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Determination of endosulfan and its metabolites in human urine using gas chromatography–tandem mass spectrometry[1]

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Abstract

A method was developed for determining the occupational exposure to endosulfan and its main metabolites (endosulfan-ether, -lactone and -sulfate) in human urine using dieldrin as an internal standard. Solid phase extraction (SPE) and gas chromatography–tandem mass spectrometry (GC–MS–MS) have been used due to their high sensitivity and selectivity in avoiding most matrix interferences. The recovery efficiencies of the tested compounds yielded more than 89.2 % at the fortification level of 10 ng ml^{-1} in urine and their relative standard deviations were between 9.1 and 12.8 %. The detection limit of each compound ranged between 6 and 18 pg ml^{-1}. Urine samples from nine pest control operators were analysed and total endosulfan concentrations between 94 and 2038 pg ml^{-1} were found.

Keywords: Endosulfan; Pesticides

1. Introduction

Endosulfan ((1,4,5,6,7,7-hexachloro-8,9,10-trinorborn-5-en-2,3-ylenebismethylene) sulfite) is a chlorinated insecticide used in many countries. It is liposoluble and persistent in the environment [1]. In the technical grade, endosulfan contains two stereoisomers: α- and β-endosulfan (α- and β-end). It is rapidly metabolised and excreted in the urine and feces as oxidation products like endosulfan-sulfate (end-sulfate), -alcohol (end-alcohol), -ether (end-ether) or -lactone (end-lactone) resulting from the cleavage of the cyclic sulfite group. Endosulfan penetrates the intact skin and is also absorbed by inhalation and from the gastrointestinal tract [2–5].

Endosulfan has shown estrogenic effects on humans [6,7]. For mammals it presents a moderate toxicity and is highly toxic to fish and some bird species. This insecticide has been analysed in natural waters [8], greenhouse air [9], fish [10], human milk [11], meat [12] and sediments [13], most of them using GC–ECD but there is no reported method for the analysis of endosulfan and its metabolites in human urine.

Previous experiments carried out [9] showed that endosulfan can be successfully sampled in air using polyurethane foam (PUF). Dissipation processes of endosulfan indicate that 24 h after application in the

[1]This article is dedicated to Dr. Antonio Arrebola Ramírez of the University of Granada, in memoriam.

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greenhouse, 7.5 % of the initial concentration remained in the greenhouse atmosphere. The dissipation and decline process may be influenced by parameters such as vapour pressure, temperature and relative humidity or the presence of volatile organic solvents in emulsifiable concentrates as in endosulfan formulations.

In this paper a method is proposed for determining endosulfan and its main metabolites in human urine using solid phase extraction (SPE) and gas chromatography–tandem mass spectrometry (GC–MS–MS) which are both very sensitive and selective. The results obtained from an analysis of urine samples from agricultural workers in Almería (Spain) are reported.

2. Experimental

2.1. Chemicals

Pesticide grade *n*-hexane, diethyl ether and methanol from Merck (Darmstadt, Germany) were used. Organic free water was prepared by distillation and then by Milli-Q SP treatment (Millipore Corporation, USA).

Pesticide analytical standard materials were purchased from Riedel-de-Haën (Seelze-Hannover, Germany) with purities higher than 99 %.

Stock solutions of individual pesticides at 400 μg ml^{-1} were prepared in *n*-hexane and stored in a freezer (−30°C). The standard working solutions were obtained by appropiate dilutions with the same solvent and stored in a refrigerator (4°C).

A buffer solution (pH=7) was prepared by dissolving 1.179 g of KH_2PO_4 and 4.303 g of Na_2HPO_4 (both from Merck) in a litre of Milli-Q water.

Sep–Pak cartridges for solid phase extraction packed with 500 mg of C_{18} purchased from Waters (Milford, MA, USA) were available.

2.2. Equipment

A Saturn 2000 ion trap mass spectrometer (Varian Instruments, Sunnyvale, CA, USA) was used. The gas chromatograph was fitted with a split/splitless programmed temperature injector SPI/1078 operated in the splitless mode and a DB5-MS 30 m×0.25 mm I.D.×0.25 μm film thickness (J&W Scientific, Folsom, CA, USA) chromatographic column. 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, other EI-MS libraries were available. The carrier gas used was helium (purity 99.999%).

A test tube shaker with a speed control was purchased from Ika-Works, Inc. (Wilmington, NC, USA).

2.3. Sample collection

Urine from nine pest control operators was collected from volunteers and stored in sterilised containers. The samples were immediately frozen and kept at −30°C until they were analysed. The operators' exposure to the pesticide was assessed by questionnaire, interview and medical examination.

2.4. Analytical procedure

2.4.1. Urine extraction procedure

Three ml of urine were mixed with 7 ml of buffer solution (pH=7) using a test tube shaker. The mixture was passed through the C_{18} cartridge previously conditioned with 5 ml of methanol and 3 ml of buffer solution (pH=7) in that order and avoiding dryness. To carry out a clean up step, 1 ml of distilled water and 0.1 ml of methanol consecutively were added. The cartridges were dried by passing air through them for 10 min by means of a vacuum pump. The analytes were eluted using 5 ml of a mixture *n*-hexane–diethyl ether (80:20). The extract obtained was evaporated under a stream of nitrogen without heat and redissolved in 0.5 ml of *n*-hexane. Thus the extract was ready to be injected into the gas chromatograph.

2.4.2. GC–MS–MS conditions

A 2 μl aliquot of the extract was injected with the split in the gas chromatograph closed for 1 min. The carrier gas was used with the head pressure set at 12 psi (36.6 cm s^{-1} at 150°C oven temperature). The injector temperature was programmed from 90°C

Table 1
Mass spectrometer conditions

Ionisation mode	EI
Multiplier voltage	1700 V
Multiplier gain	1×10^5
A/M amplitude voltage	3.6 V
Trap temperature	200°C
Manifold temperature	45°C
Transfer-line temperature	260°C
Emission current	80 μA
Automatic Gain control (AGC)	on
AGC prescan ionisation time	1500 μs
AGC target	3000 counts

(hold 0.1 min at 90°C) to 280°C at 200°C min^{-1} (hold 18 min at 280°C). The oven temperature was modified from 60°C (hold 1 min at 60°C) to 220°C at 50°C min^{-1} and then from 220°C to 300°C at 5°C min^{-1}.

The mass spectrometer was calibrated weekly. Its experimental parameters are summarised in Tables 1 and 2. For the MS–MS conditions, all the compounds were analysed using a non-resonant wave form type, except for dieldrin (resonant).

3. Results and discussion

3.1. Effect of experimental variables

3.1.1. Instrumental variables

The gas chromatographic conditions yielded a satisfactory separation of the analytes. The retention times for the insecticide and its metabolites in these chromatographic conditions are 7.07 min (end-ether), 8.48 min (end-lactone), 9.22 min (α-end), 9.75 min (dieldrin, internal standard), 10.44 min (β-end) and 11.30 min (end-sulfate). Less than 12 min were required for an adequate separation of all the compounds. For the mass spectrometer detector, Automatic Gain Control (AGC) was switched on in order to optimise sensitivity by filling completely the trap with target ions. The AGC target was fixed at 3000 counts because higher values caused electrostatic interactions between ions in the ion trap chamber. For each analyte, a parent ion was chosen by taking into consideration its m/z and its relative abundance (both as high as possible), so as to improve sensitivity. An isolation window of 2 u was used for all the compounds as most of them have ion clusters in their MS spectrum and wider windows would therefore catch additional neighbouring ions and worsen reproducibility. A non-resonant wave form (second ionisation) was selected for all the compounds except dieldrin which needed more cleavage energy to obtain a good quality secondary spectrum. The object was to generate spectra with the parent ions as their molecular peaks (between 10 and 20 % of relative abundance). The excitation amplitude was studied for this propose. The EI-MS–MS spectrum of endosulfan and its metabolites in the experimental conditions proposed are represented in Fig. 1. The base peak was selected for quantitation in all cases.

3.1.2. Extraction variables

SPE using C_{18} sorbents was selected because previous experiments have shown that liquid–liquid extraction using *n*-hexane as an organic solvent forms emulsions. A clean-up step is recommended because there are interferences from the matrix

Table 2
MS–MS conditions[a]

Compound	Activation time (min)	m/z range	Parent ion (m/z)	Mass defect (mu/100 u)	Excitation amplitude (V)	Excitation storage level (m/z)
End-ether	6.0	80–260	241	−40	83	80
End-lactone	8.0	100–340	321	−43	90	141
α-End	9.0	80–260	241	−18	83	80
Dieldrin (IS)[b]	9.5	50–300	279	−23	2	123
β-End	10.0	80–260	241	−18	83	80
End-sulfate	11.0	120–290	272	−17	95	119

[a] Excitation time=40 μs; isolation window=2 u.
[b] Non-resonant waveform.

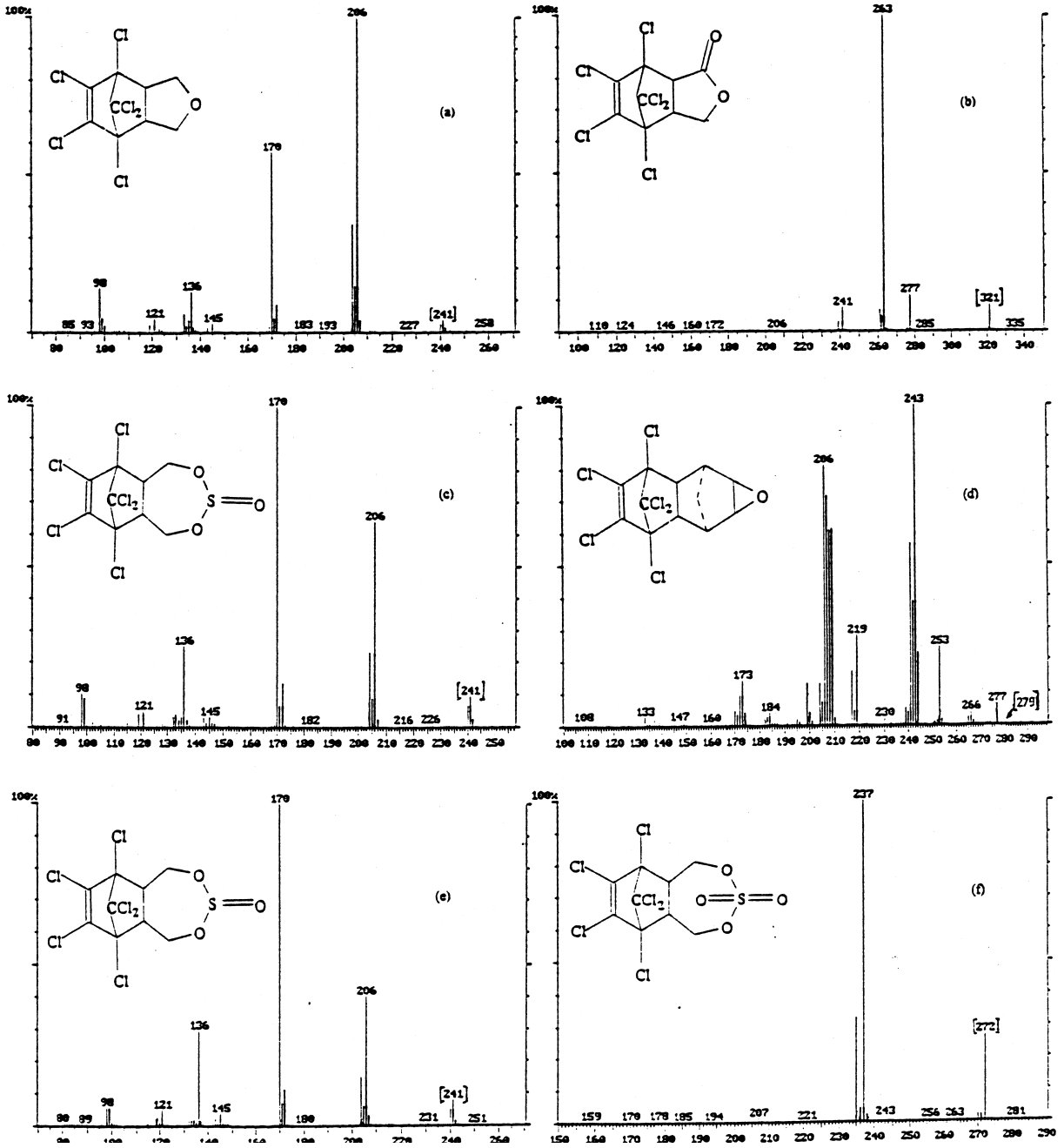

Fig. 1. EI-MS–MS spectra and structural formulae: (a) end-ether (m/z 241); (b) end-lactone (m/z 321); (c) α-end (m/z 241); (d) dieldrin (m/z 279) (IS); (e) β-end (m/z 241); (f) end-sulfate (m/z 272).

which elute with the target analytes and dirty the injector inlet, the column and the ion trap. Better recoveries and easier sample handling were observed when cartridges were dried before elution. Otherwise, water may pass through the cartridge and impede the redissolution of the analytes with the

organic solvent later added. The best results for the elution were found with a *n*-hexane–diethyl ether mixture (80:20). End-alcohol was not analysed since it requires prior derivatisation.

3.2. Identification and quantitation of target analytes

A working standard solution was analysed first to calibrate the GC–MS–MS system and to produce search files for all the analytes of interest. An analyte search and identification was carried out by using of the automatic search and identification menus of the instrument's Data System. The target analytes were searched at present retention times (±10 s window) and were identified by comparing the EI-MS–MS spectrum obtained with the known spectra of those analytes previously stored under the same experimental conditions. A positive analyte identification required a minimum spectral fit of >700 and signal-to-noise ratio (S/N) of >3 (for quantitation ions). For quantitation, S/N must be higher than 10.

Some chromatograms of endosulfans in urine obtained using EI-MS and EI-MS–MS are represented in Fig. 2. There are a substantial number of interfering chromatographic peaks and a high background when GC–MS is used, even with the selection of the quantitation ions for the compounds. The gas chromatographic conditions for GC–MS were different from those selected for GC–MS–MS for separating dieldrin and an interferent close to it. The measure of just one characteristic ion for the analyte (Selected Ion Monitoring, SIM), increases the sensitivity but reduces the qualitative information; the risk of false positives is higher. With MS–MS, if a coeluted interferent has the same identification ion as the analyte, such an interferent can be avoided using special experimental conditions for the second ionisation and quantifying with a specific ion from the analyte. The peak next to the internal standard in Fig. 2c is a matrix peak.

The instrument calibration was performed using clean urine samples fortified at 50, 100, 500 and 1000 pg ml^{-1} of each insecticide and metabolite. Dieldrin was used as the internal standard at 1000 pg ml^{-1}. It was selected because it has the properties that an internal standard should possess, such as a similar structure to the analytes, similar signal, elution between the analytes and in addition, it was never found in real samples. Calibration curves and calculations were performed using the quantitation menu of the Data System and the results are shown in Table 3.

The lack-of-fit test was used for three replicates of each concentration in order to check the linearity of the calibration graphs. The values found for the test (P_{lof}(%)) demonstrate good linearity for all the compounds in the studied range.

3.3. Recoveries and limits of detection

Recovery studies were performed three times at the 10 ng ml^{-1} level of each pesticide and metabolite in urine samples. These samples were prepared by adding 30 μl of a 1 μg ml^{-1} pesticide stock solution to 3 ml of clean urine and stirring vigorously in a test tube. The extracts were analysed as previously described and the recovery and reproducibility for each analyte are presented in Table 4.

All the compounds present recovery rates higher than 89.2 % and reproducibility expressed as relative standard deviation (RSD) lower than 12.8 %. Table 4 also summarises the detection (DL) and quantitation (QL) limits calculated for the endosulfan and its metabolites for the proposed method. The values obtained are in the low ppt range showing the high sensitivity and selectivity of EI-MS–MS. This is due basically to the drastic reduction of background of the matrix, something which is very important in dirty samples.

3.4. Applications of the method

To confirm the availability of the method, real samples from nine pest control operators (eight male and one female) were subjected to analysis. The operators were between 20 and 55 years old and with work experience that ranged from 0.6 to 40 years. Four of them had worked applying pesticides the day before and the other five a week before. All of them applied pesticides for between 2 and 5 h. Fig. 3 represents the chromatogram obtained for one of the applicators. The results obtained are listed in Table 5.

The results summarised in Table 5 show that the workers who applied pesticides the day before

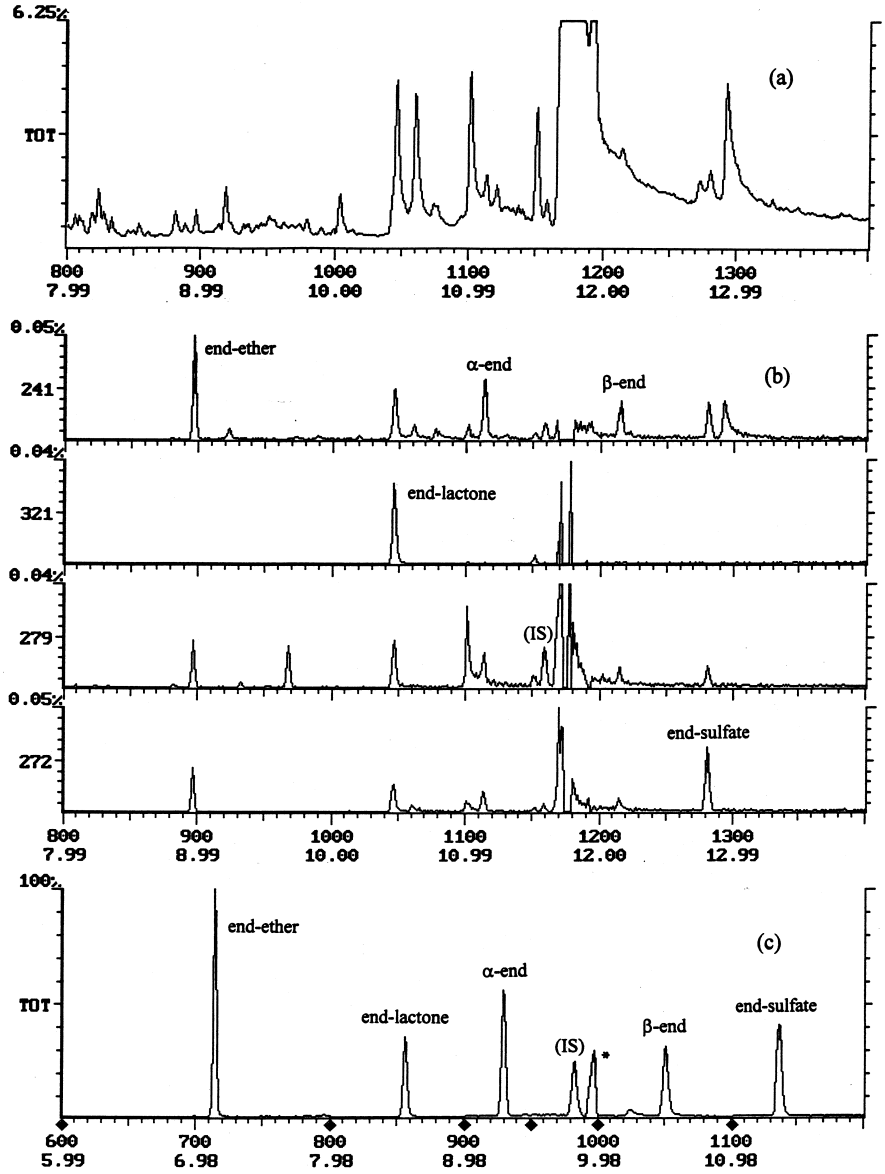

Fig. 2. Chromatograms of endosulfan and its metabolites in human urine. (a) Total ion chromatogram using EI-MS; (b) mass chromatograms using EI-MS and selecting the quantitation ions; (c) total ion chromatogram using EI-MS–MS. (*) Matrix peak.

present higher concentrations of the insecticide especially for α- and β-end. End-ether was the main metabolite in the studied urine samples and end-sulfate was not found. On the other hand, farm workers who had applied pesticides a week previous to the collection of the samples, presented lower amounts of pesticide. Operator number 7 did not show any contamination at all. Responses to the questionnaires and interviews reveal that most of them rarely use protective overalls, breathing masks or gloves when they apply the pesticides and never when they prepare the formulation applied. They do not take meals or smoke inside the greenhouse, nor do they wash their hands after a day's work. Reentry

Table 3
Calibration curves[a]

Compound	*a*	$b \cdot 10^3$	R^2	P_{lof}(%)
End-enther	0.138	4.17	99.87	0.24
End-lactone	0.038[b]	4.23	99.82	0.58
α-End	0.012[b]	2.96	99.72	0.59
β-End	0.046	2.10	99.72	0.12
End-sulfate	0.068	4.41	99.86	0.33

[a] $n=3$. *a*, intercept; *b*, slope; R^2 correlative coefficient; P_{lof} (%), Probability level of lack-of-fit.
[b] Intercept statistically non-significant.

Table 4
Recoveries (*R*%), relative standard deviations (RSD), detection (DL) and quantitation (QL) limits in urine[a]

Compound	*R*%	RSD	DL (pg ml^{-1})	QL (pg ml^{-1})
End-ether	89.2	11.6	6	18
End-lactone	90.3	12.8	9	31
α-End	111.8	10.4	9	31
β-End	97.9	9.6	18	60
End-sulfate	106.4	9.1	13	44

[a] $n=3$.

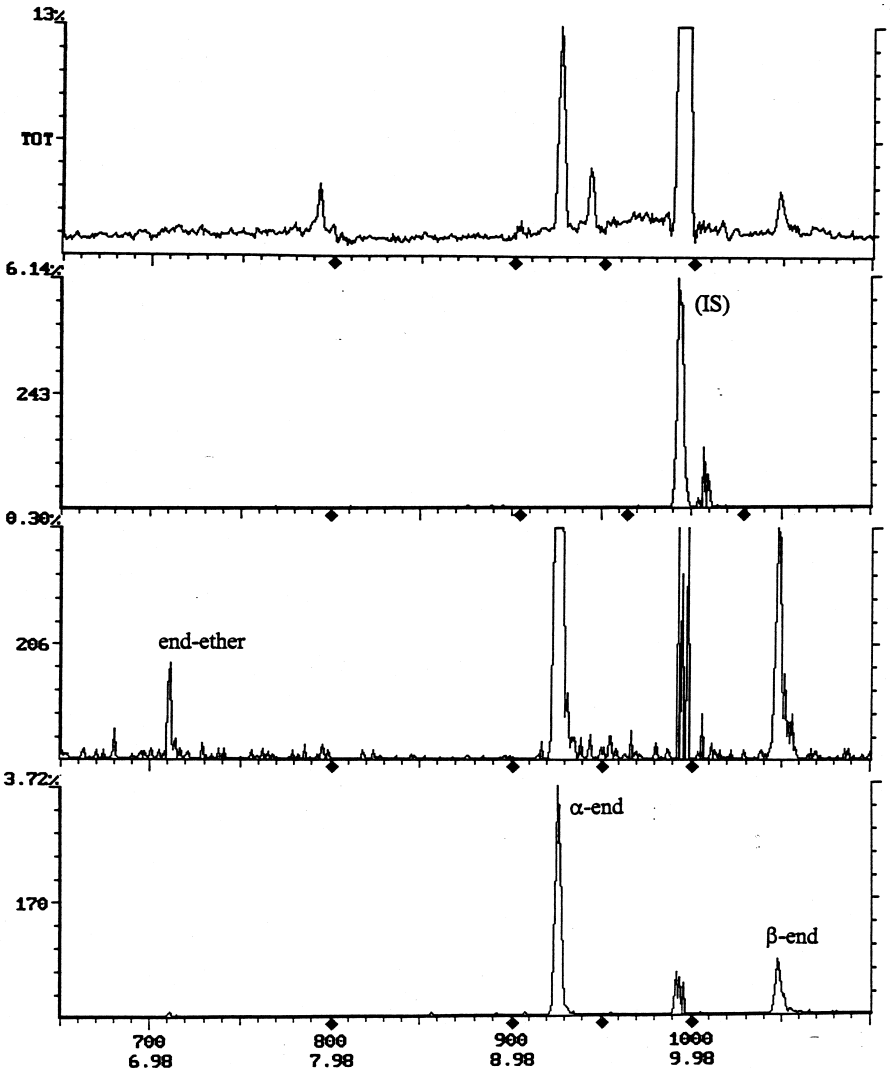

Fig. 3. Total ion and mass chromatograms using EI-MS–MS and selecting the quantitation ions of a pest control operator's urine.

Table 5
Results (pg ml^{-1}) for the biological monitoring of pest control operators[a]

PCO number	Last application	Age	Work experience (years)	End-ether	End-lactone	α-End	β-End	End-sulfate	Total
1		55	40	78	n.d.	894	896	n.d.	1868
2	1 day	35	20	72	n.d.	787	801	n.d.	1660
3	ago	37	20	n.d.	n.d.	840	826	n.d.	1666
4		40	0.6	125	222	862	829	n.d.	2038
5		50	17	n.d.	n.d.	90	n.d.	516	606
6	7 days	20	5	n.d.	n.d.	123	n.d.	n.d.	123
7	ago	51	40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8[b]		47	7	n.d.	428	84	169	n.d.	681
9		37	25	n.d.	n.d.	94	n.d.	n.d.	94

[a] PCO, pest control operator.
[b] Female operator.

field precautions are not observed by most of the workers. Non abnormal symptoms were found in the medical exploration.

4. Conclusions

A fast and accurate method with high sensitivity is reported for the occupational analysis of endosulfan and its metabolites in human urine. Solid phase extraction and GC–MS–MS have proven their selectivity in that they avoid most of the interferences from the matrix. Urine samples from nine pest control operators were analysed and total concentrations of pesticide and metabolites that ranged between 94 and 2038 pg ml^{-1} were found.

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