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Technical note

Validated analytical methodology for the simultaneous determination of a wide range of pesticides in human blood using GC–MS/MS and LC–ESI/MS/MS and its application in two poisoning cases

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ABSTRACT

Pesticides are frequently responsible for human poisoning and often the information on the involved substance is lacking. The great variety of pesticides that could be responsible for intoxication makes necessary the development of powerful and versatile analytical methodologies, which allows the identification of the unknown toxic substance. Here we developed a methodology for simultaneous identification and quantification in human blood of 109 highly toxic pesticides. The application of this analytical scheme would help in minimizing the cost of this type of chemical identification, maximizing the chances of identifying the pesticide involved. In the methodology that we present here, we use a liquid–liquid extraction, followed by one single purification step, and quantitation of analytes by a combination of liquid and gas chromatography, both coupled to triple quadrupole mass spectrometry, which is operated in the mode of multiple reaction monitoring. The methodology has been fully validated, and its applicability has been demonstrated in two recent cases involving one self-poisoning fatality and one non-fatal homicidal attempt.

1. Introduction

The large group of pesticides, which are widely used throughout the world primarily to control pests affecting crops, is often implicated in human poisoning [1]. Morbidity and mortality attributable to these substances vary from country to country, depending on many variables such as the level of socioeconomic development, accessibility to these chemicals, and the importance of the agricultural sector. Fatalities involving pesticides are a consequence of accidents, self-injury or more rarely homicides, and range from less than 1% of deaths from poisoning in EU countries [1,2] to up to 71% of all violent deaths in the Western Pacific and Southeast Asia [3–5]. In all these cases chemical analysis to investigate the poison involved is mandatory, and the forensic laboratory is facing a challenge because in most cases there is no information on what the substance involved was. The main difficulties of this type of toxicological identifications are the wide variety of biological matrices that are sent to the laboratory, often in advanced state of decomposition, along with the wide range of pesticides to which the poisoning could be attributed.

In recent years the use of chromatographic techniques (HPLC or GC) combined with detection of analytes by mass spectrometry (MS/MS) has been considered as a very useful tool in forensic toxicology laboratories, since it enables high selectivity along with a very low detection limits. Triple quadrupole mass spectrometers (QqQ) allow operating in the mode of selective multiple reaction monitoring (SRM). This allows the monitoring of parent ions fragmenting into product ions. This mode of operation improves selectivity and sensitivity of the determination, in comparison with one-stage mass spectrometry. With this technique, the virtual elimination of isobaric interferences is allowed, as well as a significant decrease in chemical noise from the matrix [6]. The use of any of these analytical techniques is currently seen as a practical way to overcome the difficulties posed by complex biological matrices, which may contain an excessive amount of potentially interfering substances, such as fat, protein, sugars, and chemicals [7]. In addition, high acquisition speed in the MRM mode allows the development of methods for the simultaneous analysis of tens or even hundreds of compounds belonging to different chemical classes [8–12].

It needs to be emphasized that when the information of the pesticide involved is lacking it is generally difficult to conduct thorough analytical investigations in complex biological matrices such as ante- or post-mortem blood, and usually several complementary analyses are needed. That is why all the techniques of high sensitivity and specificity that allow the simultaneous analysis of a wide series of chemicals of high

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toxicity may contribute to reducing the costs associated with this type of analytical, as well as to increase the chances of identifying the "unknown toxic substance". In this work we have developed an analytical method for the detection and quantification of 109 pesticides in human blood. The pesticides have been selected on the basis of both, their high toxicity to humans [13], and the frequency with which they are involved in cases of poisoning [14,15]. This methodology is based on a liquid–liquid extraction, clean-up, chromatographic separation, and detection by QqQ operated in the MRM mode, and has been successfully applied to the detection of the toxicant involved in 2 recent cases of poisoning by an unknown pesticide that were submitted to our laboratory. Besides, this methodology has been also applied in our laboratory to the identification of the pesticides in matrices other than blood [16].

2. Materials and methods

2.1. Chemicals and reagents

Acetone, acetonitrile, cyclohexane, dichloromethane, ethyl acetate, and methanol (>99.9%) were purchased from Fisher Scientific (Leicestershire, United Kingdom). Ultrapure (UP) water was obtained from a Milli-Q Gradient A10 (Millipore, Molsheim, France). Blank blood was purchased from Medichem (Medidrug® Basis Line, Medichem, Germany). All the pesticide standards (purity from 97% to 99.5%), as well as the internal standards (ISs, aldicarb-D3, carbofuran-D3, chlorfenvinphos-D10, chloropropham, chlorpyriphos-D10, diazinon-D10, heptachloro epoxide cis, and thiobencarb), were purchased from Dr Ehrenstorfer Reference Materials (Augsburg, Germany). We prepared stock solutions of target compounds (0.1 and 1 mg/mL) in cyclohexane or acetonitrile. Stock solutions were stored at −20 °C. From these stock solutions matrix-matched calibration curves were prepared (0.5 ng/mL to 500 ng/mL) using blank blood. For the fortification experiments we used mixtures of all the standards in acetone (10 μg/mL and 500 ng/mL).

2.2. Pesticide selection

A wide variety of pesticides belonging to different chemical classes are currently used in agricultural practices but, because of their high toxicity, several restrictions have been applied and most of the most toxic compounds are nowadays banned. Nevertheless, it has been shown that legal and commercial restrictions have not influenced the intentional illegal use of some pesticides as poisons [16,17]. For this reason for the selection of the 109 pesticides included in this multiresidue method we have mainly taken into account their known toxicities for either humans and other mammals (Table 1), and also according to the available data, the frequency with which these compounds have been implicated in human poisonings [14,15], regardless of whether their use is currently allowed or not.

2.3. Extraction and cleanup procedure

A liquid–liquid extraction procedure was developed for human whole blood. For the extraction, 2 mL of the sample (pH = 7.0) was placed in 50-mL polypropylene centrifuge tubes. Next, 5 mL of ultrapure water and 50 μL of the ISs solution at 1 μg/mL were added and thoroughly vortexed. 10 mL of a mixture of dichloromethane/ethyl acetate/acetone (50/30/20) were added to the tubes. The tubes were then placed in an orbital shaker for 10 min. The tubes were then sonicated for 5 min. The samples were centrifuged at 5000 rpm, 5 min, 20 °C, and the supernatant collected. The samples were then placed under a gentle nitrogen stream to evaporate the solvent. The concentrated extracts were re-dissolved in 3 mL of cyclohexane in Eppendorf tubes.

After the extraction an additional cleanup step by freezing centrifugation was performed to minimize the content of interfering substances (mainly lipids). The tubes were placed in a −82 °C freezer for 20 min, and then centrifuged at 5000 rpm, 5 min, −10 °C. The frozen lipids remained in the bottom of the tube and thus separated from the pesticides dissolved in the supernatant, which was carefully removed. This procedure was performed three times, and the resulting supernatant was divided into two 1-mL aliquots. One aliquot was directly used for GC–MS/MS. The other was evaporated and re-dissolved in acetonitrile for LC–MS/MS analysis.

2.4. GC–QqQ-MS/MS analysis

For the GC–MS–MS detection of the pesticides included in this study we used a Trace GC Ultra tandem coupled with a TSQ XLS triple quadrupole (QqQ) mass spectrometer instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA). As the stationary phase a 30 m × 0.25-mm i.d., 0.25 μm film thickness column was used (BPX5, SGE Inc., Austin, TX, USA). Helium (99.999%) was used as the carrier gas at a constant flow of 1 mL/min. The 61-min oven temperature program was: 60 °C held for 1 min, ramped to 160 °C at 15 °C/min, then to 230 °C at 2.3 °C/min, and finally to 290 °C at 5 °C/min and held for 8.9 min. The injector temperature was set al 270 °C and the transfer line was heated to 310 °C. The injection volume was 1 μl in the splitless mode.

The GC was tandem-coupled to a TSQ XLS QqQ mass spectrometer, which was used for the detection and quantification of the 90 most apolar pesticides. An electron ionization (EI)–MS/MS library was specially created for the target analytes under our experimental conditions. We calibrated the mass spectrometer scale with perfluorotributylamine on a weekly basis to ensure an optimal response over time and proper mass assignments. The instrument control, data acquisition and data analysis was performed using the Thermo Fisher Xcalibur software (Ver. 2.0.1).

We constructed a timed MRM method for the simultaneous analysis of 90 pesticides plus ISs in a single run. Matrix-matched calibration curves contained all of the target compounds except for the ISs at each level (0.5 to 500 ng/mL). The operation conditions of the mass spectrometer were: electron impact ionization (70 eV) in MRM; emission current, 50 μA; ionization source temperature, 220 °C; electron multiplier voltage, 1500 V; scan width, 0.15; scan time, 0.05 s; and peak width, *m/z* 0.7 Da. Argon (99.99%) was used as the collision gas at 0.2 Pa.

2.5. LC–MS–MS analysis

Because some of the most relevant pesticides causing poisoning in humans, such as carbofuran and aldicarb, can only be analyzed by liquid chromatography due to their high polarity (unless prior derivatization is performed), we developed a complementary method by LC–MS–MS. In this second method we included 19 compounds. Some of them can be analyzed both, by liquid chromatography and by gas chromatography (such as metamidofos, dimethoate, or pirimicarb), but we chose the technique with which a higher sensitivity for each one of them is achieved. However, we have avoided duplicating them in both methods to minimize the number of transitions and to gain sensitivity.

For the LC–MS–MS detection, we used an Accela LC tandem coupled to a TSQ Quantum Max QqQ mass spectrometer instrument equipped with an H-ESI II electrospray ionization source (Thermo Fisher Scientific Inc.). As the stationary phase we used an analytic Synergi Hydro-RP column (4.0 μm, 150 × 4.6 mm; Phenomenex, Torrance, CA, USA). We used the following mobile phases for LC: (A) 7.5 mM ammonium formate in ultrapure water; (B) methanol (HPLC–MS grade); and (C) 2% formic acid. The solvent flow was 1000 μl/min. The injection volume was 25 μl. During the entire run (26 min), solvent C was set at 2.5%. The infusion of the other two mobile phases was programmed as a gradient as follows: 0–12 min: 87.5% A → 7.5% A; 12–16 min: 7.5% A; 16.0–16.2 min: 7.5% A → 87.5% A; and 16.2–25.0 min: 87.5% A.

Table 1
Acute toxicity values (mg/kg) and toxic and lethal blood concentrations (μg/mL) of the pesticides included in the methodology.

Compound	Toxicity			Compound	Toxicity			Compound	Toxicity		
	LD_{50}[a]	[Toxic][b]	[Lethal][b]		LD_{50}	[Toxic][b]	[Lethal][b]		LD_{50}	[Toxic][b]	[Lethal][b]
Acephate	321.0	>200	–	Dieldrin	65	0.15	0.5	Mevinphos	4.0	–	–
Aldicarb	1.9	1.5	6.1	Dimefox	3.5	–	–	Monocrotophos	15.0	–	12
Aldrin	65.0	0.005	0.7	Dimethoate	220.0	–	4	Nuarimol	2450.0	–	–
Allethrin	370.0	–	–	Dioxathion	10.0	0.2	–	Omethoate	50.0	–	3.2
Amitraz	100.0	0.6	–	Disulfoton	5.0	–	–	Oxamyl	30.0	0.23	–
Azinphos ethyl	12.0	–	0.9	Ediphenphos	100.0	–	1.4	Parathion ethyl	0.9	–	0.5
Azinphos methyl	10.0	–	–	Endosulfan sulfate	18.0	–	–	Parathion methyl	57.0	–	–
Bendiocarb	35.0	1	40	Endosulfan, alpha	26.0	0.5	2.8	Phenthoate	138.0	–	–
Benfuracarb	102.0	–	–	Endosulfan, beta	26.0	0.5	2.8	Phorate	20.0	–	0.83
Bifenthrin	54.5	–	–	Endrin	3.0	0.01	–	Phosalone	112.0	–	–
Bromophos ethyl	125.0	–	1.6	EPN	20.0	–	0.8	Phosmet	40.0	–	–
Bromoxynil	78.0	20	–	Ethion	13.0	–	–	Phosphamidon	6.0	–	–
Cadusafos	71.4	5	6	Etoprophos	34.0	–	–	Phoxim	250	–	–
Carbaryl	150.0	–	2	Famphur	59.0	–	–	Pirimicarb	100.0	3.7	32.8
Carbofuran	10.2	0.06	0.4	Fenamiphos	10.0	–	–	Pirimiphos ethyl	25.0	–	–
Carbophenothion	14.0	–	–	Fenitrothion	142.0	1	–	Pirimiphos methyl	1150.0	–	–
Carbosulfan	115.0	–	–	Fensulfothion	2.2	–	–	Profenofos	116.0	–	1.2
Carboxin	430.0	–	–	Fenthion	46.2	1	–	Propachlor	392.0	–	–
Chlordane, cis	50.0	0.005	2	Flucythrinate	76.0	–	–	Propaphos	61	–	–
Chlordane, trans	50.0	0.005	2	Fonofos	3.0	–	–	Propetamphos	130.0	–	–
Chlorfenvinphos	20.0	–	0.1	Formothion	175.0	–	–	Propoxur	51.2	0.12	–
Chlormephos	12.5	–	–	Heptachlor	50.0	–	–	Pyrazophos	184.0	–	–
Chlorpyrifos	60.0	0.2	1.6	Heptenophos	117.0	–	–	Quinalphos	75.0	–	4.5
Chlorpyriphos methyl	2000.0	–	–	Imidacloprid	98.0	–	2.1	Resmethrin	250.0	–	–
Chlorthiophos	20.0	–	–	Isazophos	27.0	–	–	Sulfotep	22.0	0.08	–
Cifluthrin	300.0	3.1	37.4	Isobenzan	5.0	0.03	–	Sulprofos	70.0	–	–
Cyanazine	141.0	–	–	Isofenphos	91.5	–	–	Tebufenpyrad	210.0	–	–
Cyanophos	215.0	–	–	Isoxathion	112.0	–	–	Tefluthrin	22.0	0.9	7.1
Cyproconazole	352.0	–	–	Leptophos	65.0	–	–	TEPP	2.3	–	2.7
Dazomet	415.0	–	–	Lindane	25.0	0.3	1.3	Terbufos	3.5	–	–
DDT	200.0	1	–	Malathion	53.0	0.5	1	Tetrachlorvinphos	420.0	–	–
Deltamethrin	22.0	0.2	9.3	Mephospholan	11.0	–	–	Thiometon	37.0	–	–
Diallate	395.0	–	–	Metamidofos	18.5	–	13.5	Thionazin	5.0	–	0.8
Diazinon	76.0	0.05	0.97	Methidathion	25.0	0.1	–	Triazophos	57.0	–	–
Dichlone	160.0	–	–	Methiocarb	16.0	–	–	Trichloronat	10.0	0.15	–
Dichlorphos	61.0	–	29	Metolcarb	109.0	–	–				
Dicrotophos	11.0	0.3	2.8	Methomyl	24.9	–	0.45				

[a] Average data from different species. These data have been taken from Mineau et al. (2001) and the National Library of Medicine internet resources ChemIDplus (http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp) and Hazardous Substances Data Bank (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

[b] These data are values from non-fatal or fatal case reports of human poisonings when available in the literature and have been obtained from the National Library of Medicine Resource Toxicology Data Network (http://toxnet.nlm.nih.gov/index.html) and from the database of the Spanish National Institute of Toxicology and Forensic Sciences, available in http://busca-tox.com/.

The LC was tandem-coupled to a TSQ Quantum Max QqQ mass spectrometer, which was used for the detection and quantification of the 19 pesticides. As the ionization source we used a heated electrospray H-ESI II (Thermo Fisher Scientific Inc., Waltham, USA). The mass spectrometer was programmed according the following parameters: skimmer offset (10 V), sheath gas pressure (15 arbitrary units, a.u.), capillary temperature (250 °C), spray voltage (3000 V), and vaporization temperature (180 °C). The spectrometer was programed in the positive ionization mode. Argon (99.99%) was used as the collision gas at 0.25 Pa.

The MRM method was constructed for these pesticides plus ISs by means of the infusion of pure standard solutions in methanol into the ionization source. A matrix-matched calibration curve was constructed for all compounds (except for the ISs) from 0.5 to 500 ng/mL.

2.6. Validation

Blank whole blood (Medidrug Basis-Line VB, Medichem, Germany) was used for the validation experiments. For the fortification experiments 40 μL of a 10 μg/L or 500 ng/L of a mixture of all of the pesticides in acetone were added to 2 mL of whole blood to obtain concentrations of 200 μg/kg and 10 μg/kg, respectively. The samples were thoroughly mixed and allowed to stand at room temperature for 4 h to ensure that the analytes were homogenously distributed throughout the sample. The matrix effect was determined in quintuplicate by comparing the obtained concentrations between the spiked blood and the same concentrations of the pesticides prepared in the dissolvent. The recovery was calculated as the quotient of signals obtained from the spiked samples and the matrix matched standards. The intra- and inter-day precisions (five successive days) were also determined from the same experiments.

The analyte concentration that produced a peak signal of ten times the background noise from the chromatogram was set as the method limit of quantification (LOQ). Quantifications were based on the peak areas. Calibration curves were constructed using a least-squares linear regression from the injection of samples spiked with solutions to give final concentrations ranging from 0.5 to 500 μg/kg

2.7. Quality control

All the measurements were performed in triplicate, and the values used for calculations were the mean of the three values. In each batch of samples, two controls were included every 12 samples: a reagent blank consisting of a vial containing only cyclohexane and an internal laboratory quality control (QC) consisting of blank whole blood spiked with a mixture of all of the pesticides (20 μg/kg), and processed with the same method as the samples. The results were considered acceptable when the quantification of the analytes in the QC was within 15% of the deviation of the theoretical value.

Table 2
Method settings and validation parameters (n = 5) obtained for the 109 pesticides at the concentration of 0.2 μg/mL in human blood.

N°	Compound	Mass spectrometry settings						Validation parameters		
		RT	First transition *m/z* → *m/z*	CE (V)	Second transition *m/z* → *m/z*	CE (V)	IPs	LOQ (μg/mL)	Linearity (R^2)	Average recovery (RSD[a,b]) (%)
LC–MS/MS method										
1	Metamidofos	2.26	142.1 → 94.0	14	142.1 → 125.0	16	4	0.01	0.9985	78 (14, 11)
2	Oxamyl	2.78	237.2 → 163.0	14	237.2 → 196.0	18	4	0.03	0.9824	93 (8, 10)
3	Phoxim	4.03	300.1 → 129.3	18	300.1 → 283.0	10	4	0.01	0.9959	97 (6, 9)
4	Acephate	4.51	184.1 → 125.0	16	184.1 → 143.0	5	4	0.03	0.9816	95 (7, 11)
5	Omethoate	5.03	214.0 → 155.0	19	214.0 → 183.0	13	4	0.05	0.9904	96 (11, 14)
6	Methomyl	6.71	163.1 → 88.1	11	163.1 → 106.0	12	4	0.02	0.9934	97 (8, 12)
7	Imidacloprid	8.05	256.1 → 175.0	18	256.1 → 209.0	16	4	0.01	0.9813	88 (8, 10)
8	Dimethoate	8.64	230.0 → 125.0	23	230.0 → 199.0	11	4	0.05	0.9890	92 (8, 7)
9	Aldicarb	9.38	208.0 → 89.2	19	208.0 → 116.2	10	4	0.01	0.9906	94 (9, 11)
10	Carbofuran	10.34	222.0 → 123.1	25	222.0 → 137.5	24	4	0.01	0.9899	97 (4, 7)
11	Propoxur	10.76	210.0 → 111.2	18	210.0 → 168.0	11	4	0.01	0.9972	101 (9, 13)
12	Carbaryl	11.20	202.1 → 127.0	33	202.1 → 145.1	13	4	0.01	0.9948	82 (4, 8)
13	Pirimicarb	11.33	239.1 → 72.3	27	239.1 → 182.1	16	4	0.03	0.9982	96 (8, 11)
14	Carboxin	11.49	236.1 → 93.2	33	236.1 → 143.0	15	4	0.03	0.9929	71 (11, 9)
15	Bromoxynil	12.01	275.9 → 79.2	29	275.9 → 81.1	33	4	0.05	0.9994	92 (9, 11)
16	Methiocarb	12.97	226.0 → 121.0	19	226.0 → 169.0	8	4	0.05	0.9856	97 (12, 15)
17	Cyproconazole	13.46	292.1 → 70.3	17	292.1 → 125.1	34	4	0.01	0.9908	99 (11, 9)
18	Benfuracarb	15.02	411.2 → 190.1	13	411.2 → 252.3	15	4	0.05	0.9873	85 (5, 8)
19	Profenofos	15.52	373.0 → 302.8	18	373.0 → 344.8	13	4	0.03	0.9814	93 (14, 11)
GC–MS/MS method										
20	Dimefox	5.33	154.1 → 58.0	10	154.1 → 111.1	10	4	0.01	0.9834	91 (11, 9)
21	Dichlorphos	7.61	185.0 → 109.0	15	185.0 → 127.0	12	4	0.01	0.9921	90 (14, 12)
22	Mevinphos	9.72	192.0 → 127.0	12	192.0 → 164.0	10	4	0.01	0.9953	101 (10, 14)
23	Chlormephos	9.94	154.0 → 121.0	5	154.0 → 121.0	14	4	0.02	0.9878	105 (9,12)
24	Metolcarb	10.56	108.1 → 79.0	10	108.1 → 107.1	10	4	0.02	0.9877	103 (14, 11)
25	Heptenophos	12.26	250.0 → 124.0	10	250.0 → 215.0	4	4	0.01	0.9887	93 (6, 8)
26	Thionazin	12.96	192.0 → 96.0	10	248.0 → 140.0	10	5	0.05	0.9964	100 (8, 9)
27	TEPP	13.07	263.1 → 179.1	15	263.1 → 235.1	5	4	0.01	0.9987	94 (12, 10)
28	Propachlor	13.25	176.1 → 120.0	10	196.1 → 120.0	10	5	0.02	0.9913	96 (8, 11)
29	Etoprophos	13.70	158.0 → 114.0	10	158.0 → 130.0	10	4	0.01	0.9978	95 (6, 8)
30	Sulfotep	14.50	322.0 → 202.0	15	322.0 → 294.0	10	4	0.01	0.9889	92 (11, 14)
31	Dicrotophos	14.61	127.0 → 95.0	10	127.0 → 109.0	10	4	0.01	0.9887	101 (9, 12)
32	Bendiocarb	14.79	166.1 → 151.1	15	223.1 → 166.1	15	5	0.05	0.9856	93 (7, 9)
33	Cadusafos	14.93	159.1 → 97.0	20	159.1 → 131.0	10	4	0.01	0.9948	941 (8, 12)
34	Phorate	15.24	260.0 → 75.0	5	260.0 → 231.0	8	4	0.01	0.9932	93 (6, 8)
35	Diallate	15.30	236.0 → 152.0	20	236.0 → 194.0	15	4	0.02	0.9921	95 (10, 11)
36	Monocrotophos	15.80	127.0 → 95.0	20	127.0 → 109.3	25	4	0.01	0.9904	92 (9, 11)
37	Thiometon	15.85	88.0 → 60.0	15	248.0 → 88.0	15	5	0.01	0.9878	87 (7, 7)
38	Dazomet	16.56	89.0 → 75.0	20	162.0 → 89.0	8	5	0.05	0.9877	89 (8, 11)
39	Dioxathion	17.13	125.0 → 97.0	15	197.0 → 141.0	15	5	0.01	0.9887	101 (7, 9)
40	Lindane	17.16	216.9 → 180.9	15	218.9 → 182.9	15	5	0.005	0.9964	92 (11, 9)
41	Propetamphos	17.35	236.1 → 166.1	15	236.1 → 194.1	5	5	0.01	0.9987	94 (14, 12)
42	Terbufos	17.37	231.0 → 175.0	15	231.0 → 203.0	10	4	0.01	0.9945	101 (10, 14)
43	Chlorfenvinfos	17.60	267.0 → 159.0	15	323.0 → 269.0	10	5	0.01	0.9995	103 (14, 11)
44	Cyanofos	17.60	243.0 → 109.0	12	243.0 → 127.0	15	4	0.01	0.9883	94 (6,11)
45	Fonofos	17.69	137.0 → 109.0	10	246.0 → 137.0	10	5	0.01	0.9978	92 (4, 9)
46	Diazinon	17.81	179.1 → 127.0	15	179.1 → 137.1	15	4	0.01	0.9907	92 (9, 6)
47	Disulfoton	18.32	274.0 → 88.0	10	274.0 → 245.0	10	4	0.01	0.9889	94 (6,11)
48	Tefluthrin	18.40	197.0 → 141.0	15	197.0 → 161.0	10	4	0.02	0.9887	91 (7,5)
49	Isazophos	18.40	257.0 → 119.0	15	257.0 → 162.0	15	4	0.01	0.9856	84 (4,6)
50	Dichlone	18.58	191.0 → 135.0	15	226.0 → 191.0	10	5	0.02	0.9819	89 (8,7)
51	Formothion	19.94	224.0 → 125.0	15	224.0 → 196.0	10	4	0.01	0.9881	94 (5,9)
52	Phosphamidon	20.08	264.0 → 127.0	15	264.0 → 127.0	15	4	0.01	0.9883	91 (6,10)
53	Chlorpyrifos methyl	20.57	285.9 → 93.0	25	285.9 → 272.9	13	4	0.01	0.9890	88 (8, 11)
54	Parathion methyl	21.10	263.0 → 109.0	15	263.0 → 127.0	15	4	0.01	0.9992	89 (6, 8)
55	Heptachlor	21.36	338.8 → 267.9	15	338.8 → 303.8	15	4	0.005	0.9899	94 (8, 6)
56	Fenitrothion	22.74	277.0 → 109.0	20	277.0 → 260.0	15	4	0.01	0.9972	90 (15, 12)
57	Pirimifos methyl	23.19	290.1 → 125.0	15	290.1 → 233.1	10	4	0.01	0.9948	93 (12, 8)
58	Malathion	23.27	173.0 → 127.0	10	173.0 → 145.0	5	4	0.01	0.9982	94 (12, 14)
59	Chlorpyrifos	23.60	197.0 → 169.0	15	199.0 → 171.0	15	5	0.01	0.9929	92 (11, 13)
60	Aldrin	23.60	262.9 → 192.9	32	262.9 → 227.9	26	4	0.003	0.9994	78 (12, 8)
61	Fenthion	24.08	278.0 → 169.0	20	278.0 → 245.0	15	4	0.01	0.9856	81 (7, 11)
62	Parathion ethyl	24.26	291.0 → 109.0	15	291.0 → 263.0	10	4	0.01	0.9908	87 (9, 13)
63	Isobenzan	24.41	310.8 → 274.8	10	312.8 → 276.8	10	5	0.02	0.9873	82 (11, 13)
64	Cyanazine	24.59	225.1 → 189.1	10	225.1 → 198.1	10	4	0.05	0.9948	75 (9, 12)
65	Trichloronat	24.70	296.9 → 268.9	15	299.9 → 271.9	15	5	0.04	0.9932	93 (6, 8)
66	Pirimifos ethyl	26.08	333.1 → 288.1	20	333.1 → 318.1	15	4	0.01	0.9921	95 (8, 9)
67	Isofenphos	26.44	255.1 → 185.1	10	255.1 → 213.1	10	4	0.01	0.9904	94 (12, 10)
68	Allethrin	26.93	123.1 → 81.1	10	136.1 → 93.1	10	4	0.02	0.9987	92 (11, 14)
69	Phenthoate	27.10	274.0 → 125.0	7	274.0 → 246.0	10	4	0.05	0.9945	91 (8, 12)
70	Quinalphos	27.17	146.0 → 91.0	15	146.0 → 118.0	15	4	0.03	0.9976	90 (8, 10)

Table 2 (*continued*)

N°	Compound	Mass spectrometry settings						Validation parameters		
		RT	First transition *m/z* → *m/z*	CE (V)	Second transition *m/z* → *m/z*	CE (V)	IPs	LOQ (μg/mL)	Linearity (R^2)	Average recovery (RSD[a,b]) (%)
GC–MS/MS method										
71	Mephospholan	27.60	196.0 → 140.0	15	196.0 → 168.0	10	4	0.03	0.9995	94 (8, 7)
72	Chlordane, trans	28.04	372.8 → 265.9	15	374.8 → 267.9	16	5	0.005	0.9878	93 (9, 11)
73	Bromophos ethyl	28.07	358.9 → 302.9	20	358.9 → 330.9	10	4	0.01	0.9877	92 (4, 7)
74	Methidathion	28.23	145.0 → 58.0	15	145.0 → 85.0	10	4	0.01	0.9887	92 (9, 13)
75	Propafos	28.58	220.1 → 140.0	15	304.1 → 220.1	15	5	0.01	0.9964	77 (4, 8)
76	Tetrachlorvinphos	28.64	330.9 → 109.0	22	330.9 → 316.0	22	4	0.01	0.9819	94 (10, 8)
77	Endosulfan, alpha	28.88	195.9 → 158.9	16	195.9 → 159.9	15	4	0.01	0.9881	91 (12, 8)
78	Chlordane, cis	28.90	372.8 → 265.9	18	409.8 → 374.8	5	5	0.005	0.9883	94 (5, 9)
79	Fenamiphos	29.98	303.1 → 260.1	15	303.1 → 288.1	15	4	0.01	0.9883	94 (11, 12)
80	Dieldrin	30.87	276.9 → 206.9	20	276.9 → 240.9	10	4	0.001	0.9978	92 (4, 7)
81	Endrin	32.42	262.9 → 190.9	25	262.9 → 192.9	26	4	0.001	0.9889	93 (9, 6)
82	Isoxathion	32.47	177.0 → 130.0	15	313.0 → 177.0	15	5	0.01	0.9887	101 (6, 9)
83	Endosulfan, beta	33.50	195.9 → 158.9	16	195.9 → 159.9	15	4	0.01	0.9856	89 (11, 8)
84	Fensulfothion	33.84	293.0 → 97.0	16	293.0 → 125.0	0	4	0.01	0.9985	93 (4,11)
85	Ethion	33.96	231.0 → 175.0	15	231.0 → 203.0	15	4	0.01	0.9824	91 (14, 10)
86	Chlorthiophos	34.26	325.0 → 269.0	15	325.0 → 297.0	10	4	0.01	0.9959	101 (4, 8)
87	Sulprofos	35.31	322.0 → 139.0	15	322.0 → 156.0	15	4	0.01	0.9816	93 (5, 7)
88	Triazofos	35.55	161.0 → 105.0	13	161.0 → 134.0	10	4	0.01	0.9904	94 (9, 13)
89	Famphur	35.87	218.0 → 109.0	10	218.0 → 127.0	10	4	0.01	0.9934	97 (6, 9)
90	Carbophenothion	36.02	342.0 → 157.0	10	342.0 → 296.0	5	4	0.01	0.9813	89 (9, 6)
91	Ediphenphos	36.23	173.0 → 109.0	15	310.0 → 173.0	10	4	0.01	0.9890	88 (12, 9)
92	Endosulfan sulfate	36.38	273.9 → 236.9	10	273.9 → 239.0	15	4	0.01	0.9992	92 (6, 9)
93	DDT	36.77	234.9 → 165.0	20	234.9 → 198.9	15	4	0.002	0.9899	91 (7, 11)
94	Nuarimol	37.69	235.1 → 139.0	15	314.1 → 139.0	15	5	0.05	0.9803	90 (4, 6)
95	Resmethrin	39.00	171.1 → 128.0	9	171.1 → 143.0	9	4	0.05	0.9815	93 (5, 9)
96	Carbosulfan	39.80	163.1 → 107.1	15	163.1 → 135.1	10	4	0.01	0.9907	87 (10, 7)
97	Phosmet	40.66	160.0 → 104.0	20	160.0 → 133.0	15	4	0.01	0.9928	89 (4, 8)
98	EPN	40.75	169.0 → 77.0	16	169.0 → 141.0	10	4	0.005	0.9994	86 (6, 9)
99	Bifenthrin	40.81	181.0 → 153.0	6	181.0 → 166.0	15	4	0.04	0.9856	91 (11, 11)
100	Tebufenpyrad	41.87	333.1 → 171.1	20	333.1 → 276.1	10	4	0.04	0.9908	94 (7, 10)
101	Leptophos	42.96	374.9 → 359.9	26	376.9 → 361.9	26	5	0.01	0.9873	101 (3, 7)
102	Phosalone	43.10	182.0 → 111.0	15	182.0 → 138.0	10	4	0.02	0.9972	94 (8, 9)
103	Azinphos methyl	43.57	132.0 → 77.0	15	160.0 → 104.0	10	5	0.01	0.9948	91 (5, 9)
104	Amitraz	44.30	293.2 → 147.1	15	293.2 → 162.1	10	4	0.05	0.9982	92 (6, 4)
105	Pyrazophos	44.92	221.0 → 177.0	15	221.0 → 193.0	10	4	0.01	0.9929	94 (9, 4)
106	Azinphos ethyl	45.36	160.0 → 104.0	10	160.0 → 132.0	5	4	0.01	0.9992	91 (14, 10)
107	Cifluthrin	49.00	163.0 → 91.0	12	163.0 → 127.0	10	4	0.05	0.9836	77 (4, 8)
108	Flucythrinate	50.00	199.1 → 107.0	22	199.1 → 157.0	10	4	0.05	0.9812	79 (5, 7)
109	Deltamethrin	53.01	253.0 → 93.0	18	253.0 → 192.0	30	4	0.05	0.9801	83 (9, 13)
Internal standards										
IS1	Aldicarb-D3 (LC)	9.38	211.0 → 89.2	19	211.0 → 119.2	10	4	–	–	–
IS2	Carbofuran-D3 (LC)	10.34	225.0 → 123.1	25	225.0 → 140.5	25	4	–	–	–
IS3	Chlorfenvinphos-D10 (GC)	17.60	263.0 → 159.0	15	369.0 → 101.0	30	5	–	–	–
IS4	Chloropropham (GC)	11.32	213.0 → 127.0	15	213.0 → 171.0	10	4	–	–	–
IS5	Chlorpyrifos-D10 (GC)	23.60	197.0 → 169.0	15	362.0 → 131.0	20	5	–	–	–
IS6	Diazinon-D10 (GC)	17.81	179.1 → 137.1	15	315.0 → 170.0	20	5	–	–	–
IS7	Heptachloro epoxide *cis* (GC)	26.30	352.8 → 262.9	15	352.8 → 288.9	15	4	–	–	–
IS8	Thiobencarb (LC)	258.1	258.1 → 89.1	35	258.1 → 125.0	19	4	–	–	–

[a] Intra-day.
[b] Inter-day.

3. Results and discussion

3.1. Optimization of the instrumental method

LC–MS/MS and GC–MS/MS provided very low detection limits and could be applied to the identification and confirmation of the peak identities. Two transitions were selected for each analyte included in this study (Table 2). The combination of the transitions and their retention times allowed the pesticide identity to be confirmed.

In this work, we simultaneously investigated 90 pesticides suitable for GC (Table 2) to obtain the most efficient quantitative results with maximum separation. Additionally, we investigated 19 polar pesticides, which were separated by LC (Table 2). Although last generation QqQ analyzers permits the monitoring of co-eluted compounds with a high number of transitions simultaneously in MRM mode [18], and thus, the chromatographic separation is not always a critical stage in the development of a multi-residue method, we assayed several temperature programs (GC method), as well as various gradient programs (LC method), to achieve a good separation of the analytes. The chosen GC and LC operating conditions were those described above (Material and Methods section).

To optimize the triple quadrupole MS/MS conditions, the relevant considerations included the choices of the precursor and product ions and the optimization of the collision energies for the best response of each target compound. For the GC method, we firstly analyzed all the pesticides separately, with the aim of obtaining the full scan spectra and to select the parent ions. After that, another set of analyses was conducted at different collision energy voltages (potential on the second quadrupole) to generate the MS/MS product ions. Similarly, the parent ions described in the bibliography for each one of the 19 LC analytes (usually $M–H^+$ or $M–H^-$) were confirmed by analyzing the pesticides in separate runs to obtain their full scan spectra. Then, we selected the collision energies and MS/MS product ions after the direct infusion of a solution of each pesticide in methanol (1 μg/mL)

into the ionization source with the aid of Thermo Fisher Scientific Tune software. The collision energies (5 to 39 eV) are detailed in Table 2.

At the end of this procedure we developed two timed-MRM methods (GC–MS/MS and LC–MS/MS) with two transitions per compound (Table 2). The dwell time was adjusted so that the number of >cycles per second was 10 throughout the chromatographic run to obtain well-shaped chromatographic peaks, low detection limits, and to provide a sufficient number of chromatographic points for all compounds (>15). The peak shapes of all of the analytes in these methods were highly related to the scan time, dwell time, scan rate and number of monitored transitions [19,20]. The final MS/MS conditions used in this study are detailed in Table 2.

The concept of identification points (IPs) for the confirmation stage of mass spectrometry analysis was introduced by the European Commission Decision 2002/657/EC [21]. Meeting the requirements of this regulation, the confirmation of the pesticides included in the present protocol resulted in 4 IPs (two product ions from the same parent ion), or in 5 IPs (two product ions derived from two different parent ions). The resulting number of IPs for each pesticide is also shown in Table 2.

3.2. Optimization of sample extraction and cleanup

The liquid–liquid extraction procedure consists of shaking the samples several times in selected organic solvents to extract the pesticide residues from the bulk of the sample matrix. We considered that this method could be convenient for the extraction of pesticides from blood samples. Considering that the pesticides that can be involved in a poisoning episode can belong to different chemical classes, it is critical to select the appropriate solvents to achieve a satisfactory recovery of all of the analytes from the matrix of interest. Organic solvents such as ethanol, methanol, ethyl acetate, hexane and petroleum ether, and their mixtures, including ethanol/ethyl acetate, acetone/hexane, ethyl acetate/acetone/methanol and hexane/dichloromethane have been described for the efficient extraction of pesticides [22]. From the literature we chose various solvent mixtures that would be appropriate for pesticides included in this study, considering their range of polarities. Thus, we assayed the extraction efficacy of mixtures of acetone/hexane (50/50), ethyl acetate/acetone/acetonitrile (40/30/30) and dichloromethane/ethyl acetate/acetone (50/30/20) for all of the pesticides from fortified blood samples. The best combination of purity and recovery was obtained with the dichloromethane/ethyl acetate/acetone (50/30/20) mixture. Therefore we chose this mixture for the extraction.

According to the literature the use of sonication may improve the liquid–liquid extraction efficiency, so we assayed different times of sonication of the samples (5 to 30 min). A slight improvement in the recoveries of certain key pesticides (i.e. aldicarb, carbofuran, diazinon and methomyl) was observed with 5 min of sonication, and therefore this step was added to the extraction protocol.

We also included a cleanup step, since the liquid–liquid extraction is a non-selective method and many potentially interfering substances, such as lipids, sugars or pigments, can be co-extracted. Especially lipids should be eliminated to prevent column damage and signal reduction. There are many strategies that can be used for lipid removal: freezing centrifugation, adsorption chromatography, gel permeation chromatography, or sulfuric acid treatment, among others. We chose freezing centrifugation because blood samples yielded relatively clean extracts. Lipids possess a lower melting point than the solvent, and thus with this cleanup method, the lipids can be removed by centrifugation while pesticides remain dissolved in the solvent. According to our experiments, freezing centrifugation was an adequate single-stage cleanup method for blood samples, as it yielded extracts that were suitable for both, LC–MS/MS and GC–MS/MS analyses.

3.3. Analytical performance

After the optimization of the analytical methodology we studied the confirmation criteria, precision, linearity, method limits of quantification (LOQs) and repeatability, to evaluate its usefulness for the quantitative determination of pesticides in blood samples.

We only identified the compounds as target analytes if the chromatographic peaks satisfied all of the following criteria: 1) the retention time (tR) of the candidate was the same as that averaged plus or minus three standard deviations (SD) of the tR (tR ± 3SD) obtained when six blank samples spiked at the second level of calibration were injected, 2) there was a match with the ion ratios of the standard with a tolerance of ±30% of absolute ion abundances and 3) the S/N ratio of the target analyte was >10 for a sample extract.

To check whether there were matrix effects we carried out experiments in which blank blood samples were spiked with a mixture of the 109 analytes included in this work at two concentrations: 10.0 and 200.0 μg kg^{-1} (five replicates each). By means of the comparison between the quantifications of the recovered pesticides with those of the same concentrations of pesticides in dissolvent we calculated the recovery percentages (Table 2). The results ranged from 68% to 105%, with most of the recoveries being greater than 85% at both concentrations. As the most unfavorable RSD was below 15%, we also found that the precision was satisfactory. The inter-day measurement (recoveries and precision during five consecutive days) also yielded an RSD that was below 15%. Table 2 shows that all results were within the acceptable range and the methods were precise, with RSD values of 3.0–18.0% for all pesticides.

The quantifications were done against matrix-matched calibration curves, ranging between 0.5 and 500 μg kg^{-1} (three replicates for each level were analyzed). Calculations were performed using the peak areas. The calibration curves were constructed without including the origin point and were found to have good linearity based on the correlation coefficients (r^2), which were greater than 0.9801 for all analyses. After performing the residual analysis (values within the range of −10.618 to 11.337) we concluded that the linear regression method may be used for quantifications within the range investigated.

3.4. Application to real samples

The validated method was applied to the analysis of real samples from two recent cases (May and June of 2013) of pesticide poisoning that were received in the toxicology service of the Institute of Legal Medicine of Las Palmas (Canary Islands, Spain).

3.4.1. Case 1

A 79-year-old man was found dead by a friend who went to his house, as the man did not answer the phone. Upon entering he detected a strong odor of "chemical" and found the man on the couch with a belt tied around his neck. According to statements by the sister of the deceased, he lived alone and was being treated for prostate disease and depression. She also reports that he had attempted suicide twice, and for this reason he was being treated in the Mental Health Unit of his area. At autopsy, the remarkable features were: edematous and emphysematous lungs; bloody fluid from the parenchymal cut; presence of yellowish white mucus in the bronchial tubes and trachea; the liver appeared congested; the stomach contained a clear liquid with strong solvent odor, and walls with signs of erosive gastritis; and erosions were also observed in the esophageal mucosa. Gastric content and blood samples were submitted to our laboratory for toxicological analysis. The results of abuse of drugs and alcohol were negative. When we applied the protocol described in this paper to the blood sample in the GC–MS/MS analysis we found the organophosphate insecticide diazinon at a concentration of 6.48 μg/mL (Fig. 1A), which is more than six times higher than the value described by Repetto and Repetto (2007) in a previous fatality [15]. The proposed methodology was also applied to the gastric content sample. We first homogenized the sample and diluted it with ultrapure water (1:10), and we also performed an additional centrifugation in the clean-up step. In Fig. 1B

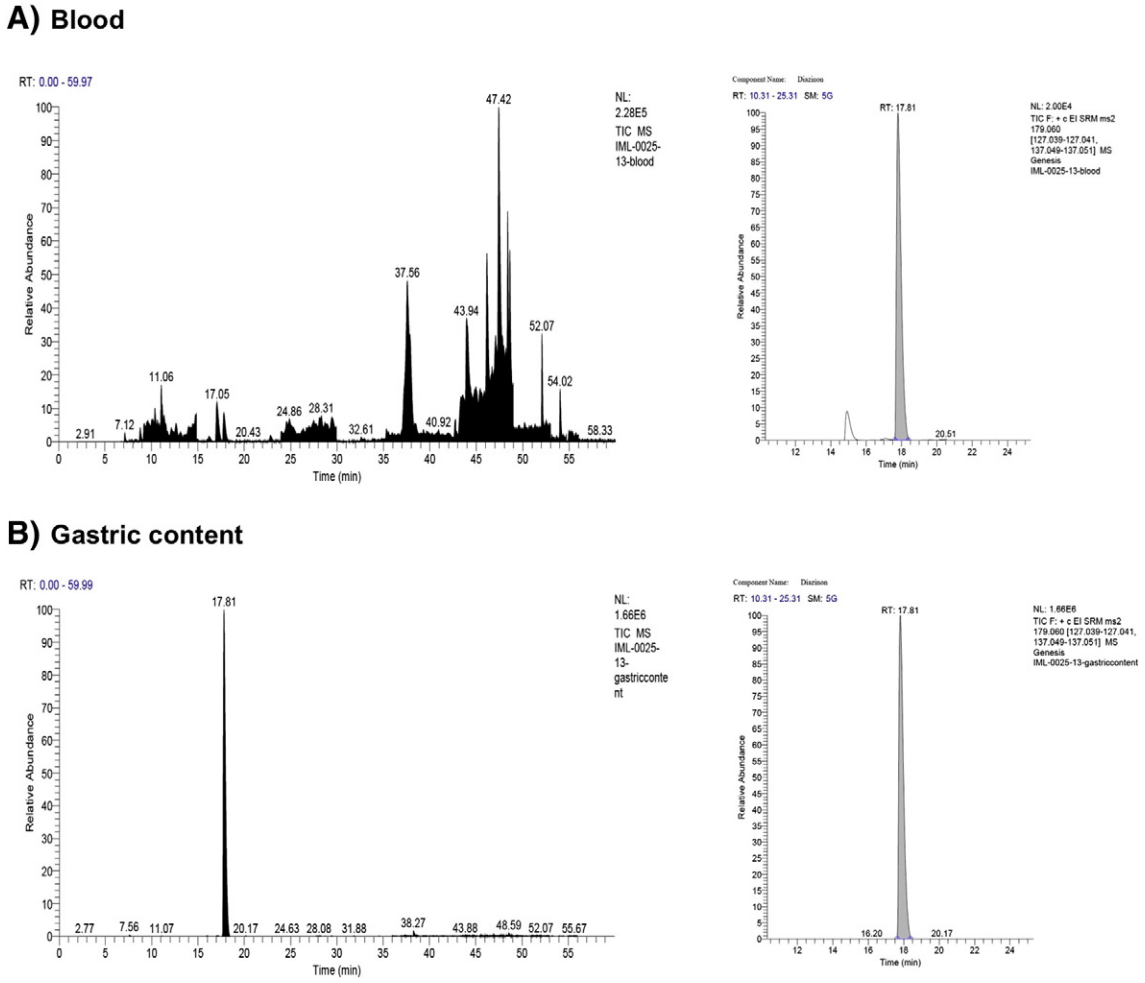

Fig. 1. (A) *Left*, GC–MS/MS total ion current chromatogram (TIC) of blood sample from case 1; *right*. Extracted chromatogram of this sample. Diazinon concentration was 6.48 μg/mL. (B) *Left*, TIC of gastric content sample from case 1. *Right*, extracted MRM chromatogram of this sample, showing the identification of diazinon.

we show the raw and the filter-extracted chromatograms that we obtained from this sample, in which we clearly identified the diazinon.

3.4.2. Case 2

A 63 year old woman was taken to the hospital after eating three tablespoons of vegetable soup that her husband had prepared for her. She did not eat more because "the soup tasted like dirt". According to her own statement, she soon vomited at home and felt strong nausea and abdominal pain. Her son took her to the hospital, and on admission to ICU she displayed marked cholinergic symptoms and was semiconscious. The patient was given pralidoxime and atropine. Her condition gradually improved on days 2 and 3 and she was discharged at 87 h after admission. A blood sample that was taken on admission was submitted to the laboratory and the described methodology was applied. In LC–ESI–MS/MS analysis the carbamate insecticide aldicarb was detected at a concentration of 2.32 μg/mL (Fig. 2). Days later the police brought to our laboratory a plastic container containing the remains of the soup, which had been located by the son in a dumpster quite out of the marital home. According to the police report, the son suspected of an attempted homicide by her father because of his strange behavior and bad relationship and frequent quarrels they had. By visual examination the soup showed abundant black colored granules (Fig. 3). One gram of this material was diluted in 10 mL of ultrapure water and subjected to the same method of extraction and chromatographic analysis and the presence of aldicarb was confirmed.

3.5. Limitations of the methodology

The proposed methodology has been successfully applied to the identification of pesticides in samples from real human poisoning episodes, allowing their quantification in the case of blood samples. Nevertheless, in spite of being quick, easy and very useful, it should be noted that this methodology has several limitations, such as the use of large amounts of expensive and hazardous organic solvents; the necessity of evaporation of solvents, which is a source of environmental contamination; the multiple steps that suppose a risk of analyte losses; and that very relevant pesticides could not be included (i.e. strychnine, paraquat, alpha-chloralose), since they are chemically very different from the rest, so that additional analysis should be specifically targeted to the identification of these compounds in particular. Besides, the whole procedure is time consuming, and because of the nature of the samples and the extraction and cleanup the methodology cannot be automated and therefore costs remain high.

4. Conclusions

We have shown in this paper the applicability of a methodology based on a liquid–liquid extraction followed by a combination of two chromatographic methods (LC and GC) with mass spectrometry detection for the identification and quantification of 109 toxic pesticides in blood samples from human pesticide poisoning episodes. The validation parameters were satisfactory. For all the pesticides we found good

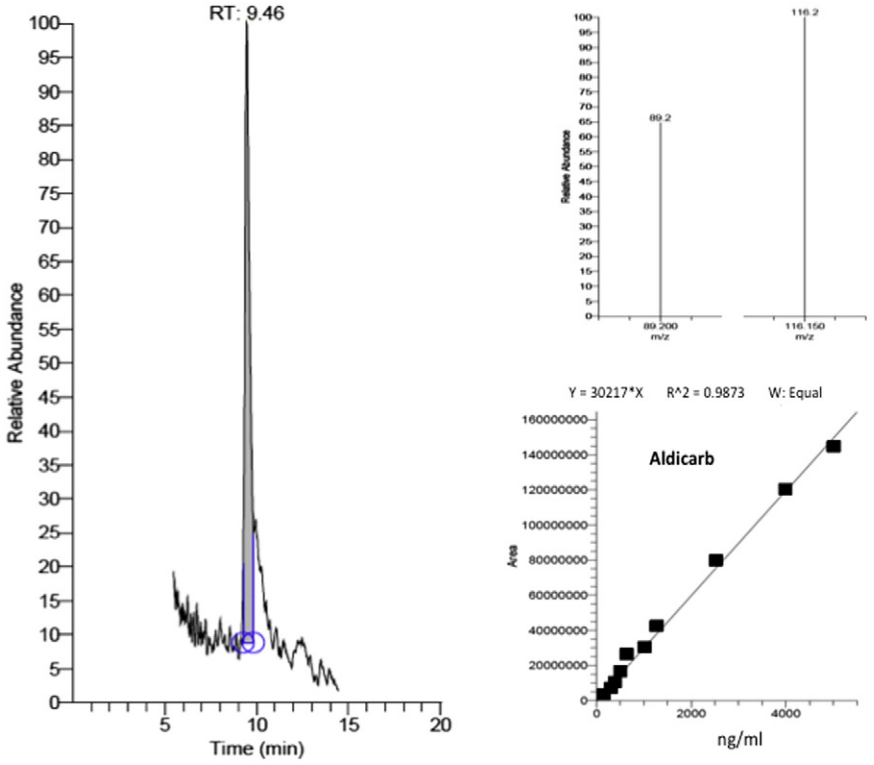

Fig. 2. *Left*, LC–MS/MS extracted MRM chromatogram of the blood sample from case 2; *right*, ion ratio, and calibration plot of aldicarb. Concentration was 2.32 μg/mL.

linearity (0.5–500 μg/mL, with $r^2 > 0.98$) and low detectability. The recoveries (68 to 105%) were good, and the precision (RSD < 15%) was acceptable. Thus we conclude that this methodology, which is simple, sensitive, and very reliable, may be recommended for its routine application in forensic toxicology laboratories. The applicability of the optimized method was proven in the analysis of samples from two recent poisoning cases. Our results also showed that this methodology is robust enough to be applied to samples different from blood, because the involved pesticide was also identified in gastric contents and food samples.

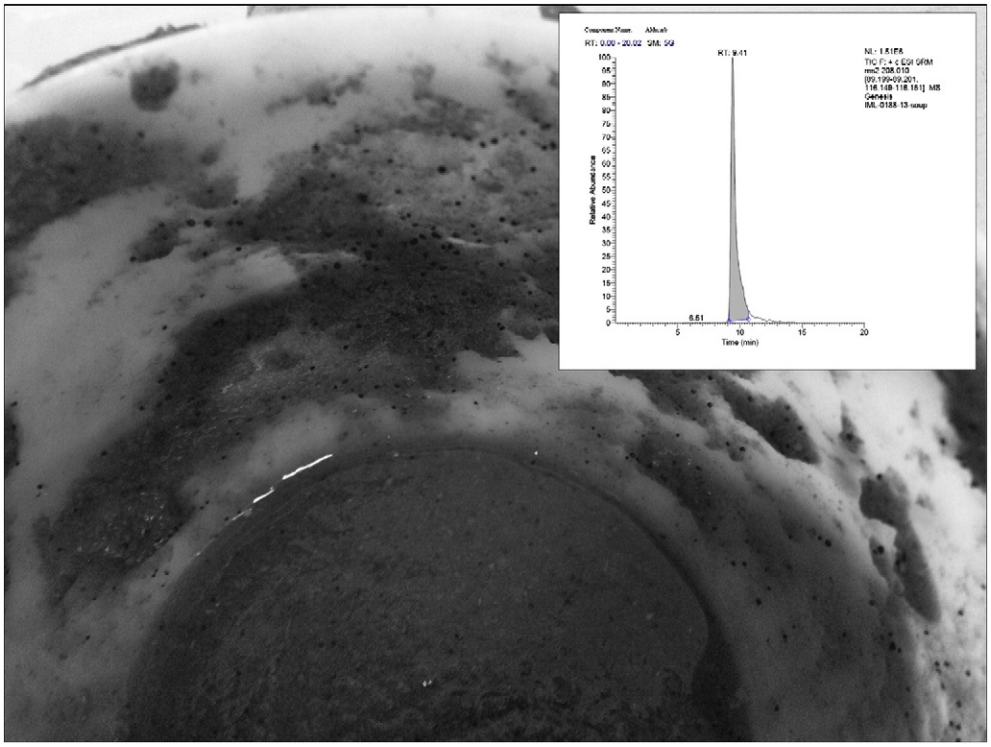

Fig. 3. Vegetable soup showing abundant black colored granules, which were positively identified as aldicarb.

Conflict of interest

There are no financial or other relations that could lead to a conflict of interest.

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