), calculated as the average of

the retention times ± 3 standard deviation (SD) of the retention times for ten measures are presented.

3.1.1. Linearity and sensitivity

In order to avoid potential matrix effect and the quantitation error associated, calibration curves were prepared in serum blank extracts. The linearity of the chromatographic method was determined injecting 1 μl of spiked blank matrix extract with concentrations ranging from 0.5 to 260 μg l^{-1}. Linear calibration graphs were constructed by least-squares regression of concentration versus peak area and height ratio (analyte/internal standard) of the calibration standards. Slightly better results were achieved using relative heights for all compounds. Table 2 summarises the slopes, intercepts and correlation coefficient values for the validation analysis from human serum extracts. Excellent linearity was found in the concentration range studied, with correlation coefficients between 0.9970 and 0.9999. Detection (LOD) and quantification (LOQ) limits were also calculated obtaining values lower than 85 ng l^{-1} for LOD and 256 ng l^{-1} for LOQ (Table 2). The exception was *p,p'*-DDT that showed higher LOD and LOQ values (104 and 500 ngl^{-1}, respectively).

Table 2
Retention time windows (RTW), %RSD for retention times and calibration data ($n = 10$) by GC–ECD method

Pesticide	RTW[a] (%RSD)	Calibration curve	r	IDL[b] (μg l^{-1})	IQL[c] (μg l^{-1})
Lindane	16.96–17.56 (0.57)	$y = 1.383x - 0.014$	0.9999	0.024	0.080
Vinclozolin	21.34–21.64 (0.46)	$y = 0.448x + 0.008$	0.9999	0.048	0.161
ISTD	22.66–23.26 (0.43)	–	–	–	–
Aldrin	25.89–26.49 (0.38)	$y = 1.139x - 0.011$	0.9999	0.018	0.059
p,p'-DDE	35.91–36.21 (0.27)	$y = 0.599x - 0.008$	0.9996	0.085	0.256
o,p'-DDT	41.60–42.20 (0.23)	$y = 0.323x - 0.008$	0.9991	0.027	0.080
p,p'-DDT	46.15–46.75 (0.21)	$y = 0.284x - 0.013$	0.9971	0.104	0.500

[a] Minutes.
[b] IDL = instrumental detection limit.
[c] IQL = instrumental quantification limit.

M. Moreno Frías et al. / J. Chromatogr. B 760 (2001) 1–15

7

3.1.2. Specificity

The specificity of the assay is demonstrated by the absence of extraneous peaks, in the blank human serum samples, interfering with the signals of the LOQ value of the compounds. Potential interferences were minimised by combining the chemical clean-up using H_2SO_4 with the separation power of the HPLC and the selectivity of the ECD detector. However, it is important to note that the acid clean up used is not a universal method for all pesticides. Dieldrin, chlorothalonil or dichloran are some examples of compounds that are removed from extracts by this acid treatment.

3.1.3. Precision

The intra- and inter-day precision was assessed with three concentrations of QC samples. Table 3 summarises the results and shows that the intra-assay precision (within-run RSD) and inter-assay precision (between run RSD) were found to be lower than 5.5% for the level concentration of 20 µg l^{-1}, and lower than 2.9% for the other QC levels. These

Table 3
Summary of within-day and between-day precision for QC samples[a] using GC–ECD method

Pesticide	Level (µg l^{-1})	Precision (%RSD)	
		within-day[a]	between-day[a]
Lindane	20	0.3	0.4
	100	0.3	0.2
	200	1.0	0.8
Vinclozolin	20	5.5	2.9
	100	1.0	1.1
	200	0.9	1.3
Aldrin	20	2.8	2.3
	100	1.4	1.5
	200	0.5	0.9
p,p'-DDE	20	3.0	2.3
	100	1.0	1.5
	200	1.0	1.0
o,p'-DDT	20	2.6	3.2
	100	1.5	1.4
	200	2.0	1.8
p,p'-DDT	20	3.4	2.8
	100	4.1	4.8
	200	3.0	2.1

[a] $n = 5$.

values indicated acceptable intra- and inter-assay precision for the assay procedure.

3.1.4. Stability

Stock solutions of the target compounds were found to be stable for a minimum of 12 weeks when prepared in *n*-hexane and stored at −20°C prior to and after each use. On the other hand, the stability of the compounds in both reconstituted and unreconstituted human serum extracts was also investigated because it is necessary to have information on the ability to store extracted samples over a period of time allowing a greater flexibility in sample processing and analysis. The stability of the analytes in the injection solvent was studied to verify that the compounds would not degrade over the course of an analysis. This was accomplished by extracting samples, storing and running them into the chromatographic system the following day with a fresh standard curve. Table 4 summarises the results obtained. Reconstituted extracts were found to be stable at ambient temperature up to 24 h. The RSD and accuracy values of a QC sample (medium concentration) at room temperature for 24 h were not significatively different compared to a 0-h sample. The target compounds were also shown to be stable after three freeze (−20°C) thaw cycles with RSD and accuracy values lower than 2.6 and 13.3%, respectively.

The stability of the unreconstituted dry extracts in a −20°C freezer was also determined. The compounds were found to be stable in unreconstituted dry extracts stored at −20°C for at least thirty days with RSD lower than 3.3%. These results are well within acceptable criteria [30] and indicate that the assay is robust under conditions likely to be encountered during sample handling.

3.1.5. Recovery

The extraction recovery of target pesticides at three QC levels was determined by comparing the peak height ratio of the analytes to the internal standard in samples which were spiked with the analyte prior to extraction with the corresponding calibration curves. In general, the recovery of the compounds was independent of the concentration in human serum. As can be seen in Table 5, the recoveries are higher than 65% and the RSD values

Table 4
Summary of precision (%RSD) for a QC sample at 160 µg l^{-1} in different conditions

Pesticide	Study 1 %RSD	Study 2 %RSD	Study 3			Study 4 %RSD
			Cycle 1 %RSD	Cycle 2 %RSD	Cycle 3 %RSD	
Lindane	0.3	1.5	0.4	1.2	1.5	1.4
Vinclozolin	0.4	1.3	0.4	1.0	0.1	1.2
Aldrin	0.2	1.8	0.2	0.8	0.7	2.1
p,p'-DDE	1.2	4.0	1.2	2.0	2.1	2.7
o,p'-DDT	2.0	3.8	2.0	1.0	1.5	3.3
p,p'-DDT	1.3	4.3	1.3	2.6	2.5	2.9

Study 1 corresponds to a just prepared QC sample; Study 2 corresponds to a QC sample after 24 h in the autosampler at room temperature; Study 3 corresponds to a QC sample after three freeze–thaw cycles; Study 4 corresponds to a QC whose unreconstituted dry extract had been stored at $-20°C$ for 8 days. In all studies $n=5$.

are below 7.4%. The exception was vinclozolin at the lower spiking level that showed a recovery 40%. Table 5 also shows the LOD and LOQ data calculated on the bases of the extraction of 10 serum blanks at a signal-to-noise ratio of 3 and 10 respectively. They allowed the analysis of pesticides in human serum samples at trace levels with LOD values ranging from 4 to 22 ng l^{-1}.

On the other hand, similar studies performed in our laboratory for other pesticides [31,32], endosufan, α, β, sulphate, ether and lactone, kepone, methoxychlor and mirex, and polychlorinated biphenyls, congeners 21, 48, 61 and 136, showed good recoveries (70–104%) applying the same extraction procedure.

3.2. GC–MS–MS analysis

In order to confirm and quantify the identity of the compounds in serum samples through the GC–ECD

chromatograms, similar samples were injected into the GC–MS–MS system. This last system, however, allows for error-free identification of the compounds if the optimisation of MS–MS parameters is properly adjusted. Fig. 2 shows a GC–MS–MS chromatogram of the standard compounds, containing both the target analytes and the internal standard at a concentration of 200 µg l^{-1}. All of them were properly separated, under the conditions described in the experimental section, in less than 28 min. RTW of the compounds is shown in Table 6.

However, the GC described method provides little resolution between target analytes and PCBs. The detection technique of MS–MS allows the resolution of overlapped signals (peaks that chromatographically coelute). For that, a MS–MS detector can apply different collision induced dissociation (CID) conditions that conmutates in a short period of time. Each CID condition is specific for each coeluted analyte. The change between CID experimental

Table 5
Recovery percentages and (RSD%) from the blank serum matrix spiked with the pesticides with GC–ECD quantification

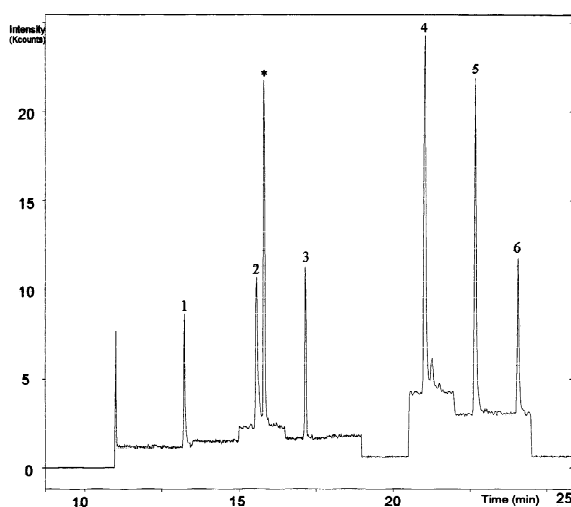| Pesticide	Recovery (RSD)			LOD (µg l^{-1}) [b]	LOQ (µg l^{-1}) [b]
	Spiking level (20 µg l^{-1}) [a]	Spiking level (100 µg l^{-1}) [a]	Spiking level (200 µg l^{-1}) [a]		
Lindane	68 (0.3)	78 (0.3)	72 (1.0)	0.006	0.020
Vinclozolin	40 (5.5)	78 (1.2)	80 (2.0)	0.012	0.040
Aldrin	77 (2.8)	65 (1.6)	65 (1.6)	0.004	0.015
p,p'-DDE	71 (4.2)	70 (3.2)	72 (3.0)	0.022	0.072
o,p'-DDT	98 (2.6)	74 (1.8)	81 (2.0)	0.007	0.022
p,p'-DDT	103 (3.3)	81 (7.4)	80 (3.0)	0.010	0.032

[a] $n=5$.
[b] $n=10$.

Fig. 2. GC–MS–MS chromatogram of a standard solution of the pesticides in *n*-hexane at 200 µg l^{-1}: 1, lindane; 2, vinclozolin; *, ISTD; 3, aldrin; 4, *p,p'*-DDE; 5, *o,p'*-DDT; 6, *p,p'*-DDT.

conditions must permit the acquisition of at least three to five points for each analyte in the time of elution of the co-eluted peak. In practice, it is possible the determination of a maximum of four compounds (under our experience) that coelute, in a single chromatographic peak, in the selected GC

conditions. After acquisition, the plotting of the experimental data obtained separately for each pesticide offers a selective chromatogram for each compound. Quantitation and identification can be conducted for these data (area or height of the GC peak, retention time, MS–MS spectrum).

The process of product ion MS–MS involves two additional steps between the formation and detection of ions. The first step is isolation of a single parent ion from the other ions present and, the second one is dissociation of the parent ion into characteristic product ions before mass analysis of the product ions. Therefore, the ions with the highest intensities in the electron-impact mass spectra are first submitted to isolation and then to fragmentation. In this way the mass–mass spectra of the target compounds were obtained. Obviously, the isolation, fragmentation and storage conditions of the ions obtained (product ions) were optimized for the compounds.

For the mass spectrometer detector, AGC was switched on in order to optimize sensitivity by filling the trap with the target ions. To program the isolation of parent ions for every compound along the chromatographic run, the overall run time was split into six segments for the separation of the pesticides and the ISTD. Each segment was assigned

Table 6
Retention time windows (RTW), %RSD for retention times and calibration data ($n=10$) by GC–MS–MS method

Pesticide	RTW (%RSD)[a]	Calibration curve	r	IDL[b] (µg l^{-1})	IQL[c] (µg l^{-1})
Lindane	12.88–13.48 (0.81)	$y = 1.520x + 0.059$	0.9970	0.5	3.0
Vinclozolin	15.18–15.78 (0.63)	$y = 1.207x - 0.007$	0.9999	2.0	4.0
ISTD	15.46−16.06 (0.63)	–	–	–	–
Aldrin	16.79–17.39 (0.58)	$y = 0.949x - 0.021$	0.9999	0.5	1.0
p,p'-DDE	20.66–21.26 (0.47)	$y = 0.841x - 0.011$	0.9999	0.5	1.0
o,p'-DDT	20.72–21.32 (0.47)	$y = 0.627x - 0.010$	0.9998	0.5	1.0
p,p'-DDT	22.26–22.86 (0.43)	$y = 0.481x - 0.007$	0.9998	2.0	4.0

[a] Minutes.
[b] IDL=instrumental detection limit.
[c] IQL=instrumental quantification limit.

to a m/z fragment according to the base ion obtained from the EI mass spectrum for each compound. The ions chosen were as follows: lindane (m/z 219), vinclozolin (m/z 212), ISTD (m/z 272), aldrin (m/z 263), p,p'-DDE (m/z 318), o,p'-DDT (m/z 235), p,p'-DDT (m/z 235), Fig. 3.

Inside each time segment, parent ions were isolated by ejecting from the trap those ions whose m/z lay outside a ±1 units window around the chosen parent m/z value.

Then, dissociation conditions were optimized for each pesticide. In general, the parent ion isolated from the first ionization was selected with the aim of achieving a compromise between both selectivity (the highest m/z ion) and sensitivity (the highest intensity ion). To do this, parent ions were CID fragmented with the goal of generating spectra with multiple ions of high relative intensity that afforded accurate quantification while preserving a certain proportion (10 and 20%) of the parent ions. This aim can be achieved by appropriate selection of the non-resonant excitation amplitude and excitation storage level.

The excitation storage level, which is related to the trapping field that stabilises the parent ion, was selected as the minimum value that allowed the dissociation of the parent ion. If a higher excitation storage level was applied the ions were held in the trapping field more strongly allowing the application of a higher excitation voltage, which was able to produce more product ions. Nevertheless, if the excitation storage level was too high, the lower weight product ions will not be trapped and will not be observed in the spectrum.

The MS–MS spectra obtained in the final experimental conditions were stored in an home-made MS–MS library. The main ions are shown in Fig. 3. Criteria for validation of the GC–ECD method were also used for establishing the GC–MS–MS method.

3.2.1. Linearity and sensitivity

The linearity of the chromatographic method was determined by injecting 2 μl of spiked blank matrix extract with concentrations ranging from 0.1 to 260 μg l^{-1}. Calibration graphs and the different parameters associated with them were studied using both areas and heights relative to the internal standard. Better results were achieved using relative areas. All

the pesticides, showed good linearity with correlation coefficients between 0.997 and 0.999 (Table 6). Detection (LOD) and quantification (LOQ) limits were also calculated with values lower than 2 μg l^{-1} for LOD and 4 μg l^{-1} for LOQ.

3.2.2. Precision

The data for the within and between-day precision was assessed by analysing five replicates, at three concentrations of QC samples, as in the GC–ECD method (Table 3). The results obtained at the higher concentration level (200 μg l^{-1}) are summarised in Table 7. The within and between-day precision were found to be <12.0% for the level concentration of 20 μg l^{-1}, and lower than 10% for the other QC concentration levels. These measures indicated acceptable intra- and inter-assay precision for the assay procedure.

3.2.3. Stability

Stability studies were also performed in GC–MS–MS. They showed similar results to the ones obtained in GC–ECD, demonstrating that for these pesticides the stability does not depend on the analytical technique tested.

3.2.4. Recovery

The extraction recovery of target pesticides at three QC levels was determined by comparing the peak area and height ratio of the analytes to the internal standard in samples which were spiked with the analyte prior to extraction with the corresponding calibration curves. Each experiment was repeated three times. The recovery and RSD data were calculated at three concentration levels (20, 100 and 200 μg l^{-1}) and the ones obtained at the higher level using relative peak areas are reported in Table 7. Recoveries were higher than 73% and the RSD values are below 10% in all cases. Table 7 shows also the LOD and LOQ data calculated on the basis of the extraction of 10 serum blanks at a signal-to-noise ratio of 3 and 10 respectively. They allowed the analysis of pesticides in human serum samples at trace levels.

On the other hand, the proposed method showed to be effective in the recovery of other pesticides, endosufan, α, β, sulphate, ether and lactone, kepone, methoxychlor and mirex, and polychlorinated bi-
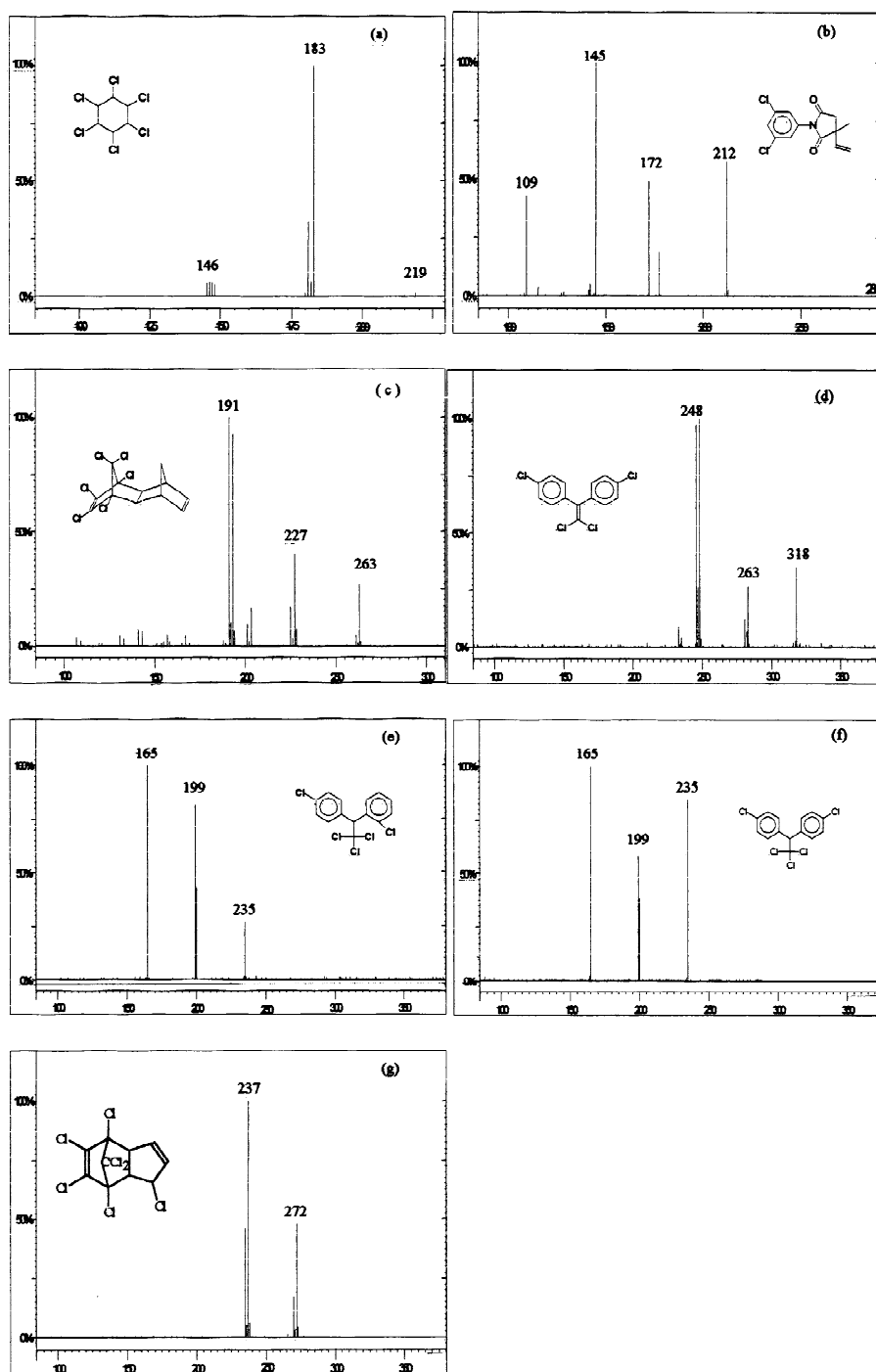
Fig. 3. EI–MS–MS spectra and structural formulas of: (a) lindane; (b) vinclozolin; (c) aldrin; (d) *p,p′*-DDE; (e) *o,p′*-DDT; (f) *p,p′*-DDT; (g) ISTD.

Table 7
Summary of precision (%RSD), recovery percentages, RSD%, LOD and LOQ by the GC–MS–MS method

Pesticide	%RSD*		Recovery[a]	LOD (μg l^{-1})[b]	LOQ (μg l^{-1})[b]
	intra[a]	inter[a]			
Lindane	3.8	4.3	75 (6.8)	0.1	0.3
Vinclozolin	2.9	7.3	76 (8.5)	0.5	1.5
ISTD	–	–	–	–	–
Aldrin	4.2	7.9	75 (5.1)	0.1	0.3
p,p'-DDE	3.2	6.0	77 (6.6)	0.1	0.3
o,p'-DDT	4.5	7.3	76 (10.0)	0.1	0.3
p,p'-DDT	3.6	6.8	73 (7.9)	0.5	1.3

* Data obtained with a QC sample of 200 μg l^{-1}.
[a] $n = 5$.
[b] $n = 10$.

phenyls, congeners 21, 48, 61 and 136, with values ranging from 70–114% [31,32].

In short, a comparison of the GC–ECD and GC–MS–MS methods shows a good linearity of the pesticides in the studied concentration ranges, with $r > 0.99$. The GC–ECD method showed better precision values than the GC–MS–MS method.

4. Application of the method to the analysis of human serum samples

To assess the applicability of the methodology, six human serum samples from patients affected with breast cancer were analysed. Analysis of laboratory blank serum samples, laboratory spiked serum blank and laboratory spiked matrix samples were performed together with the set of samples, in addition to calibration graphs. Laboratory blanks rejected any contamination of interference due to reagents during processing samples. Analysis of samples was carried out if recoveries were between 70 and 80% in both laboratory spiked blank and laboratory spiked matrix samples.

The presence of the pesticides in the extracts was quantified and confirmed taking into account that those compounds were monitored in two detectors, ECD and tandem MS. No target compounds were found in the chromatograms corresponding to the laboratory blanks processed.

An interfering peak was revealed near the retention time of p,p'-DDE in two serum samples by the GC–ECD method, which complicated a reliable analysis of this compound. This problem was avoided when using GC–MS–MS analysis. Figs. 4a and b show, respectively, the chromatograms obtained for the same extract in GC–ECD and GC–MS–MS.

In addition, p,p'-DDT was detected in one serum sample by GC–ECD, but with a very low signal that was easily confused with the background signal (Fig. 5a). In order to confirm the presence of the cited peak the sample was analysed by the GC–MS–MS method, which allowed its identification and quantification at a concentration of 15.5 μg l^{-1} (Fig. 5b).

Therefore, it is evident the great advantage of GC–MS–MS over GC–ECD in the elimination of matrix interferences in the analysis of complex biological samples.

5. Conclusions

Validated GC–ECD and GC–MS–MS assays have been developed to determine trace levels of endocrine-disrupting compounds in human serum samples. Both methods offer good linearity and sensitivity and meet all method validation criteria. GC–ECD method shows better precision and accurate and, on the contrary, the GC–MS–MS method presents a higher selectivity.

The tandem capabilities of the ion trap allow the positive confirmation of the nature of each peak, being possible, on one hand, the isolation of the
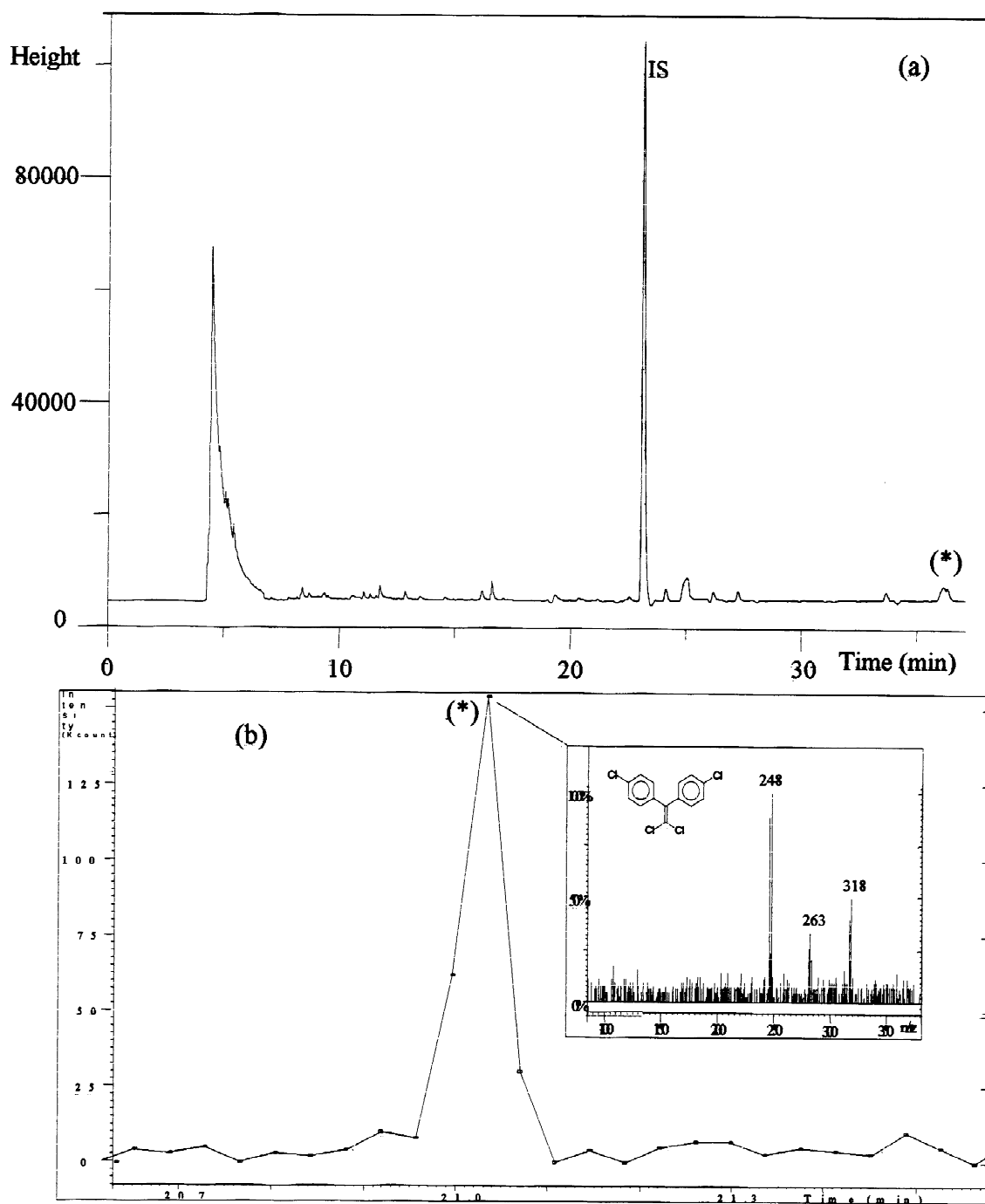
Fig. 4. Gas chromatogram of a human serum sample using (a) ECD and (b) MS–MS monitoring the quantification ion (m/z 248) of p,p'-DDE (*).
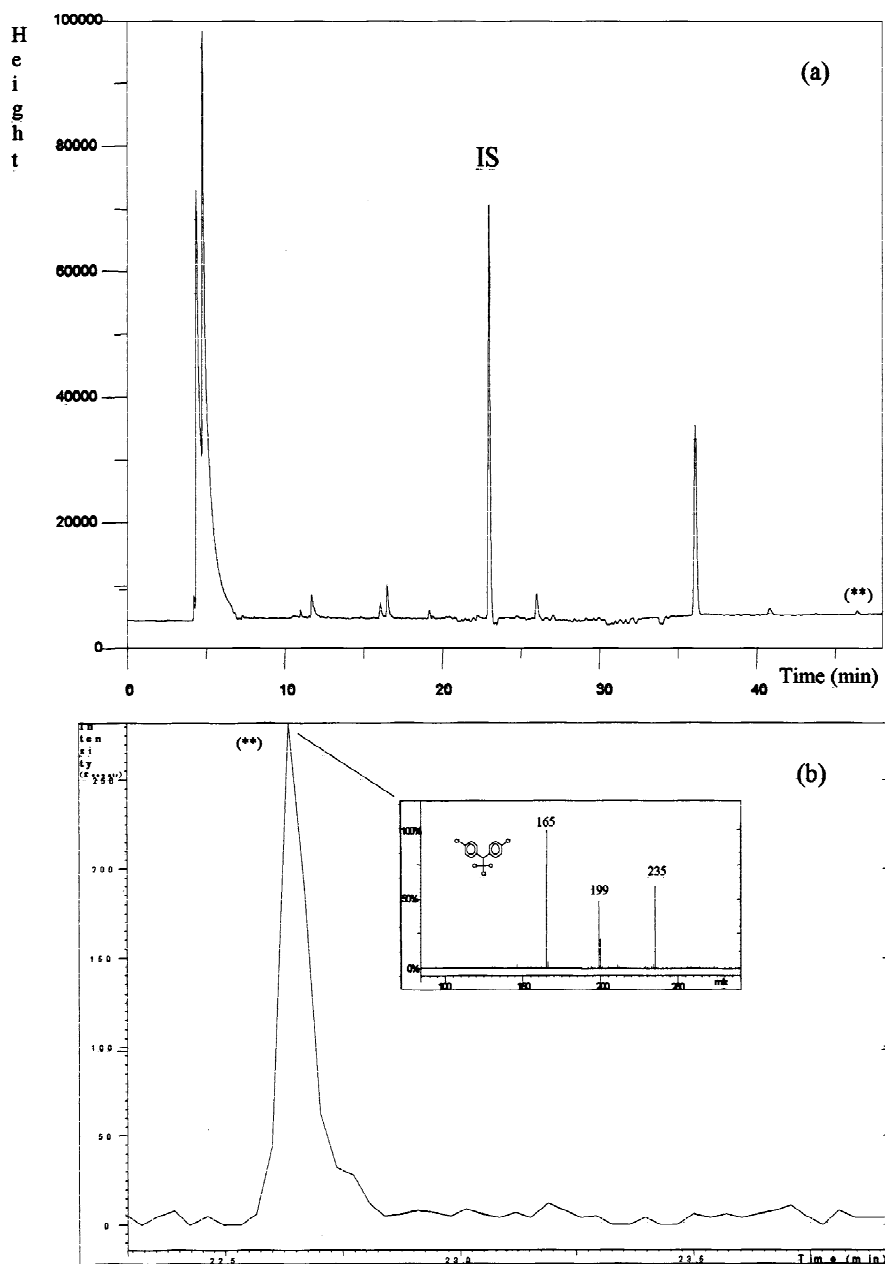
Fig. 5. Gas chromatogram of a human serum sample using (a) ECD and (b) MS–MS monitoring the quantification ion (m/z 165) of p,p'-DDT (**).

target signal from the complex matrix permitting reproducible quantification and, on the other hand, the elimination of matrix interferences by monitoring of secondary fragmentation products avoiding false positive results.

Acknowledgements

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