Brief Report

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A simple and economical method of gas chromatography-mass spectrometry to determine the presence of 6 pesticides in human plasma and its clinical application in patients with acute poisoning

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Summary **An economical, rapid, and sensitive method of gas chromatography-mass spectrometry (GC-MS) was developed and validated to determine the presence of six pesticides (dichlorvos, acetochlor, atrazine, chlorpyrifos, α-endosulfan, and β-endosulfan) in human plasma. The pesticides were extracted with acetonitrile and concentrated using anhydrous sodium sulfate. Then, the target compounds were analyzed and quantified with GC-MS using borneol as an internal standard. Separation was performed on a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) with temperature programming. Detection was accomplished under electro-spray ionization (ESI) in selected ion monitoring (SIM) mode. Under optimized conditions, satisfactory linear ranges of 0.05-10 μg/mL were obtained for all of the analyzed pesticides. The linear correlation coefficients were greater than 0.99. The average recovery was between 86.8 and 106.5%. The inter- and intra-day precision ranged from 1.7-14.5% and 4.2-13.8%, respectively. Dichlorvos was unstable in plasma both at room temperature and when frozen. The other five pesticides were stable after storage at –20°C for 17 days and two freeze-thaw cycles. Thirty-five plasma samples from 15 patients with acute self-poisoning were analyzed using this method. Dichlorvos was found in 13 plasma samples with a mean concentration of 0.289 μg/mL, and atrazine was found in 6 with a mean concentration of 0.261 μg/mL. Acetochlor was found in one plasma sample (0.153 μg/mL). This method is simple, reliable and cost-effective. It takes little time and does not waste solvents, and it can be used to routinely detect six pesticides in patients with acute poisoning.**

Keywords: Gas chromatography-mass spectrometry, pesticides, determination, plasma

1. Introduction

Numerous pesticides have been widely used in agriculture, industry, and medicine. However, the long-term and large-scale use of these chemicals inevitably contaminates the environment, thereby posing a serious threat to human health (*1,2*). Pesticides are leading causes of morbidity and mortality following intentional self-poisoning or in cases of occupational or environmental exposure (*3,4*). Self-poisoning with pesticides is a major problem (*5*). Thus, sensitive and efficient methods to detect pesticides in human blood or urine need to be developed.

Rapid identification and quantification of causal pesticides would provide useful information to clinicians so that they can make appropriate treatment decisions (*6*). In cases of acute poisoning, determining the presence of a pesticide in human blood can provide information to identify the type of pesticide, the absorbed dose, and the degree of exposure to target tissues prior to elimination from the body (*7,8*). The volatility, thermal stability, and low polarity of pesticides render them suitable for gas chromatographic analysis, and particularly for the determination of their presence in biological matrices (*9*). Gas chromatography coupled with mass spectrometry (GC-MS or GC-MS/MS), which can avoid most matrix interference, has been used when a highly selective

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detection is required (10,11).

A few methods of gas chromatography with specific detectors including a flame ionization detector (FID) (*12,13*), a nitrogen-phosphorus detector (NPD) (14,15), a flame photometric detector (FPD) (*16,17*), and mass spectrometer detectors (*18-20*) have been used to determine the presence of pesticides in human blood, plasma, or serum. However, these methods were mainly developed to determine the presence of organophosphate pesticides and not other types of pesticides, and the extraction procedure used was mainly solid phase extraction (SPE), which is complex, takes time, and is expensive. A simple and rapid method of sample extraction needs to be developed to determine the presence of pesticide in order to provide useful information to clinicians for accurate diagnosis and treatment.

The aim of this study was to develop, optimize, and validate a simple and rapid method for simultaneous determination of the presence of six pesticides including dichlorvos, acetochlor, atrazine, chlorpyrifos, α-endosulfan, and β-endosulfan in human plasma. These pesticides different from those detected using the methods mentioned above, and they were selected based on the frequency of cases of poisoning, clinical requirements, and their physical and chemical properties. Hence, a new and sensitive method for simultaneous determination of the presence of these pesticides in human plasma was developed using a combination of rapid protein precipitation (PPT) extraction and GC-MS. The method is simple, rapid, economical, and successfully detects the selected pesticides in patients with acute self-poisoning.

2. Materials and Methods

2.1. *Reagents and materials*

Dichlorvos (99.2%, Lot No. 20161121), acetochlor (99.2%, Lot No. 20161125), atrazine (99.4%, Lot No. 20161207), and chlorpyrifos (98.0%, Lot No. 20160810) were purchased from Shanghai Pesticide Research Institute Co., Ltd. (Shanghai, China). α-endosulfan (99.0%, Lot No. 41020) and β-endosulfan (99.0%, Lot No. 41013) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Borneol (internal standard, Lot No. 110881-200706) was provided by the National Institutes for Food and Drug Control (Beijing, China). Chromatographic-grade acetonitrile and hexanes were purchased from J.T. Baker (Phillipsburg, New Jersey, USA). Anhydrous sodium sulfate (99%, Lot C1410040) was purchased from Aladdin Industrial Corporation (Shanghai, China). Blank human plasma was collected from healthy donors and stored immediately at –20°C until analysis. This study was approved by the ethics committee of Qilu Hospital of Shandong University. Signed informed consent was obtained from each donor.

2.2. *Instrumentation and analytical conditions*

The GC apparatus used in this study was an Agilent 7890A equipped with an Agilent 7683 autosampler, and the split/splitless injector was operated in splitless mode. The apparatus was coupled to an Agilent 5975C mass spectrometer (Agilent Technologies, Inc, Santa Clara, CA, USA). The analytical column was an HP-5MS Agilent column (30 m × 0.25 mm × 0.25 μm, Santa Clara, CA, USA). The chromatograph was programmed for an initial temperature of 80°C for 4 min. The temperature was increased to 200°C at a rate of 70°C/min and then increased to 250°C at a rate of 5°C/min, held for 2 min, and then increased to 300°C at a rate of 50°C/min, held for 5 min. The total run time was 23.7 min. Helium was used as the carrier gas (flow rate: 2 mL/min). The mass spectrometer was operated in the electron impact (70 ev) and selected ion monitoring (SIM) mode both for qualitative and for quantitative analysis, with a solvent delay of 3.75 min. For each analyte, the most abundant and characteristic mass fragment ion was chosen for quantitation along with two others for confirmation, as shown in Table 1. The compounds were subsequently identified based on their relative retention times and the ratio of their respective confirmation ions to their quantitation ion.

2.3. *Preparation of standard solutions, calibration standards, and quality control samples*

A stock solution for each pesticide was prepared at a concentration of 2 mg/mL in acetonitrile. Mixed working solutions of the six pesticides were prepared at concentrations of 1, 2, 4, 10, 20, 40, 100, and 200 μg/mL in acetonitrile. Calibration standards and quality control (QC) samples were prepared by appropriate dilution of the above working solutions in blank plasma to obtain concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg/mL, and 0.15, 0.8, and 8 μg/mL. An IS solution of borneol was prepared at 1 mg/mL in acetonitrile and further diluted with acetonitrile to reach a concentration of 3 μg/mL. All stock and working solutions in acetonitrile were stored at 4°C.

Table 1. GC parameters used for the selected pesticides

Pesticide	Retention time (min)	Quantitation ion (m/z)	Confirmation ions (m/z)
Borneol	6.00	95.1	110.1, 138.2
Dichlorvos	6.49	109.0	185.0, 79.0
Atrazine	9.12	314.0	196.9, 257.9
Acetochlor	10.17	146.1	162.1, 223.1
Chlorpyrifos	11.11	200.0	215.1, 173.0
α-Endosulfan	12.75	194.9	240.9, 264.9
β-Endosulfan	14.16	194.9	240.9, 264.9

2.4. *Sample extraction*

A 200-μL aliquot of plasma was transferred to a labeled conical plastic Eppendorf tube. Ten μL of the IS (3 μg/mL) solution and 200 μL of acetonitrile were added, vortexed for 2 min, and then centrifuged at 10,800 rpm for 5 min. The supernatant portion was transferred into another labeled conical plastic Eppendorf tube containing 250 mg of anhydrous sodium sulfate, vortexed for 2 min, and then centrifuged at 10,800 rpm for 5 min. The supernatant portion was transferred into a glass vial and 1 μL was introduced into the GC-MS system.

2.5. *Method validation*

The method was validated in terms of selectivity, extraction recovery, calibration curves, the lower limit of quantitation (LLOQ), precision and accuracy, carryover, and stability according to the US Food and Drug Administration and Chinese State Food and Drug Administration guidelines for the validation of bioanalytical methods.

Selectivity was studied by analyzing six blank plasma samples and plasma samples spiked with six pesticides to identify the potential interference of endogenous substances at the retention time of each pesticide and the IS under the GC-MS conditions described.

Extraction recovery was investigated at two concentrations (0.15 and 8 μg/mL) in triplicate for each pesticide. Recovery was calculated by dividing the peak area for the pre-spiked sample by the peak area for the post-spiked sample and multiplying by 100%.

A calibration curve was generated for each pesticide using eight non-zero concentrations, typically described by the equation $y = ax + b$, where y corresponds to the peak area ratio and x to the ratio of the concentration of each pesticide to that of the IS. The calibration curves were obtained using quadratic least-squares regression with the reciprocal of the concentration squared ($1/x^2$) as the weighting factor. The LLOQ of each pesticide was defined as the lowest concentration on the calibration curve with a precision of < 20%, expressed as the relative standard deviation (RSD) and an accuracy between 80 and 120%.

The intra-day precision was assessed at 0.05, 0.15, 0.8, and 8 μg/mL through extraction and analysis of five replicates for each concentration on the same day. The inter-day precision was studied at the same concentrations on three consecutive days. The accuracy was determined by comparing the mean measured concentration to its theoretical value, and the result was expressed as the mean relative error (RE). Precision was expressed as the RSD. The RE and RSD should be less than ± 20% for a concentration of 0.05 μg/mL and ± 15% for concentrations of 0.15, 0.8, and 8 μg/mL.

Stability was studied using two concentrations (0.15 and 8 μg/mL) in triplicate stored or processed under different conditions, *i.e*. storage at –20°C for 17 days, freezing (–20°C) and thawing (room temperature) for two cycles, leaving plasma samples to stand on the bench top for 2 h, and leaving the post extraction samples in the auto sampler for 24 h at room temperature. The accuracy expressed as RE% should be less than ± 15%.

2.6. *Application*

This method was used to determine the concentrations of pesticides in the plasma of patients with suspected poisoning seen in the Emergency Department of Qilu Hospital of Shandong University between May and September 2017. Three mL of venous blood was collected into vacutainer tubes containing disodium ethylenediamine tetraacetic acid (EDTA-Na) as the anticoagulant. The tubes were centrifuged at 5,000 rpm for 5 min. The plasma was removed and placed in an Eppendorf tube and stored at –20°C until analysis. This study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All protocols were reviewed and approved by the scientific research ethics committee of Qilu Hospital of Shandong University. Signed informed consent forms were obtained from all patients who participated in this study or their caregivers.

3. Results and Discussion

3.1. *Optimization of GC-MS conditions*

Gas chromatography conditions were optimized by changing the initial temperature and heating rate. When the initial temperature was set at 50°C, dichlorvos and atrazine produced markedly tailed peaks. This situation was improved by increasing the initial temperature to 80°C. Heating had to progress at a reasonable rate, or else the separation and response of the pesticides would be seriously affected. Caffeine and borneol were tried as ISes, but there was endogenous substance interference at the retention time of caffeine. Ultimately, borneol was used as the IS because of the appropriate retention time and lack of endogenous interference.

3.2. *Development of a procedure for sample extraction*

The choice of a procedure for sample extraction started with liquid-liquid extraction with ethyl acetate as the extraction solvent. After centrifugation, the supernatant was transferred into a tube and dried in a water bath under a gentle N_2 flow at 40°C. Then the residue was re-dissolved using acetonitrile and analyzed using GC-MS. However, no or a very small amount of dichlorvos was found at concentration of 0.1 or 0.2 μg/mL depending on the drying time. When protein

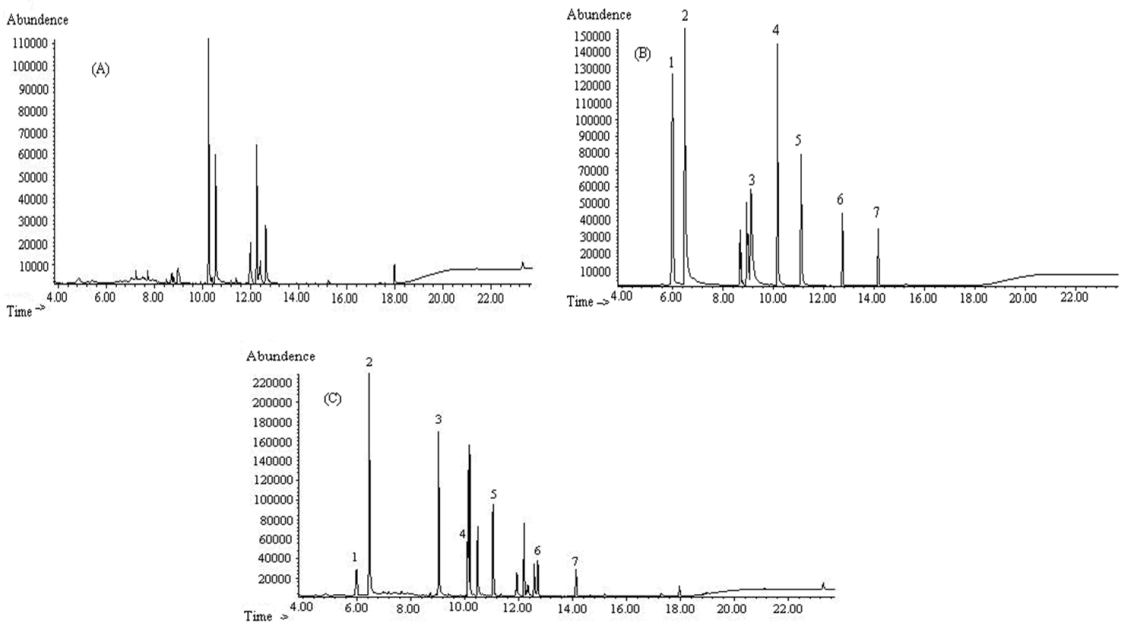

Figure 1. The total ion current chromatograms of blank plasma (A), six standard pesticides and the IS (B), and blank plasma spiked with six pesticides and the IS (C). (1-Borneol; 2-Dichlorvos; 3-Atrazine; 4-Acetochlor; 5-Chlorpyrifos; 6-α-Endosulfan; 7-β-Endosulfan).

precipitation with acetonitrile as precipitant was used, dichlorvos was detected, and the peak areas were concentration-dependent. This indicates that dichlorvos readily volatilizes when dried with N_2. Thus, protein precipitation was selected as the method for sample preparation. In order to reduce the LLOQ, anhydrous sodium sulfate was used for dehydration. With this method of sample preparation, the LLOQs for the six pesticides reached 0.05 μg/mL, and the extraction recovery ranged from 86.8%-106.5%. This method was simpler, faster, and more cost-effective than solid phase extraction, which is often used in literature related to the trace analysis of pesticides.

3.3. *Method validation*

All spiked and blank samples were free of co-eluting peaks at the retention times of the pesticides and the IS. The total ion current chromatograms of blank plasma, six standard pesticides and the IS, and blank plasma spiked with six pesticides and the IS are shown in Figure 1. There were no interfering peaks for all selected ions from the sample matrix (absence of any interfering peaks). This indicated that the method was selective to the six pesticides.

The extraction recovery of the six pesticides and the IS is shown in Table 2. In general, an average recovery greater than 86.8% was obtained, and the recovery was often close to 100%. This indicates that the recovery was good and reproducible, so it was thus deemed fit for purpose.

Calibration curves ranging from 0.05 to 10 μg/mL were obtained for all of the pesticides. Linearity was noted with a correlation coefficient (r^2) greater than 0.99.

Table 2. The extraction recovery (%) for the six pesticides

Pesticide	0.15 μg/mL	8 μg/mL
Dichlorvos	96.3 ± 2.9	97.9 ± 4.4
Atrazine	106.5 ± 13.9	94.1 ± 6.4
Acetochlor	104.2 ± 11.5	96.0 ± 6.1
Chlorpyrifos	103.9 ± 10.0	92.6 ± 5.9
α-Endosulfan	102.6 ± 12.5	86.8 ± 5.7
β-Endosulfan	102.3 ± 4.3	88.7 ± 6.7

The regression equation, correlation coefficient, and RSDs for LLOQ are shown in Table 3.

The results of precision and accuracy experiments at four different concentrations are shown in Table 4. Intra-day accuracy (RE%) of the six analytes ranged from 0.0 to 11.7% while inter-day accuracy ranged from 0.7 to 7.8%. The intra- and inter-day precision (RSD%) varied from 1.7 to 14.5%, and 4.2 to 13.8%, respectively. Overall, results indicated that the method was accurate and precise at detecting each pesticide.

The stability results for the six pesticides are summarized in Table 5. Atrazine, acetochlor, chlorpyrifos, α-endosulfan, and β-endosulfan were stable in human plasma when left on the bench top for 2 h before processing, in the autosampler for 24 h after processing, and after storage at –20°C for 17 days and two freeze-thaw cycles. Dichlorvos was unstable in human plasma when left on the bench top for 2 h before processing, and a degradation of about 50% was noted. It was also unstable after storage at –20°C for 17 days and two freeze-thaw cycles, and a degradation of about 20% was noted. However, dichlorvos was stable in the autosampler for 24 h after processing. This indicates that some endogenous components of plasma may

Table 3. The regression equation, correlation coefficient, and RSDs for LLOQ

Pesticide	Regression equation	Correlation coefficient (r^2)	RSDs for LLOQ (%)
Dichlorvos	y = 0.6355x - 7.556e - 003	0.9956	5.2
Atrazine	y = 0.3016x - 9.134e - 004	0.9964	8.4
Acetochlor	y = 0.1769x + 1.095e - 003	0.9912	8.3
Chlorpyrifos	y = 0.1018x - 1.193e - 003	0.9952	7.5
α-Endosulfan	y = 0.03923x + 5.972e - 003	0.9953	9.5
β-Endosulfan	y = 0.03969x + 1.938e - 003	0.9960	9.8

Table 4. The precision and accuracy with which six pesticides were detected

Pesticide	Concentration (μg/mL)	Intra-day		Inter-day	
		Accuracy (RE%)	Precision (RSD%)	Accuracy (RE%)	Precision (RSD%)
Dichlorvos	0.05	0.0	5.7	2.0	9.0
	0.15	-3.3	4.0	2.0	5.7
	0.8	7.4	2.2	6.0	6.2
	8	-11.7	3.7	-4.5	7.4
Atrazine	0.05	-2.0	7.8	-2.0	8.1
	0.15	-7.3	2.4	-2.7	5.1
	0.8	2.1	2.7	3.7	4.7
	8	-10.7	4.4	-2.2	7.7
Acetochlor	0.05	-10.0	7.6	-6.0	10.4
	0.15	-6.0	4.4	-4.7	6.6
	0.8	5.9	8.6	3.6	5.4
	8	9.4	4.0	1.7	7.5
Chlorpyrifos	0.05	-6.0	5.3	-2.0	9.2
	0.15	-8.7	4.4	-5.3	7.2
	0.8	0.3	7.5	0.8	4.8
	8	6.2	5.0	-3.6	8.6
α-Endosulfan	0.05	-4.0	14.5	-4.0	13.8
	0.15	0.7	4.6	-0.7	5.1
	0.8	5.3	6.8	3.4	4.4
	8	-1.1	4.4	-7.8	7.1
β-Endosulfan	0.05	8.0	6.0	-2.0	12.1
	0.15	6.7	4.4	-1.3	8.2
	0.8	-1.0	1.7	1.3	4.2
	8	-5.0	2.2	-5.2	8.3

Table 5. The stability (RE %) of the six pesticides in human plasma under various conditions

Pesticide	Concentration (μg/mL)	Left on bench top (2 h)	Left in autosampler (24 h)	Two freeze-thaw cycles	17 days at -20°C
Dichlorvos	0.15	-54.0	0.6	-24.7	-21.3
	8	-47.4	-10.2	-18.3	-15.6
Atrazine	0.15	-0.7	13.3	3.3	13.3
	8	5.5	0.04	4.7	15.0
Acetochlor	0.15	-0.7	5.3	4.7	13.3
	8	10.9	-2.5	13.2	12.6
Chlorpyrifos	0.15	-11.3	4.7	-12.0	11.3
	8	7.6	-3.5	10.1	11.9
α-Endosulfan	0.15	-8.7	14.0	3.3	13.3
	8	-6.3	-6.3	-7.1	7.4
β-Endosulfan	0.15	-10.0	6.0	-4.0	11.3
	8	7.3	-1.4	-1.7	6.8

affect the stability of dichlorvos, so it should be detected immediately after collecting blood from a patient.

3.4. *Application*

This method was used to determine the presence of pesticides in 35 plasma samples from 15 patients with acute self-poisoning seen in the Emergency Department of Qilu Hospital of Shandong University.

The plasma was collected before and after treatment. Dichlorvos was found in 13 plasma samples at a concentration ranging from 0.05-0.725 μg/mL with a mean concentration of 0.289 μg/mL. Atrazine was found in six plasma samples from two patients before and after treatment at a concentration ranging from 0.070-0.240 μg/mL with a mean concentration of 0.261

μg/mL. Acetochlor was found in one plasma sample at a concentration of 0.153 μg/mL. The determined concentrations of pesticides in samples obtained from patients with acute self-poisoning are shown in Table 6. The total ion current chromatograms of patients with acute self-poisoning are shown in Figure 2. In addition, another peak (marked with "x") appeared near the retention time of dichlorvos in eight plasma samples obtained from patients with suspected dichlorvos poisoning, as shown in Figure 2B. This peak has the same ions of m/z 109 and 79 but no ion of m/z 185. This may be a degradation product or metabolite of dichlorvos and needs to be studied further.

Table 6. The determined concentrations of samples from patients with acute self-poisoning

Samples	Concentration (μg/mL)		
	dichlorvos	atrazine	acetochlor
1	0.725	0.240	0.153
2	0.700	0.180	-
3	0.270	0.700	-
4	0.050	0.277	-
5	0.185	0.091	-
6	0.050	0.079	-
7	0.356	-	-
8	0.120	-	-
9	0.193	-	-
10	0.558	-	-
11	0.321	-	-
12	0.150	-	-
13	0.075	-	-
Mean	0.289	0.261	-

Note: The samples were obtained from different patients poisoned with dichlorvos, atrazine, or acetochlor.

Some methods such as GC-NPD, GC-MS, and liquid chromatography-mass spectrometry (LC-MS) have been used to determine the presence of dichlorvos (*21*), acetochlor (*22*), atrazine (*23*), chlorpyrifos (*24*), and α-endosulfan and β-endosulfan (*25*) in plasma or serum. However, these methods can only detect one or two pesticides, and liquid-liquid extraction or SPE requires a somewhat complicated method of sample preparation. In this study, a method of GC-MS was developed and fully validated for simultaneous determination of the presence of the six pesticides mentioned above. It is simple since samples are prepared using PPT, economical since acetonitrile is used as the protein precipitator, and sensitive with an LLOQ of 0.05 μg/mL. To the extent known, this is the first study to report a method for simultaneous determination of the presence of six pesticides in patients with acute poisoning.

4. Conclusion

In this study, a GC-MS method was developed to simultaneously quantify the concentrations of six pesticides including dichlorvos, atrazine, acetochlor, chlorpyrifos, α-endosulfan, and β-endosulfan in human plasma. The method is both sensitive and selective, and it is readily validated. Sample preparation is accomplished through convenient and economical protein precipitation and only requires 200 μL of plasma. This method can be used for routine clinical detection of dichlorvos, atrazine, acetochlor, chlorpyrifos, α-endosulfan, and β-endosulfan in patients with acute self-poisoning.

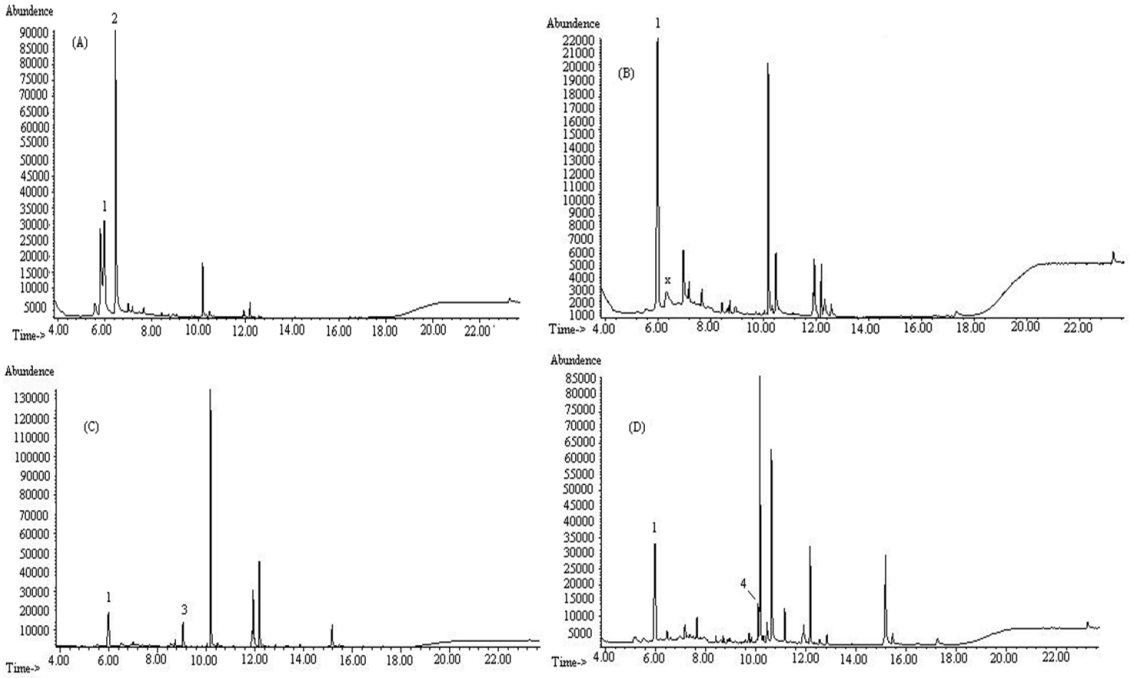

Figure 2. The total ion current chromatograms of dichlorvos (A), suspected dichlorvos (B), atrazine (C), and acetochlor (D) in patients with acute poisoning. (1-Borneol; 2-Dichlorvos; 3-Atrazine; 4-Acetochlor; x-uncertain compound).

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