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Article

Methodology for the Identification of 117 Pesticides Commonly Involved in the Poisoning of Wildlife Using GC–MS-MS and LC–MS-MS

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Poisoning with agricultural chemicals is a major threat for wildlife all over the world. We have developed and validated an analytical scheme aimed to the identification and quantification of a wide range of pesticides in fresh liver from wildlife specimens that are routinely delivered to the forensic laboratories for toxicological investigation. The proposed method is comprised of a general solid–liquid extraction followed by purification steps and three complementary liquid or gas chromatographic analyses with triple quadrupole mass spectrometry detection. The developed methodology allows for the determination of 117 highly toxic pesticides in a variety of samples from wildlife poisoning incidents. The validity of the method has been demonstrated in samples from 98 real cases submitted to our laboratory between 2010 and 2012. This method allowed the identification and quantification of poison in 78 of 94 fresh liver samples from wild animals and was successfully used for the identification of pesticides in 35 of 46 non-liver samples. Therefore, the extraction and cleanup method with minor modifications and the potency of triple quadrupole mass spectrometry allow this method to be used to simultaneously detect and quantify or semi-quantify a majority of the most toxic pesticides in a variety of complex and degraded matrices.

Introduction

Accidental or malicious poisoning episodes in wildlife are very common all over the world and represent a major research challenge for toxicologists and forensic laboratories (1). Poisoned animals are usually found dead by hunters, hikers or wildlife protection agents. The investigation of an incident that involves the death of wildlife specimens generally consists of a field inquiry, a postmortem examination and, when necessary, chemical analysis to determine whether a poison might be responsible (2). In addition to biological samples from wildlife specimens, any bait or recipients found at the scene are also usually sent to the laboratory for chemical identification. The variety and complexity of biological samples in different states of decomposition and the wide range of chemicals that can be responsible for a lethal poisoning in wildlife are major sources of difficulty in these toxicological investigations. The development of powerful, sensitive multiresidue identification methodologies is necessary to identify unknown toxicants in this context.

Multistage mass spectrometry (MS-MS) is considered a very useful tool to detect low levels of analyte when coupled with chromatographic techniques. The use of triple quadrupole mass spectrometry (QqQ) analyzers operated in the selective reaction monitoring (SRM) mode significantly improves both the sensitivity and selectivity of the analytical determination, when compared with single-stage mass spectrometry (MS). This is mainly due to the elimination of isobaric interferences and a substantial reduction of background noise (3). Currently, the use of this technique in forensic toxicology laboratories represents a practical way to overcome the complexity that represents the identification of the target analytes in difficult matrices (4). Moreover, the high speed of the electronics of the QqQ analyzers permits the simultaneous acquisition of several transitions and thus the monitoring of co-eluted compounds. This allows the development of multiclass, multiresidue methods that can include dozens or even hundreds of compounds that can be analyzed simultaneously (5).

Among all the chemicals that threaten wildlife, pesticides are particularly important. It has been described that the great majority of all the pesticides used in the agriculture is dispersed into the environment never reaching their target organisms (6). In addition, the deliberate poisoning of prey species that compete with hunters and poachers usually involves bait material that has been laced with pesticides due to their high toxicity (2). It has been estimated that the illegal use of pesticides can be involved in as much as 68% of all suspected poisoning cases (7). Epidemiological studies have revealed that pesticides account for ~52.5% of bird poisonings, and that pesticides are also major agents in wild mammal deaths by poisoning (1). Not all pesticides have the same relevance in wildlife poisonings, with most primary and secondary cases being caused by anticholinesterase agents and anticoagulant rodenticides (7–10).

It is noteworthy that, in general, it is difficult to carry out thorough analytical investigations in the usually decayed samples that are sent to the laboratory, where information on the toxic substance involved is lacking in most cases. Many authors have published methods for the analysis of pesticides in wildlife samples, but most of them have been designed for the analysis of a few pesticides belonging to the same chemical group, making it necessary to use several of these methods in a complementary manner (2, 11–13). For this reason, sensitive and specific multiresidue techniques that cover a wide spectrum of highly toxic substances can substantially contribute to minimizing the costs and maximizing the chance of identifying the toxicant involved. Thus, we have developed a highly sensitive methodology for the extraction, detection and quantification of 117 pesticides that have been selected based on either their high toxicity for wildlife (7, 14), or their frequent use (14). As far as we know to the present, no work has been published that cover such a wide range of highly toxic pesticides in a single analytical scheme and that have been specifically oriented to wildlife forensic samples.

Materials and methods

Ethical statement

The chicken liver samples used in the validation experiments were purchased from a butcher and came from poultry that had been slaughtered in accordance with European legislation. The rest of the samples analyzed came from poisoned animals found dead in the countryside or that died during their stay in the Wildlife Recovery Centers of the Canary Islands, Spain. No animal was killed for the purposes of this study, and no experiments on living animals or with samples coming from them were performed.

Chemicals and reagents

All the solvents (>99.9%) were purchased from Fisher Scientific (Leicestershire, UK). The quality of solvents was: Optima™ LC/MS for acetonitrile and methanol, and Pesticide grade for the rest. Diatomaceous earth Celite® 503 and Bio-Beads SX3 were purchased from Sigma-Aldrich (St. Louis, USA) and BioRad Laboratories (Hercules, USA), respectively. About 0.20-μm polyester syringe filters were from Macherey-Nagel (Düren, Germany). Fifty- and 15-mL polypropylene conical centrifuge tubes were from VWR International (Radnor, PA, USA). Neat standards (97–99.5%) of the analytes included in this method were purchased from Dr. Ehrenstorfer Reference Materials (Augsburg, Germany). Surrogate standards were used to monitor the entire analytical process, especially the step of sample preparation (extraction and clean-up), but also the instrument performance. We used aldicarb-d_3, carbofuran-d_3, chlorfenvinphos-d_{10} and chlorpyriphos-d_{10} for this purpose. We also used a mixture of (±)-warfarin-d_5, thiobencarb, chloropropham, diazinon-d_{10} and heptachloro epoxide, *cis* as internal standards (ISs), since we used the IS method of quantification. Surrogates and ISs were also purchased from Dr. Ehrenstorfer Reference Materials. Diluted working solutions of each compound (0.5 to 500 ng/mL) either in acetonitrile or cyclohexane were used for the calibration curves. Two mixtures of all the standards were prepared in acetone at 10 μg/L or 500 ng/L for the fortification experiments.

Extraction and cleanup procedure

For the extraction, 5 mL of ultrapure water were added to the 2 g of the sample (animal tissue, meat, plant or insects), and homogenization was performed using a disperser at 10,000 rpm (Ultra-turrax T 25, IKA Laboratory Equipment, China). About 25 μL of the mixture of surrogates (40 μg/mL in acetone) were added to give a final concentration of 500 ng/g sample. Diatomaceous earth (10 g) was added to absorb the moisture in the sample, and 10 mL of dichloromethane/ethyl acetate/acetone (50/30/20) were added. The samples were placed in an orbital shaker (Cel-Gro Tissue Culture Rotator, Thermo Fisher Scientific, CA, USA) for 10 min and then sonicated for 5 min. The whole extraction procedure is summarized in Figure 1.

Owing to the usually high content of interfering substances in the extracts one or two additional cleanup steps were needed, depending on the matrix and its degradation status. The fastest

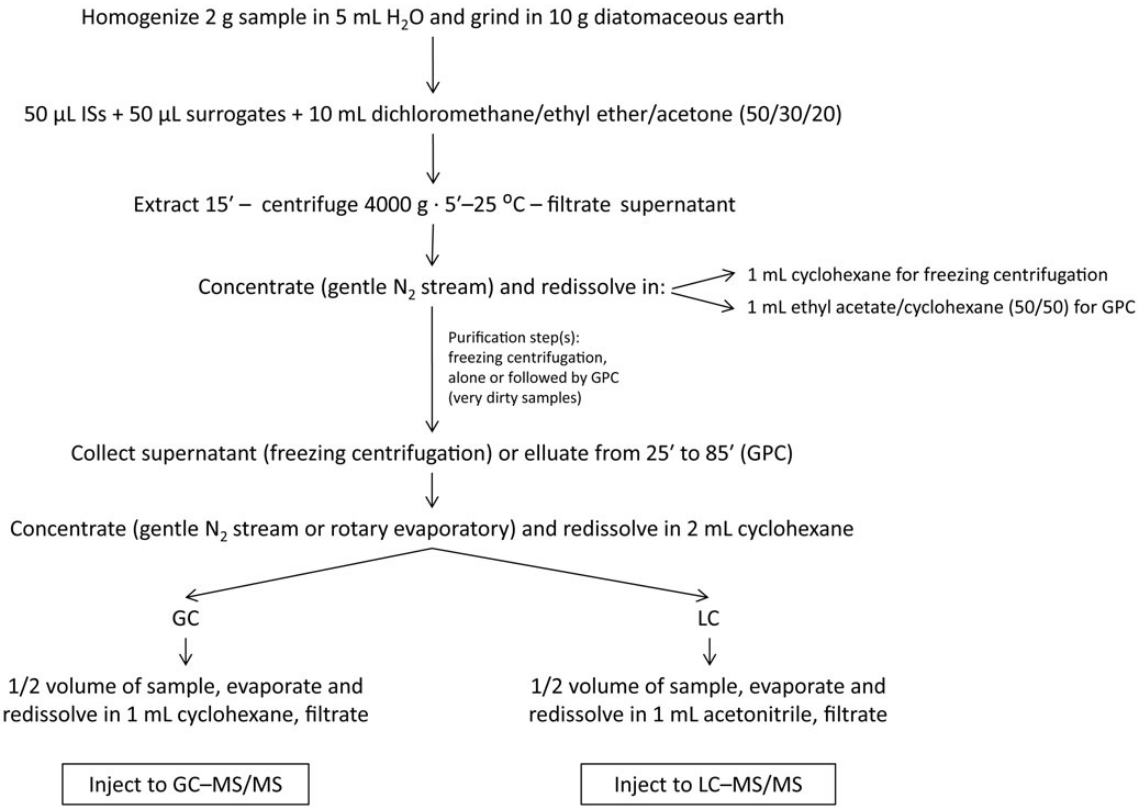

Figure 1. Scheme of the extraction and cleanup method.

and most economical purification method was freezing centrifugation in which the concentrated extract was redissolved in 2 mL of cyclohexane in an Eppendorf tube and placed in an −82°C freezer for 20 min. After this period, the sample was centrifuged (4,000 g, 5 min, −10°C), and the supernatant was carefully removed and separated from the frozen lipids in the bottom of the tube. This purification step was repeated three times, and the resulting supernatant was divided into two aliquots, which were evaporated under a gentle nitrogen stream. The one of them was redissolved in 1 mL of cyclohexane and used for GC–MS-MS and the other, redissolved in acetonitrile and used for LC–MS-MS. This method was applied as the unique cleanup step for extracts from fresh animal tissues, plants or blood. For highly degraded matrices or insect homogenates, which yielded very dirty extracts, freezing centrifugation was also used as a pre-cleaning step, but in these cases, the pre-cleaned extracts were evaporated to dryness, redissolved in 1 mL of ethyl acetate/cyclohexane (50/50) and subjected to an additional purification step by gel permeation chromatography (GPC). We used a 500 × 25 mm column (Omnifit, New York, USA) packed with 30 g Bio-beads S-X3 as the stationary phase. The eluent used was ethyl acetate/cyclohexane (50/50) at a continuous flow of 2 mL/min. Larger molecules (>600 Da) were discarded with the first 25 min of elution (50 mL). The following 120 mL were collected (from min 25 to 85) and concentrated in a rotary evaporator (Heidolph, Schwabach, Germany) to a volume of 10 mL. This concentrate was split into two 5-mL aliquots that were filtrated and evaporated under a gentle nitrogen stream. One of the aliquots was redissolved in cyclohexane and used for GC–MS-MS, and the other was redissolved in acetonitrile and used for LC–MS-MS. Previous to the chromatographic analysis, 10 μL of the mixture of ISs (50 μg/mL in acetone) were added to each vial to reach a final concentration of 500 ng/mL. The efficacy of this purification method for fatty samples has been demonstrated previously in our laboratory (15, 16).

GC-QqQ-MS-MS analysis

We used a Thermo Trace GC Ultra with split/splitless injector for the chromatographic analyses (Thermo Fisher Scientific, Inc., USA). We used as the stationary phase a column of 30 m × 0.25 mm, 0.25 μm film thickness (BPX5, SGE, Inc., USA). As the carrier gas we used helium (99.999%) that was set at constant flow (1.0 mL/min). The 61-min oven temperature program was: 60°C held for 1 min, ramped to 210°C at 12°C/min and then to 320°C at 8°C/min with a 6-min hold time. The injector temperature was set at 270°C, and the transfer line was heated to 310°C. All the injections (1 μL) were done in the splitless mode.

The 91 pesticides that were separated by GC were detected with a triple quadrupole TSQ XLS mass spectrometer (QqQ, Thermo Fisher Scientific, Inc., USA). We first determined their retention times (RTs) in the full scan mode (range: m/z 45–500), and then a timed-SRM method was constructed to analyze all the target compounds, surrogates and ISs in a single run. The calibration curve ranged from 0.5 to 500 ng/mL and included all the compounds in each calibration standard level. The surrogates and ISs were excluded from the calibration mix. As the collision gas in the collision cell we used argon (99.99%) that was set at 0.2 Pa. The operating conditions of the mass spectrometer were: electron impact ionization at 70 eV in SRM (emission current of 50 μA); ionization source temperature 220°C; electron multiplier voltage 1,500 V; scan width 0.15; scan time 0.05 s; peak widths m/z 0.7 Da (first and third quadrupole).

LC-QqQ-MS-MS analysis

We performed two chromatographic methods using a Thermo LC Accela Ultra instrument (Thermo Fisher Scientific, Inc., USA).

For LC–MS-MS method 1 (Table I), we used an analytic Accucore C18 column (2.6 μm, 150 × 3 mm; Thermo Fisher Scientific, Inc., USA) as the stationary phase. The mobile phases were (A) ultrapure water as the aqueous phase and (B) methanol (HPLC–MS grade) as the organic phase. The flow was set at 800 μL/min. The injection volume was 25 μL, and the total run time was 5 min. The gradient program was programmed as follows: 0–1 min: 50% A; 1–1.5 min: 50% A → 5% A; 1.5–3.5 min: 5% A; 3.5–3.7 min: 5% A → 50% A; 3.7–5 min: 50% A.

For LC–MS-MS method 2 (Table I), an analytic Synergi Hydro-RP column (4.0 μm, 150 × 4.6 mm; Phenomenex, Torrance, USA) was used as the stationary phase. The mobile phases were (A) 7.5 mM ammonium formate in ultrapure water as the aqueous phase, (B) methanol (HPLC–MS grade) as the organic phase and (C) 2% formic acid. The solvent flow was 1,000 μL/min. The injection volume was 25 μL, and the total run time was 26 min. During the entire run, 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; 16.2–25.0 min: 87.5% A.

The 26 most polar pesticides that were separated by LC were detected using a TSQ Quantum Max QqQ mass spectrometer equipped with the H-ESI II heated electrospray ionization source (Thermo Fisher Scientific, Inc., USA). For LC–MS-MS methods 1 and 2, the mass spectrometer and the ionization source were programmed according to the following parameters, respectively: skimmer offset (4 and 10 V), sheat gas pressure (10 and 15 arbitrary units, a.u.), ion sweep gas pressure (8 and 0 a.u.), capillary temperature (250°C in both cases), spray voltage (3,500 and 3,000 V) and vaporization temperature (200 and 180°C). The spectrometer was programmed in the negative ionization mode for method 1 and the positive mode for method 2.

We first determined their RTs in the full scan mode (range: m/z 45–500), and then we constructed a timed-SRM method by directly infusing pure standard methanolic solutions into the source to analyze 26 target compounds as well as surrogates and ISs in two separate runs. The calibration curve included 10 levels that ranged from 0.5 to 500 ng/mL for all compounds, excluding the surrogates and ISs. The gas in the collision cell was argon (99.99%) at a pressure of 0.25 Pa.

Validation

Chicken liver samples that were purchased from a butcher were used for the validation experiments. We added 40 μL of a 10-μg/mL or 500-ng/mL working standard solution in acetone (containing all the pesticides included in the method) to 2 g of liver tissue homogenized in 5 mL of ultrapure water to obtain concentrations of 200 and 10 ng/g, respectively. We determined the recoveries from spiked liver tissue in quintuplicate experiments by comparing the obtained concentrations with the same concentrations of the pesticides prepared in the dissolvent. In the same experiments, we also determined the intra- and inter-day precision

Table I

Toxicities of the pesticides detected by LC–MS-MS, method settings and results from recovery experiments

No.	Compound	Toxicity (LD_{50}, mg/kg)[a]		Mass spectrometry settings							Validation parameters			
		Birds	Mammals	RT (min)	CV (V)	First transition ($m/z \rightarrow m/z$)	CE (V)	Second transition ($m/z \rightarrow m/z$)	CE (V)	IPs	LOD (μg/mL)	LOQ (μg/mL)	Average recovery % (RSD)	IS
LC–MS-MS method 1														
1	Coumatetralyl	38.3	42.5	1.57	65	291.1 → 140.9	28	291.1 → 247.0	22	4	0.01	0.03	89.2 (13.6)	1
2	Warfarin	942.0	6.5	1.71	56	307.1 → 116.9	39	307.1 → 250.0	24	4	0.005	0.02	92.7 (8.3)	1
3	Chlorophacinone	430.0	7.5	1.76	123	373.1 → 116.0	50	373.1 → 200.9	25	4	0.01	0.03	87.9 (12.4)	1
4	Difenacoum	50.0	50.0	1.83	90	443.2 → 134.9	36	443.2 → 293.0	33	4	0.005	0.01	91.3 (11.7)	1
5	Brodifacoum	4.5	2.5	1.88	108	521.1 → 135.0	44	521.1 → 186.9	39	4	0.005	0.01	97.4 (5.8)	1
6	Bromadiolone	138	16.5	2.02	96	525.1 → 180.9	37	525.1 → 249.9	37	4	0.005	0.01	94.3 (8.9)	1
7	Difethialone	0.9	4.0	2.08	100	537.1 → 150.9	45	537.1 → 370.9	36	4	0.01	0.03	86.9 (13.4)	1
LC–MS-MS method 2														
8	Metamidofos	14.3	18.5	2.26	148	142.1 → 94.0	14	142.1 → 125.0	16	4	0.005	0.02	62.8 (14.6)	2
9	Oxamyl	4.2	30.0	2.78	85	237.2 → 163.0	14	237.2 → 196.0	18	4	0.01	0.05	63.2 (11.6)	2
10	Phoxim	5.6	250	4.03	117	300.1 → 129.3	18	300.1 → 283.0	10	4	0.005	0.02	61.9 (15.5)	2
11	Acephate	125.0	321.0	4.51	100	184.1 → 125.0	16	184.1 → 143.0	5	4	0.01	0.05	89.7 (12.4)	2
12	Omethoate	125.0	50.0	5.03	106	214.0 → 155.0	19	214.0 → 183.0	13	4	0.01	0.05	93.3 (13.7)	2
13	Metomil	20.5	24.9	6.71	98	163.1 → 88.1	11	163.1 → 106.0	12	4	0.005	0.02	74.6 (9.8)	2
14	Imidacloprid	152.0	98.0	8.05	99	256.1 → 175.0	18	256.1 → 209.0	16	4	0.005	0.02	97.3 (4.3)	2
15	Dimethoate	45.6	220.0	8.64	150	230.0 → 125.0	23	230.0 → 199.0	11	4	0.01	0.05	88.2 (9.7)	2
16	Aldicarb	3.8	1.9	9.98	115	208.0 → 89.2	19	208.0 → 116.2	10	4	0.005	0.02	67.2 (12.4)	2
17	Carbofuran	22.4	10.2	10.34	147	222.0 → 123.1	25	222.0 → 137.5	24	4	0.005	0.03	98.4 (3.1)	2
18	Propoxur	19.9	51.2	10.76	123	210.0 → 111.2	18	210.0 → 168.0	11	4	0.005	0.02	70.1 (10.0)	2
19	Carbaryl	56.0	150.0	11.20	145	202.1 → 127.0	33	202.1 → 145.1	13	4	0.005	0.02	64.1 (12.9)	2
20	Pirimicarb	45.5	100.0	11.33	92	239.1 → 72.3	27	239.1 → 182.1	16	4	0.01	0.05	84.3 (10.7)	2
21	Carboxin	42.2	430.0	11.49	92	236.1 → 93.2	33	236.1 → 143.0	15	4	0.01	0.05	65.2 (12.7)	2
22	Bromoxynil	50.0	78.0	12.01	95	275.9 → 79.2	29	275.9 → 81.1	33	4	0.01	0.05	85.7 (9.1)	2
23	Methiocarb	2.4	16.0	12.97	135	226.0 → 121.0	19	226.0 → 169.0	8	4	0.01	0.05	62.8 (14.3)	2
24	Cyproconazole	150.0	352.0	13.46	107	292.1 → 70.3	17	292.1 → 125.1	34	4	0.01	0.02	58.4 (11.1)	2
25	Benfuracarb	92.0	102.0	15.02	111	411.2 → 190.1	13	411.2 →252.3	15	4	0.01	0.05	66.2 (16.5)	2
26	Profenofos	1.9	116.0	15.52	122	373.0 →302.8	18	373.0 →344.8	13	4	0.01	0.03	69.9 (13.4)	2
Surrogates														
S1	Aldicarb-d_3			9.98	115	211.0 → 89.2	19	211.0 → 119.2	10	4	–	–	–	
S2	Carbofuran-d_3			10.34	147	225.0 → 123.1	25	225.0 → 140.5	25	4	–	–	–	
Internal standards														
IS1	(±)-Warfarin-d_5			1.71	56	312.1 → 116.9	39	312.1 → 250.0	24	4	–	–	–	
IS2	Thiobencarb			8.65	132	258.1 → 89.1	35	258.1 → 125.0	19	4	–	–	–	

RT: retention time; CV: cone voltage; CE: collision energy; IPs: identification points; LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation.

[a]Average data from different species. These data have been taken from Mineau *et al.* (14) 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).

(5 successive days). The matrix effect was evaluated by comparing spiked samples after the extraction of liver tissue with the spiked-extracted samples at the same concentrations.

Those concentrations of analytes, which produced a signal peak of 10-fold the background noise of the chromatogram, were set as the limit of quantification (LOQ) of the method. We quantified on the basis of the peak areas. Least squares linear regressions were constructed from the areas of each of the 10 calibration levels (0.5–500 μg/kg).

Quality control

In each batch of samples, two controls were included: a reagent blank consisting of a vial containing only cyclohexane and an internal laboratory quality control (QC) consisting of melted butter spiked at 20 μg/kg of each of the analytes processed using the same method as the samples. The batch analyses were considered valid when the values of the analytes in the QC were within a 10% of deviation of the theoretical value.

Results and discussion

Optimization of the instrumental method

In this study, 91 apolar pesticides suitable for gas chromatography (Table II) were investigated under the same optimized temperature program and analysis time to obtain the most efficient quantitative results with maximum separation. Twenty-six polar pesticides (Table I) were separated by liquid chromatography in two different chromatographic runs because the conditions that yielded the optimal ionization of the parent compounds for MS were different. The complete chromatographic separation of all the analytes is not always necessary when using QqQ analyzers, since the high-speed acquisition of these spectrometers (high number of simultaneous SRM transitions) allows the identification of co-eluted compounds (17). Nevertheless, to achieve a good separation, various changes in the temperature program were assayed in the gas chromatography method, and various gradient programs were assayed in the liquid chromatography methods. The chosen GC and LC operating conditions were described in the Material and Methods section.

For the optimization of the conditions of the triple quadrupole MS-MS, the precursor ion for each of the 91 GC analytes was first selected after analyzing the pesticides separately to obtain the full scan spectra. The product ions from the precursor ion were selected from another set of experiments at different collision energy (CE) voltages. The precursor ions of each of the 26 LC analytes (usually $M-H^+$ or $M-H^-$) were selected from the bibliography, and the CEs and MS-MS product ions were chosen

Table II

Toxicities of the pesticides detected by GC–MS-MS, method settings and results from recovery experiments

No.	Compound	Toxicity (LD_{50}, mg/kg)[a]		Mass spectrometry settings						Validation parameters			
		Birds	Mammals	RT (min)	First transition ($m/z \rightarrow m/z$)	CE (V)	Second transition ($m/z \rightarrow m/z$)	CE (V)	IPs	LOD (μg/mL)	LOQ (μg/mL)	Average recovery % (RSD)	IS
27	Dimefox	1.7	3.5	5.33	154.1 → 58.0	10	154.1 → 111.1	10	4	0.001	0.01	62.5 (13.3)	3
28	Dichlorphos	8.8	61.0	7.61	185.0 → 109.0	15	185.0 → 127.0	12	4	0.001	0.01	78.7 (9.5)	3
29	Metamidophos	8.0	18.0	9.32	141.0 → 80.0	10	141.0 → 95.0	10	4	0.001	0.01	93.2 (7.6)	3
30	Mevinphos	1.4	4.0	9.72	192.0 → 127.0	12	192.0 → 164.0	10	4	0.001	0.02	68.7 (11.4)	4
31	Chlormephos	65.0	12.5	9.94	154.0 → 121.0	5	154.0 → 121.0	14	4	0.001	0.02	82.3 (9.9)	4
32	Metolcarb	100.0	109.0	10.56	108.1 → 79.0	10	108.1 → 107.1	10	4	0.001	0.01	62.4 (15.7)	4
33	Heptenophos	17.0	117.0	12.26	250.0 → 124.0	10	250.0 → 215.0	4	4	0.001	0.05	76.5 (9.3)	4
34	Thionazin	2.4	5.0	12.96	192.0 → 96.0	10	248.0 → 140.0	10	5	0.001	0.01	79.2 (8.2)	4
35	TEPP	1.3	2.3	13.07	263.1 → 179.1	15	263.1 → 235.1	5	4	0.001	0.02	97.6 (7.5)	3
36	Propachlor	91.0	392.0	13.25	176.1 → 120.0	10	196.1 → 120.0	10	5	0.001	0.01	78.3 (11.2)	3
37	Etoprophos	4.2	34.0	13.70	158.0 → 114.0	10	158.0 → 130.0	10	4	0.001	0.01	63.3 (11.5)	4
38	Sulfotep	25.0	22.0	14.50	322.0 → 202.0	15	322.0 → 294.0	10	4	0.001	0.01	81.5 (10.7)	3
49	Dicrotophos	1.2	11.0	14.61	127.0 → 95.0	10	127.0 → 109.0	10	4	0.001	0.05	92.4 (9.3)	4
40	Bendiocarb	21.0	35.0	14.79	166.1 → 151.1	15	223.1 → 166.1	15	5	0.001	0.01	94.5 (4.5)	3
41	Cadusafos	16.0	71.4	14.93	159.1 → 97.0	20	159.1 → 131.0	10	4	0.001	0.01	96.7 (8.9)	4
42	Phorate	1.0	20.0	15.24	260.0 → 75.0	5	260.0 → 231.0	8	4	0.001	0.02	88.3 (11.8)	4
43	Diallate	167.0	395.0	15.30	236.0 → 152.0	20	236.0 → 194.0	15	4	0.001	0.01	99.3 (10.6)	4
44	Monocrotophos	0.8	15.0	15.80	127.0 → 95.0	20	127.0 → 109.3	25	4	0.001	0.01	88.5 (14.3)	3
45	Thiometon	100.0	37.0	15.85	88.0 → 60.0	15	248.0 → 88.0	15	5	0.001	0.05	91.2 (11.0)	4
46	Dazomet	424.0	415.0	16.56	89.0 → 75.0	20	162.0 → 89.0	8	5	0.001	0.01	87.6 (6.8)	4
47	Dioxathion	200.0	10.0	17.13	125.0 → 97.0	15	197.0 → 141.0	15	5	0.0005	0.005	97.6 (8.2)	4
48	Lindane	127.0	25.0	17.16	216.9 → 180.9	15	218.9 → 182.9	15	5	0.001	0.01	97.7 (13.2)	5
49	Propetamphos	49.0	130.0	17.35	236.1 → 166.1	15	236.1 → 194.1	5	5	0.001	0.01	94.7 (6.4)	4
50	Terbufos	15.0	3.5	17.37	231.0 → 175.0	15	231.0 → 203.0	10	4	0.001	0.01	99.1 (3.4)	4
51	Diazinon	2.0	76.0	17.57	179.1 → 127.0	15	179.1 → 137.1	15	4	0.001	0.01	92.1 (9.9)	5
52	Chlorfenvinphos	13.0	20.0	17.60	267.0 → 159.0	15	323.0 → 269.0	10	5	0.001	0.01	97.9 (11.6)	4
53	Cyanophos	3.0	215.0	17.60	243.0 → 109.0	12	243.0 → 127.0	15	4	0.001	0.01	89.7 (12.2)	4
54	Fonofos	10.0	3.0	17.69	137.0 → 109.0	10	246.0 → 137.0	10	5	0.001	0.01	93.3 (7.9)	4
55	Disulfoton	2.4	5.0	18.32	274.0 → 88.0	10	274.0 → 245.0	10	4	0.001	0.02	99.7 (5.8)	4
56	Tefluthrin	267.0	22.0	18.40	197.0 → 141.0	15	197.0 → 161.0	10	4	0.001	0.01	79.3 (12.1)	3
57	Isazophos	244.0	27.0	18.40	257.0 → 119.0	15	257.0 → 162.0	15	4	0.001	0.02	95.2 (7.7)	4
58	Dichlone	–	160.0	18.58	191.0 → 135.0	15	226.0 → 191.0	10	5	0.001	0.01	88.6 (12.0)	3
59	Formothion	630.0	175.0	19.94	224.0 → 125.0	15	224.0 → 196.0	10	4	0.001	0.01	97.2 (3.7)	4
60	Phosphamidon	1.8	6.0	20.08	264.0 → 127.0	15	264.0 → 127.0	15	4	0.001	0.01	98.6 (6.8)	4
61	Chlorpyriphos methyl	13.0	2000.0	20.57	285.9 → 93.0	25	285.9 → 272.9	13	4	0.001	0.01	93.2 (12.4)	4
62	Parathion methyl	5.0	57.0	21.10	263.0 → 109.0	15	263.0 → 127.0	15	4	0.0005	0.005	95.4 (12.1)	4
63	Heptachlor	125.0	50.0	21.36	338.8 → 267.9	15	338.8 → 303.8	15	4	0.001	0.01	85.6 (9.7)	5
64	Fenitrothion	11.0	142.0	22.74	277.0 → 109.0	20	277.0 → 260.0	15	4	0.001	0.01	96.7 (7.6)	4
65	Pirimiphos methyl	30.0	1150.0	23.19	290.1 → 125.0	15	290.1 → 233.1	10	4	0.001	0.01	96.1 (8.4)	4
66	Malathion	400.0	53.0	23.27	173.0 → 127.0	10	173.0 → 145.0	5	4	0.001	0.01	92.2 (6.3)	4
67	Chlorpyrifos	5.2	60.0	23.60	197.0 → 169.0	15	199.0 → 171.0	15	5	0.0005	0.003	95.3 (11.2)	4
68	Aldrin	7.2	65.0	23.60	262.9 → 192.9	32	262.9 → 227.9	26	4	0.001	0.01	97.3 (13.7)	5
69	Fenthion	1.4	46.2	24.08	278.0 → 169.0	20	278.0 → 245.0	15	4	0.001	0.01	91.5 (10.0)	4
70	Parathion ethyl	1.3	0.9	24.26	291.0 → 109.0	15	291.0 → 263.0	10	4	0.005	0.02	96.3 (7.8)	4
71	Isobenzan	1.0	5.0	24.41	310.8 → 274.8	10	312.8 → 276.8	10	5	0.005	0.05	76.4 (8.9)	4
72	Cyanazine	400.0	141.0	24.59	225.1 → 189.1	10	225.1 → 198.1	10	4	0.005	0.04	78.3 (9.1)	4
73	Trichloronat	1.6	10.0	24.70	296.9 → 268.9	15	299.9 → 271.9	15	5	0.001	0.01	63.9 (13.2)	4
74	Pirimiphos ethyl	3.0	25.0	26.08	333.1 → 288.1	20	333.1 → 318.1	15	4	0.001	0.01	92.1 (11.6)	4
75	Isofenphos	3.0	91.5	26.44	255.1 → 185.1	10	255.1 → 213.1	10	4	0.001	0.02	95.2 (6.7)	4
76	Allethrin	2030.0	370.0	26.93	123.1 → 81.1	10	136.1 → 93.1	10	4	0.001	0.05	78.9 (8.8)	4
77	Phenthoate	58.6	138.0	27.10	274.0 → 125.0	7	274.0 → 246.0	10	4	0.001	0.03	76.5 (9.6)	4
78	Quinalphos	20.0	75.0	27.17	146.0 → 91.0	15	146.0 → 118.0	15	4	0.001	0.03	92.1 (13.2)	4
79	Mephospholan	2.8	11.0	27.60	196.0 → 140.0	15	196.0 → 168.0	10	4	0.0005	0.005	98.3 (3.6)	4
80	Chlordane, *trans*	220.0	50.0	28.04	372.8 → 265.9	15	374.8 → 267.9	16	5	0.001	0.01	92.1 (7.9)	5
81	Bromophos ethyl	20.5	125.0	28.07	358.9 → 302.9	20	358.9 → 330.9	10	4	0.001	0.01	94.4 (9.5)	4
82	Methidathion	80.0	25.0	28.23	145.0 → 58.0	15	145.0 → 85.0	10	4	0.001	0.01	98.7 (9.3)	4
83	Propaphos	2.5	61	28.58	220.1 → 140.0	15	304.1 → 220.1	15	5	0.001	0.01	92.2 (10.9)	4
84	Tetrachlorvinphos	100.0	4200.0	28.64	330.9 → 109.0	22	330.9 → 316.0	22	4	0.001	0.01	97.7 (2.5)	4
85	Endosulfan, alpha	35.0	26.0	28.88	195.9 → 158.9	16	195.9 → 159.9	15	4	0.0005	0.005	93.6 (9.9)	5
86	Chlordane, *cis*	220.0	50.0	28.90	372.8 → 265.9	18	409.8 → 374.8	5	5	0.001	0.01	95.9 (10.0)	5
87	Fenamiphos	2.4	10.0	29.98	303.1 →260.1	15	303.1 → 288.1	15	4	0.0005	0.005	96.6 (10.0)	4
88	Dieldrin	13.3	65	30.87	276.9 → 206.9	20	276.9 → 240.9	10	4	0.0005	0.005	93.3 (9.3)	5
89	Endrin	1.7	3.0	32.42	262.9 → 190.9	25	262.9 → 192.9	26	4	0.001	0.01	95.8 (9.5)	5
90	Isoxathion	21.6	112.0	32.47	177.0 → 130.0	15	313.0 → 177.0	15	5	0.001	0.01	95.7 (6.7)	4
91	Endosulfan, beta	35.0	26.0	33.50	195.9 → 158.9	16	195.9 → 159.9	15	4	0.001	0.01	93.9 (8.9)	5
92	Fensulfothion	0.3	2.2	33.84	293.0 → 97.0	16	293.0 → 125.0	0	4	0.001	0.01	99.1 (9.1)	4
93	Ethion	45.0	13.0	33.96	231.0 → 175.0	15	231.0 → 203.0	15	4	0.001	0.01	89.9 (11.7)	4
94	Chlorthiophos	45.0	20.0	34.26	325.0 → 269.0	15	325.0 → 297.0	10	4	0.001	0.01	94.3 (6.6)	4
95	Sulprofos	65.0	70.0	35.31	322.0 → 139.0	15	322.0 → 156.0	15	4	0.001	0.01	94.1 (7.9)	4
96	Triazophos	4.2	57.0	35.55	161.0 → 105.0	13	161.0 → 134.0	10	4	0.001	0.01	96.4 (7.3)	4
97	Famphur	1.8	59.0	35.87	218.0 → 109.0	10	218.0 → 127.0	10	4	0.001	0.01	95.2 (7.9)	4

(*continued*)

Table II Continued

No.	Compound	Toxicity (LD_{50}, mg/kg)[a]		Mass spectrometry settings					IPs	Validation parameters			
		Birds	Mammals	RT (min)	First transition ($m/z \rightarrow m/z$)	CE (V)	Second transition ($m/z \rightarrow m/z$)	CE (V)		LOD (μg/mL)	LOQ (μg/mL)	Average recovery % (RSD)	IS
98	Carbophenothion	5.8	14.0	36.02	342.0 → 157.0	10	342.0 → 296.0	5	4	0.001	0.01	95.8 (11.0)	4
99	Ediphenphos	350.0	100.0	36.23	173.0 → 109.0	15	310.0 → 173.0	10	4	0.001	0.01	98.3 (9.3)	4
100	Endosulfan sulphate	52.4	18.0	36.38	273.9 → 236.9	10	273.9 → 239.0	15	4	0.0005	0.005	89.7 (8.7)	5
101	DDT	1135.0	200.0	36.77	234.9 → 165.0	20	234.9 → 198.9	15	4	0.001	0.05	93.3 (7.8)	5
102	Nuarimol	200.0	2450.0	37.69	235.1 → 139.0	15	314.1 → 139.0	15	5	0.001	0.05	68.9 (5.6)	4
103	Resmethrin	75.0	250.0	39.00	171.1 → 128.0	9	171.1 → 143.0	9	4	0.001	0.01	79.4 (11.3)	4
104	Carbosulfan	120.0	115.0	39.80	163.1 → 107.1	15	163.1 → 135.1	10	4	0.001	0.01	79.1 (5.4)	4
105	Phosmet	18.0	40.0	40.66	160.0 → 104.0	20	160.0 → 133.0	15	4	0.0005	0.005	96.7 (9.1)	4
106	EPN	2.4	20.0	40.75	169.0 → 77.0	16	169.0 → 141.0	10	4	0.003	0.04	98.2 (9.9)	4
107	Bifenthrin	1975.0	54.5	40.81	181.0 → 153.0	6	181.0 → 166.0	15	4	0.003	0.04	75.9 (10.2)	4
108	Tebufenpyrad	2000.0	210.0	41.87	333.1 → 171.1	20	333.1 → 276.1	10	4	0.001	0.01	76.9 (17.9)	4
109	Leptophos	268.8	65.0	42.96	374.9 → 359.9	26	376.9 → 361.9	26	5	0.001	0.02	96.8 (4.5)	4
110	Phosalone	–	112.0	43.10	182.0 → 111.0	15	182.0 → 138.0	10	4	0.001	0.01	91.0 (9.2)	4
111	Azinphos methyl	8.5	10.0	43.57	132.0 → 77.0	15	160.0 → 104.0	10	5	0.003	0.05	97.3 (9.4)	4
112	Amitraz	–	100.0	44.30	293.2 → 147.1	15	293.2 → 162.1	10	4	0.001	0.01	85.9 (9.2)	4
113	Pyrazophos	118.0	184.0	44.92	221 .0→ 177.0	15	221.0 → 193.0	10	4	0.001	0.01	98.2 (4.3)	4
114	Azinphos ethyl	34.4	12.0	45.36	160.0 → 104.0	10	160.0 → 132.0	5	4	0.005	0.05	91.7 (11.5)	4
115	Cifluthrin	250.0	300.0	49.00	163.0 → 91.0	12	163.0 → 127.0	10	4	0.005	0.05	78.5 (13.2)	4
116	Flucythrinate	2708.0	76.0	50.00	199.1 → 107.0	22	199.1 → 157.0	10	4	0.005	0.05	77.0 (5.4)	4
117	Deltamethrin	1000.0	22.0	53.01	253.0 → 93.0	18	253.0 → 192.0	30	4	0.005	0.05	81.4 (9.4)	4
Surrogates													
S3	Chlorfenvinphos-d_{10}			17.60	263.0 → 159.0	15	369.0 → 101.0	30	5	–	–	–	–
S4	Chlorpyrifos-d_{10}			23.60	197.0 → 169.0	15	362.0 → 131.0	20	5	–	–	–	–
Internal standards													
IS3	Chloropropham			11.32	213.0 → 127.0	15	213.0 → 171.0	10	4	–	–	–	–
IS4	Diazinon-d_{10}			17.81	179.1 → 137.1	15	315.0 → 170.0	20	5	–	–	–	–
IS5	Heptachloro epoxide, *cis*			26.30	352.8 → 262.9	15	352.8 → 288.9	15	4	–	–	–	–

RT: retention time; CE: collision energy; IPs: identification points; LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation.

[a]Average data from different species. These data have been taken from Mineau *et al.* (14) 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).

by the direct infusion of a 1-μg/mL of methanolic solution of each pesticide into the ionization source. All the selected CEs of these complementary methods ranged from 5 to 39 eV (Tables I and II). The final goal was to develop three timed-SRM methods with two reactions or transitions per compound. The dwell time was adjusted to 10 cycles per second throughout the chromatogram to obtain low detection limits and well-shaped chromatographic peaks. The peak shapes were satisfactory and were highly related to the number of monitored transitions, the scan and dwell times, and the scan rate (18, 19). According to the European Commission Decision 2002/657/EC (20), which introduced the concept of identification points (IPs) for the confirmation stage, the confirmation of the analytes included in this study involved the monitoring of two product ions from the same precursor ion, which resulted in four IPs, or two product ions derived from two different precursor ions, which resulted in five IPs. Therefore, the timed-SRM methods used in this study meet the requirements of the aforementioned regulation (20). The resulting number of IPs and also the final MS-MS conditions used in this study for each analyte are summarized in Tables I and II.

Optimization of sample extraction and cleanup

In the present study, solid–liquid principles were adopted for the extraction of contaminants from samples coming from wildlife poisoning episodes. When considering the simultaneous extraction of analytes belonging to different chemical classes, selecting the appropriate solvents and extraction methods is critical to achieving a satisfactory recovery from the matrix. Many organic solvents are applied in the literature for the extraction of pesticides (21). We selected mixtures of solvents that have been applied to the extraction of pesticides of each chemical group in the literature due to the wide range of polarities of the pesticides included in this method. Thus, we assayed the extraction efficacy of various mixtures of solvents of different polarities, and dichloromethane/ethyl acetate/acetone (50/30/20) provided the best combination of extract recovery and purity and was therefore chosen for the extraction. The use of sonication has also been described in the literature, as it may improve the extraction efficiency. In our case, we observed a slight improvement in the recoveries of certain key pesticides, such as carbofuran. Therefore, a 5-min sonication step was added to the extraction protocol.

The samples sent to the laboratory for poison identification are usually matrices with a relatively high content of fat and other interfering substances derived from degradation processes, a cleanup step was included to eliminate substances that could reduce the signal or cause column damage. Many strategies can be used for lipid removal, such as freezing centrifugation, partitioning lipid extraction, adsorption chromatography, GPC and sulfuric acid treatment. We chose GPC for those extracts from complex matrices (e.g., animal tissues, insects and laced baits) because its efficacy had been proven previously in our laboratory, yielding residual lipid concentrations of $<3\%$ of the initial amount (15, 16). Nevertheless, GPC is a solvent- and time-consuming method; thus, in extracts from such samples as blood or fresh animal tissue, we preferred to assay the efficacy of freezing centrifugation, where pesticides remain dissolved in

the solvent while frozen lipids can be removed by centrifugation due to their lower melting points relative to the solvent. Our experiments showed that freezing centrifugation alone was an adequate cleanup method for these samples, because it gave suitable extracts for chromatographic analysis with MS detection.

Analytical performance

To evaluate the usefulness of this methodology for the quantitative determination of pesticides in fresh liver samples, the confirmation criteria, precision, linearity and method limits of detection (LODs) and quantification (LOQs) were studied.

The obtained chromatographic peaks were identified as target analytes only if satisfied all of the following criteria: (i) the RT of the unknown peak coincided with that obtained from six replicates of the second level of calibration (RT $\pm$ 3 SD); (ii) there was a match with the ion ratios of the standard. We applied the tolerances of absolute ion abundances that are specified in the 2002/657/EC Directive [$\pm$20% tolerance for >50% relative intensity (% of base peak); $\pm$25% for > 20–50%; $\pm$30% for >10–20% and $\pm$50% for <10%] and (iii) we obtained a S/N ratio higher than 10 for a sample extract.

The extraction efficiency of the proposed methodology was evaluated by spiking five chicken liver samples with a mixture of the 117 pesticides at two concentrations (10.0 and 200.0 μg/kg). We calculated the recovery values and their relative standard deviations (RSDs) for each level by comparing the areas of the analytes in the extracted spiked samples with those of the same concentrations in the dissolvent (Tables I and II). The results ranged from 61.0 to 99.7%, with most of the recoveries being >90% at both concentrations. The precision was satisfactory, with the most unfavorable RSD being <18%. We also evaluated the recoveries and precision during 5 consecutive days (inter-day measurement), which yielded an RSD that was also <18% (Tables I and II).

To evaluate the possible existence of matrix effects, the same comparison was performed with chicken liver samples that were extracted without having being spiked, and where the spiking was performed after the extraction to evaluate if the extracted components of the matrix had an enhancement/suppression effect on the signal of the target pesticides. We did not found significant differences. Thus, we could conclude that there were no significant matrix effects, and the rest of the studies and calculations were performed against calibration curves of standards (0.5 and 500 μg/kg). We used the peak areas for performing the calculations. The origin point was not included when constructing the calibration curves. A good linearity was found and we concluded that it is possible to use the linear regression method to calculate the concentrations of these analytes since the correlation coefficients (r^2) were >0.9804 for all the analyses and the residual analysis showed values within the range of −11.32 to 9.42%.

Application to real samples

The validated methodology has been applied to the routine analysis of 140 real samples from 98 wildlife poisoning incidents that were submitted between 2010 and 2012 to the Clinical and Analytical Toxicology Service of the University of Las Palmas de Gran Canaria (SERTOX, Canary Islands, Spain).

Table III presents the results of the analysis of well-preserved fresh liver samples from suspected wild animal poisonings. We positively identified a pesticide in 78 of 94 liver samples, and their quantified levels were compatible with death by poisoning. The most frequently detected pesticides belonged to the group of anticoagulant rodenticides (brodifacoum, bromadiolone and

Table III

Summary of the positive detection of pesticides in fresh liver samples from wildlife poisoning episodes (2010–2012) with the validated methodology, and application to the identification and semi-quantification of pesticides in degraded liver tissue, laced baits and other samples from the poisoning scenarios

Liver	No. of animals	Principal toxicant(s)				
		Anticoagulants	Carbamates	OPs[a]	Pyrethroids	Others
Western Canaries Lizard (*Gallotia galloti*)	12		12			
Barn Owl (*Tyto alba*)	12	12				
Common Kestrel (*Falco tinnunculus*)	11	8		2	1	
Long-eared Owl (*Asio otus*)	8	8				
Common Buzzard (*Buteo buteo*)[a]	7		6	1		1
European hedgehog (*Erinaceus europaeus*)	6		6			
Common Raven (*Corvus corax*)	5		4		1	
European Turtle Dove (*Streptopelia turtur*)	5	5				
Barbary Falcon (*Falco pelegrinoides*)	4	2	2			
European Sparrowhawk (*Accipiter nisus*)	4	2	2			
Long-eared Bat (*Plecotus teneriffae*)	3					3
Cory's Shearwater (*Calonectris diomedea*)	1			1		
Other samples	No. of samples					
Degraded liver tissue (different species)	9	3	3	1	1	1
Meat	4		3	1		1
Containers and plastic[b]	4		3	1	1	
Gastrointestinal content	4		3	1		
Chicken carcasses	3		3			
Feed	3		2	1		
Insects	3		2	1		
Soil	3		1	1	1	
Bones and flesh	2		2			

[a]In one animal, two pesticides were found (carbofuran and aldicarb).

[b]In one plastic container, two pesticides were found (carbofuran and methomyl).

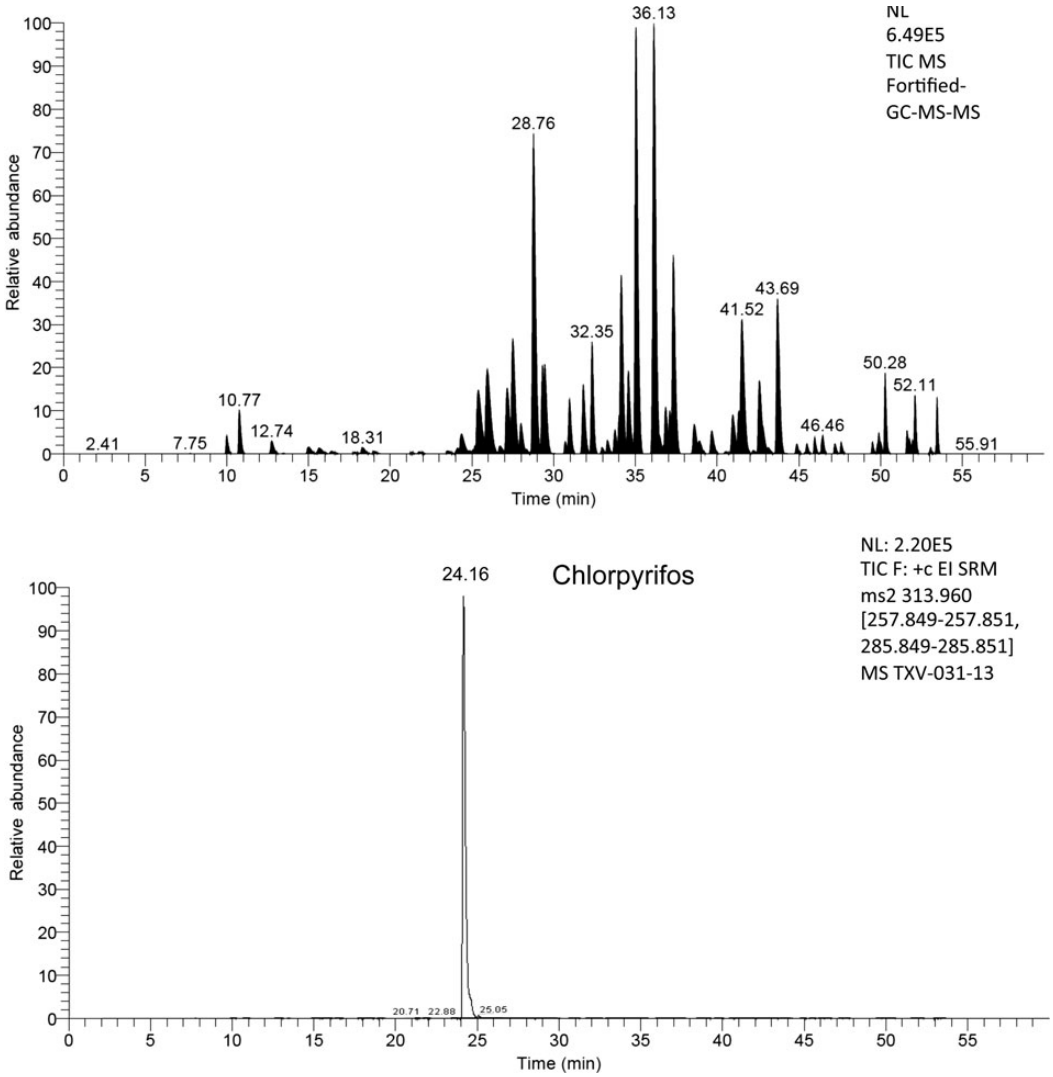

Figure 2. Representative GC–MS-MS chromatogram (A) sample spiked with the 91 apolar pesticides analyzed by gas chromatography; (B) SRM chromatogram of chlorpyrifos from a real sample. This SRM chromatogram results from the product ions spectra (m/z 169.0 and 171.0) from the precursor ion at m/z 197.0 for chlorpyrifos.

difenacoum), followed by carbamate insecticides (mainly carbofuran but also aldicarb and methomyl) and organophosphorus pesticides (chlorpyrifos, chlorpyrifos-methyl and diazinon). In some of the livers, multiple pesticides were detected and quantified. A representative chromatogram is shown in Figure 2.

The proposed methodology was also applied to degraded liver, other animal tissues, materials suspected of being bait and other samples that were submitted to our laboratory. We applied minor modifications to the extraction and cleanup procedures depending on the moisture content, adding enough water in the homogenization step to obtain a homogenate of similar physical consistency than that obtained from liver, and also depending on the state of conservation of the samples and the dirtiness of the extracts (necessity of GPC or not). As summarized in Table III, its application allowed the detection of a pesticide in 35 of 46 samples of very different natures, from biological samples to plastic materials.

We must note that, although the results of the validation experiments were satisfactory for chicken liver, given the wide range and conservation status of the samples from cases of wildlife poisoning, the usual rigorous method validation standards cannot be applied to all the real samples. Thus, many measurements of pesticides using this method must be regarded as only semi-quantitative in samples other than fresh liver (2).

Conclusions

This study reports the application of a solid–liquid extraction procedure in combination with triple quadrupole GC– and LC–MS-MS for the simultaneous detection and quantification or semi-quantification of 117 pesticides in a variety of samples from wildlife poisoning. The results show satisfactory validation parameters in liver tissue. All the pesticides could be detected at very low concentrations, with a good linearity of the calibration curves within the investigated calibration range (0.5–500 μg/kg, with $r^2 > 0.98$). The recovery rates were between 62 and 99%, with very good precision (RSD <18%). The proposed method can be recommended for routine application in environmental

forensic studies because it is simple, sensitive and very useful. Our results show that our methodology represents a valuable tool in the task of identifying unknown toxicants, as at least one pesticide was identified in 113 samples at levels compatible with death by poisoning.

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