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ABSTRACT

A generic method for the determination of linuron, chlorfenvinphos and ethion in liver samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS) is described. In vitro sample treatment was performed by using solid phase extraction (SPE) after protein precipitation. The lowest elution solvent volumes providing the highest recoveries were obtained with Sola^{TM} polymeric reversed-phase cartridges. Gradient elution using 10 mM ammonium formate in methanol (A) and 10 mM ammonium formate in water (B) was used for chromatographic separation of analytes on a Hypersil^{TM} end-capped Gold PFP column (100 mm×2.1 mm, 3 µm). All analytes were quantified without interference, in positive mode using multiple reaction monitoring (MRM) with chlorfenvinphos-d10 as internal standard. The whole procedure was successfully validated according to the Food and Drugs Administration (FDA) guidelines for bioanalytical methods. The calibration curves for all compounds were linear over the concentration range of 0.005 – 2 µM, with coefficients of determination higher than 0.998. A Lower limit of quantification of 0.005 µM was achieved for all analytes. Compounds extraction recovery rates ranged from 92.9 to 99.5% with a maximum relative standard deviation (RSD) of 2.3%. Intra-- and inter--day accuracies were within 90.9% and 100%, and imprecision varied from 0.8 to 8.2%. Stability tests proved all analytes were stable in liver extracts during instrumental analysis (+12°C in autosampler tray for 72 h) at the end of

three successive freeze-thaw cycles and at −20°C for up to 9 months. This accurate and robust analytical method is therefore suitable for metabolism studies of pesticide mixtures.

Keywords:

Pesticide mixtures, Liver samples, LC-MS/MS, Method validation

1. Introduction

For many years, pesticides have been used on a broad scale for pest control in agriculture. Despite their outstanding positive influence on farm productivity, these active ingredients are harmful for the environment. Owing to their physicochemical properties and their wide use, many of the pesticide residues end-up in water resources and in agricultural products. Consequently, the entire food chain is exposed to such toxic molecules, which may ultimately reach human beings through bioaccumulation or directly by the consumption of contaminated water or foodstuffs (Cao et al., 2011; Damalas et al., 2011; Ding, 2014). Most of studies aiming to estimate dietary exposure of the general population highlighted that the consumers were simultaneously exposed to different residues (Iñigo-Nuñez et al., 2010, Claeys et al., 2011, Chen et al., 2011, Nougadère et al., 2012, Bakırcı et al., 2014, Betsy et al., 2014, Lozowicka, 2015, Szpyrka et al., 2015 and Lemos et al., 2016). In France, Crepet et al. (2013) established that the general population was mainly exposed to 7 different pesticide mixtures consisting of 2 to 6 compounds. Among them, a mixture including chlorfenvinphos, linuron and ethion was significantly correlated to basic food items such as carrots and potatoes. After the consumption of these potentially contaminated vegetables, and once these xenobiotics have passed into the body, the blood flow delivers them to the liver for degradation and subsequent elimination.

Thus, to evaluate the importance of the liver contamination, a sensitive and reliable analytical method is required. Unfortunately, to the best of our knowledge, no analytical procedure determining simultaneously chlorfenvinphos, linuron and ethion in a liver matrix has been published so far.

To date, literature survey reveals that very few articles have been reported on the analysis of linuron, chlorfenvinphos or ethion in biological samples. Nguyen et al. (2007) proposed a methodology based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for the quantification of

linuron in urine sample. Cazorla-Reyes et al. (2011) also developed a method using LC-MS/MS for the determination of this polar herbicide in the same matrix. By contrast, the same authors quantified the non-polar compounds chlorfenvinphos and ethion thanks to gas chromatography coupled to tandem mass spectrometry (GC-MS/MS). Pitarch et al. (2003) and Raposo et al. (2010) also used GC-MS/MS for the determination of ethion in blood samples. Even if gas chromatography is adequate (Deme et al., 2012; Sinha et al., 2012) for the separation of organophosphorus, it is a less suitable option for phenyl urea herbicides, since these are thermolabiles (Liska and Slobodnik, 1996). As a result, an analytical protocol which uses a LC separation followed by MS/MS detection would be suitable to estimate the pesticides liver contamination.

Thus, the aim of this work was to develop, optimize and fully validate a simple, sensitive and reproducible analytical method for quantitative determination of linuron, chlorfenvinphos and ethion in human liver samples (hepatocytes, microsomes…).

2. Experimental

2.1. Chemicals, materials and biological samples

Trichloroacetic acid, ammonium sulfate salts of research grade purity and, anhydrous dimethylsulfoxide were supplied by Sigma Aldrich (Saint-Quentin Fallavier, France). LC-MS grade methanol and acetonitrile were obtained from Carlo Erba (Val de Reuil, France). Chlorfenvinphos, chlorfenvinphos-d10 (internal standard; IS), ethion and linuron certified standards of purity higher than 99.5% were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Standard stock solutions were prepared by dissolving the pure compounds in acetonitrile and further diluted as required in acetonitrile, for calibration standards and sample treatment. Sample extracts were centrifuged using a Thermo IEC Micromax™ RF benchtop centrifuge acquired from Thermo Fisher Scientific (Illkirch, France). Oasis™ HLB (10 mg/1 mL), Strata X® (10 mg/1 mL) and Sola™ (10 mg/1 mL) solid phase extraction (SPE) cartridges were provided by Waters (Guyancourt, France), Phenomenex (Le Pecq, France) and Thermo Fisher Scientific (Courtaboeuf, France), respectively. A 12 ports SPE manifold (J.T. Baker®) connected to a KNF Neuberger LABOPORT® filtration pump (VWR, Paris, France) was used for conditioning, sample loading, drying of the cartridges and elution of the targeted compounds.

All experiments on human tissue were carried out according to the ethical standards of the responsible committee on human experimentation and the Helsinki Declaration. Liver tissue can be mechanically decomposed into cellular (hepatocytes) or subcellular (S9, cytosolic and microsomal) fractions. Here we chose to carry out the study with thermally inactivated hepatocytes (100 °C for 3 min) previously isolated as described by Berry and Friend (1969).

2.5. Sample treatment

400 µL of thermally inactivated liver cells at a total protein concentration of 0.5 mg/mL in 100 mM phosphate potassium buffer (pH 7.4) were pipetted into 1.8 mL Eppendorf® tubes. The samples were spiked with the required amounts of chlorfenvinphos, ethion, linuron and ITSD before being briefly vortex-mixed. Then, 400 µL of glacial acetonitrile was added to the tubes. Centrifugation performed at 16000g for 5 min allowed the denatured proteins to precipitate. The supernatant was purified according to the optimized following SPE protocol. The samples were diluted in 6 mL borosilicate glass tubes by addition of purified water in order to obtain a ratio of acetonitrile–water (25:75, *v/v*) in the mixture. The samples were then loaded onto Thermo Sola™ extraction cartridges, which had been pre-cleaned using 1mL of methanol, followed by 1 mL of acetonitrile and finally conditioned using 1 mL of acetonitrile–water (25:75, *v/v*). Compounds of interest were trapped on the cartridges while interferents were successively eluted with 1 mL of acetonitrile–water (25:75, *v/v*) and 1 mL of purified water. After 5 min SPE manifold vacuum drying (-10 PSI) of the cartridges, compounds of interest were eluted under vacuum with 2×0.2 mL of pure acetonitrile. Finally, the eluates were diluted 1:1 in purified water prior to analysis.

2.6. LC-MS/MS analysis

Compounds were separated and quantified using a Surveyor HPLC analytical system purchased from Thermo Fisher Scientific (Courtaboeuf, France). It consisted in a quaternary low pressure mixing pump equipped with an integrated degasser, a 20 µL injection loop, a temperature controlled autosampler set at 10 °C and a column oven kept at 25 °C. A Hypersil end-capped Gold PFP reversed phase column (100 mm×2.1 mm, 3 µm) purchased from Thermo Fisher Scientific (Gif-sur-Yvette,

France) equipped the Surveyor module. LC separation was achieved at a flow rate of 280 µL.min^{-1} using a mobile phase composed of 10 mM ammonium formate in methanol (solvent A) and 10 mM ammonium formate in water (solvent B). The gradient program was run as follows: maintain 35% A from 0 to 7 min, linear increase to 100% A from 7 to 9 min, hold 100% A from 9 to 12 min, return to the initial conditions from 12 to 17 min and stabilization during 3 min before the next injection.

The detection and quantification were performed using a TSQ Quantum triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) source. To prevent a contamination of the ESI source, from 0 to 6 min, the column effluent was systematically diverted to the waste by means of a motorized Divert/Inject valve.

The mass spectrometer was operated in multiple reaction monitoring (MRM) positive acquisition mode. The ion capillary temperature was heated to 350 °C and the ESI needle voltage was set at 4000 V. The sheath and auxiliary gas (N_2) pressures were respectively tuned at 40 and 30 (arbitrary units). A 4 V source collision induced dissociation (CID) offset and a 1.5 mTorr collision gas (Ar) pressure were applied at the collision cell. For each compound, sensitive quantitative determination was performed using an addition of the MRM transitions displayed in Table 1.

All MS parameters were optimized by direct infusion and the source parameters were subsequently adjusted by flow injection. Data analysis was accomplished using Xcalibur™ 2.1 software.

2.7. Analytical method validation

Validation of the analytical method was carried out in accordance with the general guidelines for bioanalytical methods established by the FDA (US Food and drug Administration, 2013). Validation criteria including lower limit of quantification (LLOQ) sensitivity, linearity, selectivity, accuracy, precision, recovery and stability were investigated.

2.7.1. Limit of quantification

The LLOQ is defined as the lowest concentration that can be measured with acceptable precision and accuracy. For its assessment, four serial dilutions of sample containing 0.400 µM mL of each analyte were made by mixing equal volumes of spiked microsomal sample with blank microsomal sample (six

replicates). The peak areas of these fortified extracts should be at least five times higher than the background of blank samples (*i.e.* signal-to-noise ratio, S/N=5/1) to be considered as a proven LLOQ. The precision and mean accuracy of back-calculated LLOQ replicate concentrations must be of <20% and ±20%, respectively.

2.7.2. Selectivity and matrix effects

The evaluation of the selectivity was conducted after the pretreatment and instrumental analysis of ten different blank human microsomes samples. Selectivity was assessed to ensure the absence of any potential endogenous interference co-eluting with analytes, including the chlorfenvinphos-d10 (IS). Chromatographic signals of pesticides were discriminated on the basis of their specific retention times and MRM responses.

In addition, to assess matrix effects, ten different blank matrices were extracted, further spiked with the standard solution at the LLOQ level and compared with aqueous standards of the same concentration level. According to the guidance the difference of response should not exceed ±5%.

2.7.3. Linearity

For each compound, the calibration curve range varied from the validated LLOQ (0.005 µM) to 2.0 µM. Calibration curve standard samples were prepared in replicates (n=6) in a mixture of purified water and acetonitrile (50:50, *v/v*), and then analyzed. Data was reprocessed and validity of the linearity was checked through ANOVA statistical analyses (Microsoft Excel). The goodness of fit (GoF) and lack of fit (LoF) were determined and correlated with the corresponding Fisher theoretical table value. The fitting of the calibration curves was obtained with a 1/x weighted least squares linear regression.

2.7.4. Recovery

Recovery rates of pesticides from thermally inactivated human hepatocytes samples were assessed at three concentration levels: low (0.05 µM), medium (5.0 µM) and high (50.0 µM). Three replicates were prepared for each level and extracted. After their analysis, the peak areas from these samples were compared to those from post-extracted blank inactivated hepatocytes samples fortified with the

targeted compounds at the same concentration and analyzed. The ratio of mean peak areas of pre-extracted samples to mean post-extracted spiked samples enabled to calculate individual percentage recovery.

2.7.5. Precision and accuracy

Thanks to freshly prepared calibration curves, imprecision (intra- and inter-day) and accuracy were back calculated for the mixture respectively at four concentration levels: low (0.05 µM), medium 1 (2.0 µM), medium 2 (5.0 µM) and high (50.0 µM).

For intra-day imprecision and accuracy, five replicate samples per concentration were prepared and consecutively analyzed on the same day. For inter-day imprecision, the samples' preparation and analysis were carried out in duplicate at the same spiking levels, and repeated on six different days. Imprecision was expressed as the relative standard deviation (RSD%) and accuracy was calculated as the mean percentage deviation (A%) from the spiked value. The acceptance criteria for intra- and inter-day imprecision were ≤15% and, for accuracy, were between 85 and 115% of the nominal concentrations.

2.7.6. Stability of pesticides in the matrix extract

Stability tests were conducted in triplicate with processed samples that had been previously spiked at a concentration level of 5 µM. Different storage conditions were tested: 72 h in the autosampler tray at +12 °C, after 15 h of three cycles of freezing (–20 °C) and thawing (room temperature) for either 1 month, 3 months or 9 months at –20 °C. The results were calculated using freshly prepared calibration curves. The imprecision and accuracy calculated for samples' stability should be below 15% and between 85 and 115% of their nominal levels, respectively.

3. Results and discussion

3.1. Method development

3.1.2. Mass spectrometry optimization

Direct infusion of individual compound and IS solutions at a concentration of 1 mg.L^{-1} in water–methanol (50:50, *v/v*), 10 mM ammonium acetate in water–methanol (50:50, *v/v*) or 10 mM ammonium formate in water–methanol (50:50, *v/v*) was carried out to select the best solvent mixture phase, precursor and product ions. The individual mass spectra of each molecule obtained in positive ion mode showed the presence of both an abundant pseudo-molecular ion $[M+H]^+$ and a reproducible stable sodium adduct $[M+Na]^+$ signal. For all the compounds, the best $[M+H]^+/[M+Na]^+$ signal ratio was obtained with the standard solution containing 10 mM ammonium formate. Once the precursor ion was chosen, the optimum tube lens voltage was automatically optimized. Then, analytes were fragmented by applying the collision energy giving the highest abundance for each product ion. The optimized source parameters, MRM transitions and settings were then included in the mass spectrometry acquisition method.

3.1.3. Chromatographic conditions

After optimization of the mass spectrometry parameters, different liquid chromatography columns were tested. The first evaluations were achieved on two core-shell LC columns from Phenomenex (Le Pecq, France): a Kinetex® C_{18} and a Kinetex® PFP (100 mm × 2.1 mm, 3 µm). Both columns enabled a satisfactory separation of linuron, chlorfenvinphos, ethion and IS. Less peak tailing was nevertheless obtained with the Kinetex PFP, therefore contributing to a significant improvement on sensitivity, especially for the chlorinated molecules such as chlorfenvinphos and linuron. This column was initially selected, but the drawback of using this specific stationary phase appeared later during the study. In fact, a high back pressure was observed only after a short time use. As a consequence, a fully porous Thermo Hypersil™ Gold PFP column was used instead. A new elution gradient was therefore optimized for this column and enabled to achieve optimum resolution and compound detection, as already depicted in "Experimental - LC-MS/MS analysis". Finally, the analytes' signals were appropriately separated and chromatograms displayed good peak shapes as presented on Fig. 1. The carry-over in the chromatographic system was measured by injecting three blank solvents after the highest calibration standard.

3.1.4. Sample purification

A suitable optimization of the extraction step was then needed to achieve a satisfactory LLOQ and selectivity for the detection of the pesticides mixture in such small volumes of human liver extracts. For this purpose, after the protein precipitation step described above, an additional solid phase extraction step was preferred against a liquid–liquid extraction to remove potential additional endogenous interferents such as phospholipids or inorganic salts contained in the cell seeding medium (Yaroshenko and Kartsova, 2014) and polyethylene glycol leached from plastic containers (Weaver and Riley, 2006).

SPE sorbent types such as polymeric and silica based reversed-phase sorbents seemed appropriate for the simultaneous extraction of organophosphorus and neutral phenylurea pesticides from biological matrices (Cazorla-Reyes et al., 2011). The few studies dedicated to their purification from human body fluids indicated that both polymeric reversed-phase cartridges (Nguyen et al., 2007; Raposo et al., 2010) and silica based reversed-phase sorbent cartridges (Pitarch et al., 2003) could be used. In this study, the aim was to minimize elution volumes in order to simplify sample treatment by avoiding the concentration step after the elution of the analytes. As a result, the polymeric reversed-phase (PRP) sorbents were chosen for their higher loading capacities which allowed the use of lower sorbent amounts, associated with reduced elution volumes. In this work, three different commercial PRP cartridges were compared (Oasis™ HLB, Strata X® and Sola™) with the goal to choose the one which could give the maximum recoveries with the minimum elution volume. Aiming at obtaining comparable results, a common SPE methodology was applied for all the cartridges. First, after being successively rinsed using 1 mL of methanol and 1 mL acetonitrile, the cartridges were conditioned with 1 mL of purified water. Then, a blank liver cells sample containing all the test compounds at 20 µM was loaded on the cartridge. Then, a washing step consisting of 1 mL of purified water, followed by a 5 min drying step and an elution of the target compounds with 2×0.2 mL of pure acetonitrile under vacuum (-5 PSI), were applied. Acetonitrile was chosen for its LC-MS compatibility and its higher elution strength in comparison with methanol. Indeed, the latter did not allow for satisfactory elution of ethion even if a larger volume was used (up to 2 mL). After dilution (50:50, *v/v*)

in purified water, the extract was transferred to an injection vial for analysis. The performance of the SPE cartridges tested are displayed in Table 2. The best results, in terms of relative recoveries and RSD values, were achieved using Sola™ cartridges with values ranging from 97 to 100%.

As incubation of both hepatocytes and microsomes samples was stopped by adding cold-acetonitrile (50/50, v/v), further optimization was performed to determine the maximum acceptable amount of acetonitrile in the liver extracts to be purified by SPE. To this goal, as described above, blank samples were prepared and loaded onto Sola™ cartridges. Then, an elution/retention profile of the analytes was established with the successive addition of mixtures of water and acetonitrile, as shown in Table 3. The experiments were performed in duplicate. All the analytes were retained with acetonitrile proportion below 30%. Linuron, as the most polar compound, was the first one to be desorbed. As a consequence, we considered that the liver extracts from kinetic experiments should be diluted with the appropriate amount of water (25:75, *v/v*) prior to the solid phase extraction.

3.2. Performance of the analytical method

The chromatograms of liver cells samples were visually checked and compared with chromatogram obtained from standard references in neat solvents. As they showed no disturbing peaks, the selectivity was approved. Besides, the matrix effect assessed at the LLOQ level did not exceed 0.6% and was considered negligible for all the pesticides. Additionally, no cross-contamination was observed when three blank solvent samples were injected consecutively to the highest calibration standard.

The results of the linearity, the intra- and inter-day precision and accuracy, as well as the stability of pesticides in the matrix extracts are summarized in Table 4.

The results from the Goodness of Fit and Lack of Fit of the Fisher significance tests (GoF-LoF) indicated that the linear regression model was validated in the defined range of concentrations for all compounds. In addition, the determination coefficient was systematically verified and always gave satisfactory values (r^2>0.998).

Recovery data collected on three replicates of three wide range covering levels ranged from 92.9% to 99.5%, with a maximum RSD of 2.3%, demonstrating the efficiency of the SPE purification process. Moreover, intra- and inter-day imprecision and accuracy were all within the established ranges of

acceptance. Finally, stability data revealed that whichever test used, no significant loss was noticed, indicating that all the analytes were stable within the studied working conditions.

All the evaluated performance parameters were in accordance with FDA recommendations, making this method reliable and rugged for future in vitro liver metabolism studies.

4. Conclusions

To our knowledge, this is the first reported analytical method for determination of linuron, chlorfenvinphos and ethion in liver samples. This LC-MS/MS method developed with one stable isotope-labeled internal standard exhibited very satisfactory performance in terms of selectivity, limit of quantification, linearity, recovery, precision and accuracy, in compliance with current FDA requirements. A user-friendly sample treatment process providing excellent recoveries and high sample cleanliness was obtained after appropriate optimization of conditions. Indeed, protein precipitation, solid phase extraction sorbent type, volume and solvent elution strength were optimized. This method is optimal for conducting metabolism studies through the accurate monitoring of the parent compound loss in in-vitro human liver samples. Furthermore, it would probably be also convenient for the determination of the above-mentioned pesticides mixture in human liver biopsies or mammalian liver samples.

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Table 1

Ions monitored under the MRM mode by LC-MS/MS[a] and their relative intensities (%).

Compound	precursor ion[b] (m/z)	product ion 1 (m/z)	product ion 2 (m/z)	collision energy (V)
Linuron	249	182(100)	161(23)	20-25
chlorfenvinphos	359	155(100)	127(45)	18-22
chlorfenvinphos-d10 (IS)	369	165(100)	133(66)	16-25
Ethion	385	143(100)	97(88)	35-45

[a] The compounds were quantified with both product 1 and product 2 ions.
[b] Ionized in the positive mode with a 4 V CID offset.

Table 2

Percentage recoveries and associated RSD (in brackets) of the target analytes testing different SPE cartridges (n=3).

	Oasis™ HLB	Strata X®	Thermo Sola™
Linuron	92 (4)	87 (11)	100 (2)
chlorfenvinphos	90 (8)	83 (12)	97 (2)
Ethion	85 (9)	43 (3)	98 (5)

Table 3

Elution of the pesticides mixture from Sola™ sorbent cartridge (n=2).

elution mixture acetonitrile/water (*v:v*, 1 mL)	Elution rate from (%)		
	linuron	chlorfenvinphos	Ethion
0/1	0	0	0
0.05/0.95	0	0	0
0.10/0.90	0	0	0
0.15/0.85	0	0	0
0.20/0.80	0	0	0
0.25/0.75	0	0	0
0.30/0.70	1	0	0
0.35/0.65	14	3	0
0.40/0.60	56	29	0
1/0	100	100	100

Table 4

Results of the analytical method validation: linearity (n=6), recovery (n=3), intra-day accuracy (n=5), inter-day accuracy (n=2, 6 days), stability (n=3).

Parameter	Linuron		chlorfenvinphos		Ethion		Limits
Linearity	GoF-LoF						
LoF	0.164		1.019		0.055		<4.51
GoF	4840		21868		3084		>>5.39
Recovery	R%	RSD%					
Low	99.2	1.0	97.0	2.0	92.9	1.9	
Medium	98.7	2.2	95.1	1.8	94.2	1.6	n.a
High	99.2	1.3	97.6	1.7	95.0	1.2	
Accuracy							
Intra-day	Ar%	RSD%					
Low	100.0	1.3	96.6	4.9	93.8	2.6	±20, ≤20 %
Medium 1	98.4	3.5	99.6	4.2	93.6	0.8	±15, ≤15 %
Medium 2	97.6	6.2	94.6	7.1	93.9	3.7	
High	100.0	0.8	99.0	1.6	93.3	4.2	
Inter-day	Br%	RSD%					
Low	99.4	4.1	98.2	6.9	92.8	4.8	±20%, ≤20 %
Medium 1	99.8	6.7	100	6.6	91.6	2.9	±15%, ≤15 %
Medium 2	98.9	8.1	97.2	8.2	90.9	5.9	
High	99.5	2.6	100.0	3.1	93.1	6.3	
Stability							
Freeze-thaw	SFt%						
-20/20 °C-15 h	101.1	5.3	99.9	5.7	100.0	4.2	±15 %, ≤15 %
Long term	SLt%						
1 month	100.7	6.1	99.0	5.5	97.8	3.0	±15 %, ≤15 %
3 months	102.1	5.7	101.1	5.8	104.2	4.1	
9 months	99.8	6.0	99.5	5.1	102.2	3.7	
Autosampler	SA%						
72 h	99.9	5.8	101.2	4.9	100.2	4.6	±15 %, ≤15 %

GoF-LoF : Goodness of Fit - Lack of Fit

Figure

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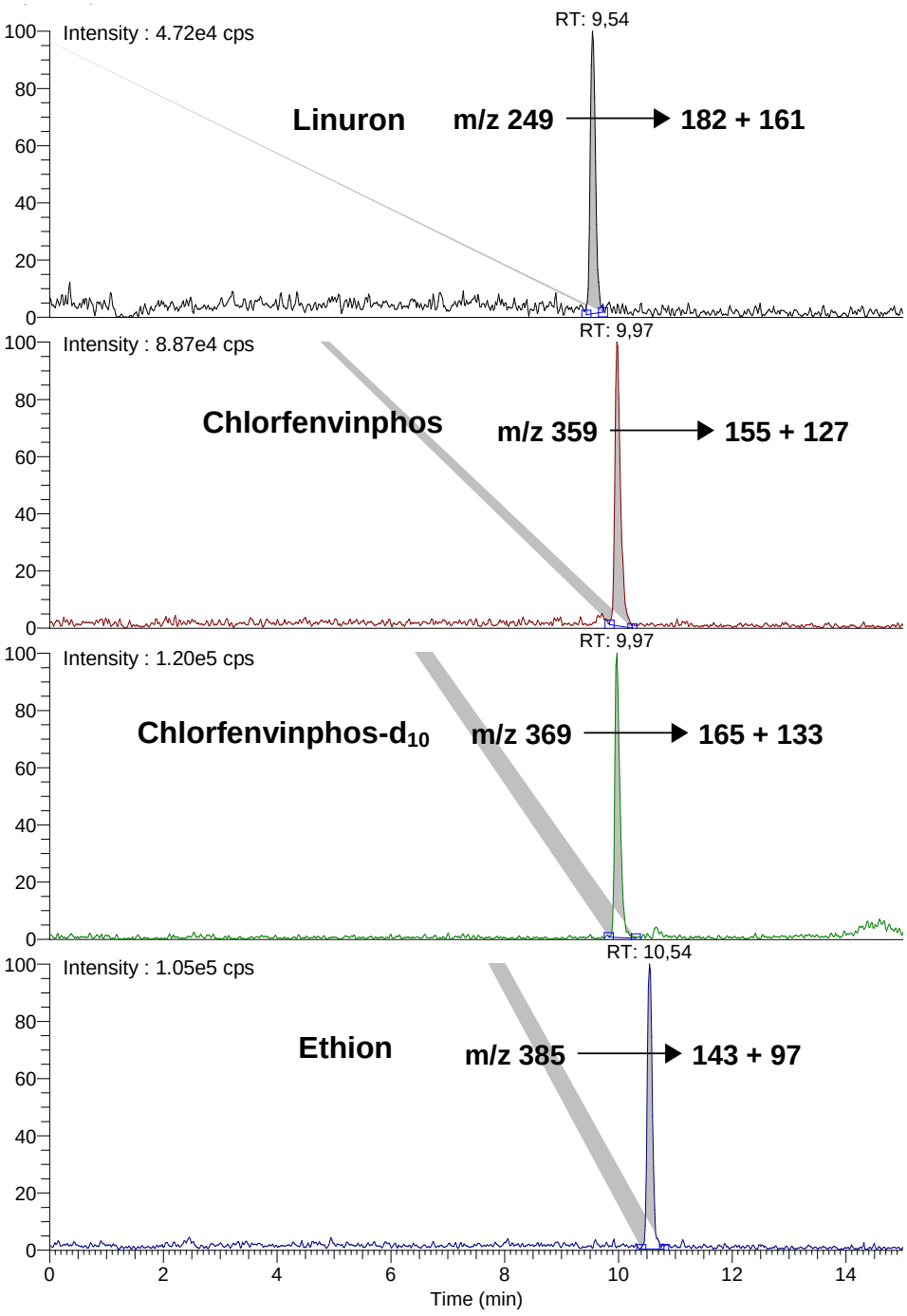

Fig. 1. LC-MS/MS chromatograms obtained from fortified inactivated hepatocytes extract (LLOQ)

ABSTRACT

A generic method for the determination of linuron, chlorfenvinphos and ethion in liver samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS) is described. In vitro sample treatment was performed by using solid phase extraction (SPE) after protein precipitation. The lowest elution solvent volumes providing the highest recoveries were obtained with Sola™ polymeric reversed-phase cartridges. Gradient elution using 10 mM ammonium formate in methanol (A) and 10 mM ammonium formate in water (B) was used for chromatographic separation of analytes on a Hypersil™ end-capped Gold PFP column (100 mm×2.1 mm, 3 µm). All analytes were quantified without interference, in positive mode using multiple reaction monitoring (MRM) with chlorfenvinphos-d10 as internal standard. The whole procedure was successfully validated according to the Food and Drugs Administration (FDA) guidelines for bioanalytical methods. The calibration curves for all compounds were linear over the concentration range of 0.005 – 2 µM, with coefficients of determination higher than 0.998. A Lower limit of quantification of 0.005 µM was achieved for all analytes. Compounds extraction recovery rates ranged from 92.9 to 99.5% with a maximum relative standard deviation (RSD) of 2.3%. Intra-- and inter--day accuracies were within 90.9% and 100%, and imprecision varied from 0.8 to 8.2%. Stability tests proved all analytes were stable in liver extracts during instrumental analysis (+12°C in autosampler tray for 72 h) at the end of three successive freeze-thaw cycles and at −20°C for up to 9 months. This accurate and robust analytical method is therefore suitable for metabolism studies of pesticide mixtures.