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An accurate and robust LC-MS/MS method for the quantification of chlorfenvinphos, ethion and linuron in liver samples

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HIGHLIGHTS

- Development of an analytical method for the analysis of chlorfenvinphos, ethion and linuron in liver samples.
- Accuracy and robustness of the method were validated according to a FDA guideline.
- This method should be useful for measuring these pesticides in human or animals liver samples.

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ABSTRACT

A method for the determination of chlorfenvinphos, ethion and linuron in liver samples by LC-MS/MS is described. Sample treatment was performed by using Sola™ polymeric reverse phase SPE cartridges after protein precipitation. 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 reverse phase column (100 mm × 2.1 mm, 3 μm). All analytes were quantified without interference, in positive ionization mode using multiple reaction monitoring (MRM) with chlorfenvinphos-d10 as internal standard. The whole procedure was validated according to the FDA guidelines for bioanalytical methods. The calibration curves for chlorfenvinphos, linuron and ethion compounds were linear over the concentration range of 0.005–2 μM (i.e. 0.0018–0.720 μg/mL, 0.0019 –0.770 μg/mL and 0.0012–0.500 μg/mL respectively) with coefficients of determination higher than 0.998. A Lower limit of quantification of 0.005 μM was achieved for all analytes, i.e. 5.76, 6.08 and 3.84 μg/kg of liver for chlorfenvinphos, ethion and linuron respectively. Compounds extraction recovery rates ranged from 92.9 to 99.5% with a 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 contamination or metabolism studies.

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 (Gassmann et al., 2015; Farha et al., 2016), many of the pesticide residues end-up in water resources (Helbling, 2015; Ouyang et al., 2016) and in agricultural products. Consequently, the entire food chain is exposed to such toxic molecules (Dorne and Fink-Gremmels, 2013), which may ultimately reach human beings through bioaccumulation or directly by the consumption of contaminated water or foodstuffs (Cao et al., 2011; Damalas and Eleftherohorinos, 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; Bakirci et al., 2014; Betsy et al., 2014;

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Lozowicka, 2015; Szpyrka et al., 2015; 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 2–6 compounds. Among them, a mixture including chlorfenvinphos, ethion and linuron 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.

To the best of our knowledge, literature survey reveals that no article has been reported on the simultaneous analysis of chlorfenvinphos, ethion and linuron in human biological samples. However, Kaczyński et al. (2017) have recently published an analytical method for the analysis of multiclass pesticides including chlorfenvinphos, ethion and linuron in fish liver. Before that, several studies reported the analysis of chlorfenvinphos or ethion in bovine liver by either liquid chromatography coupled UV detector (García de Llasera and Reyes-Reyes, 2009; Gutiérrez Valencia and García de Llasera, 2011) or, more recently, by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Souza et al., 2016).

To the best of our knowledge the only study dealing with real human liver samples was presented by Russo et al. (2002). It depicted the development of a methodology for the determination of several organophosphorus pesticides including chlorfenvinphos and ethion using gas chromatography–negative chemical ionization mass spectrometry analysis.

Nguyen et al. (2007) proposed a methodology based on 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) used respectively GC-MS/MS and GC-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).

For sample purification, Solid Phase Extraction showed to be suitable. 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.

As chosen by Kaczyński et al. (2017) 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 chlorfenvinphos, ethion and linuron in human liver samples (hepatocytes).

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).

2.2. Sample treatment

A sample consisting in 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) was pipetted into 1.8 mL Eppendorf® tubes. The samples were spiked with the required amounts of chlorfenvinphos, ethion, linuron and IS before being briefly vortex-mixed. Then, 400 μL of glacial acetonitrile was added to the tubes. Centrifugation performed at 16000*g* 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 1 mL 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 (−5 PSI) with 2 × 0.2 mL of pure acetonitrile. Finally, the eluates were diluted (50:50, *v/v*) in purified water prior to analysis.

2.3. 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.

We deliberately delayed the retention time of the native pesticides to make possible a future use of the present analytical method for the separation and the analysis of their metabolites. 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 detection and quantification were performed using a TSQ Quantum triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) source.

The mass spectrometer was operated in multiple MRM positive ionization 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 ion source parameters were subsequently adjusted by flow injection. Data analysis was accomplished using Xcalibur™ 2.1 software.

2.4. 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.

2.4.1. 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 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) 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.

2.4.2. Selectivity and matrix effects

The evaluation of the selectivity was conducted after the pretreatment and instrumental analysis of ten different blank human cells 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. For this criterion the FDA guidance did not specified acceptable limits. But González et al. (2014) specified that the deviation between the calculated and the nominal values should be within ±15%. Here, this difference was set at ±5%.

2.4.3. Linearity

For chlorfenvinphos, ethion and linuron, the calibration curve range varied from the validated LLOQ (0.005 μM) to 2.0 μM, i.e. 0.0018–0.720 μg/mL, 0.0019–0.770 μg/mL and 0.0012–0.500 μg/mL respectively. The LLOQ achieved for chlorfenvinphos, ethion and linuron corresponds to 5.76, 6.08 and 3.84 μg/kg of liver respectively. Calibration curve standard samples were prepared in replicates (n = 6) in a mixture of hepatocytes extract in 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.

2.4.4. 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.

2.4.5. Precision and accuracy

The use of freshly prepared calibration curves allowed imprecision (intra- and inter-day) and accuracy to be 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 precision, 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 (Ar% and Br%) 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.

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.

2.4.6. 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. For this criterion also, the FDA guidance did not set acceptable limits. Nevertheless, González et al. (2014) considered that for stability, accuracy should be within ±15%. The imprecision and accuracy calculated for samples' stability should be below 15% and between 85 and 115% of their nominal levels, respectively.

To check the absence of any pollution or significant drift of the instrument sensitivity, a blank sample and a standard at 0.05 μM were analyzed after each set of 8 samples run. A maximum drift of 10% based on the sensitivity of the first calibration standard (0.05 μM) was considered acceptable for the aim of the validation study.

3. Results and discussion

3.1. Method development

3.1.1. Mass spectrometry optimization

Direct infusion of individual compound and IS solutions at a concentration of 1 mg/L 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 ionization 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. Indeed with the use of this additive, chlorfenvinphos, ethion and linuron $[M+H]^+/[M+Na]^+$ ratio increased by approximately a 5- 16- and 13-fold respectively. 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.

3.1.2. 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. It enabled to achieve very similar 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.

3.1.3. 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).

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 of acetonitrile, the cartridges were conditioned with 1 mL of purified water. Then, a blank liver cells sample spiked with all the test compounds at 20 μM was loaded on the 3 cartridges under study. Afterwards, a washing step consisting of 1 mL of purified water, followed by a 5 min drying step (−10 PSI) and an elution of the target compounds with 1 mL of pure acetonitrile under vacuum (−5 PSI) were applied. Acetonitrile was preferred to methanol for its higher elution strength. Indeed, methanol 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%.

For these cartridges, further optimization was performed to determine which proportion of acetonitrile in the sample extract allowed the compounds to be fully retained by the sorbent. To this goal, as described above, spiked blank liver extract samples were prepared in duplicate and loaded onto different Sola™ cartridges. Then, an elution/retention profile of the analytes was established after collecting and analyzing the 1 mL eluate composed of mixtures of water and acetonitrile, as shown in Table 3. All the analytes remained retained with acetonitrile proportion below 30%. Linuron, as the most polar compound, was the first one to be desorbed and ethion the most difficult to elute. As a consequence, following the protein precipitation step, all the samples should be diluted in water from (50:50, *v/v*) to (25:75, *v/v*).

Finally, in order to optimize the elution volume, a similar spiked liver extract was prepared in duplicate. Elution was realized with successive additions of 0.2 mL of pure acetonitrile. The results presented in Table 4 permitted to conclude that 2 × 0.2 mL of pure acetonitrile were necessary to elute the total amount of pesticides.

3.2. Performance of the analytical method

All the sequence analyses were validated as neither significant drift nor pollution was observed.

The chromatograms of liver cells samples were visually checked and compared with chromatogram obtained from standard

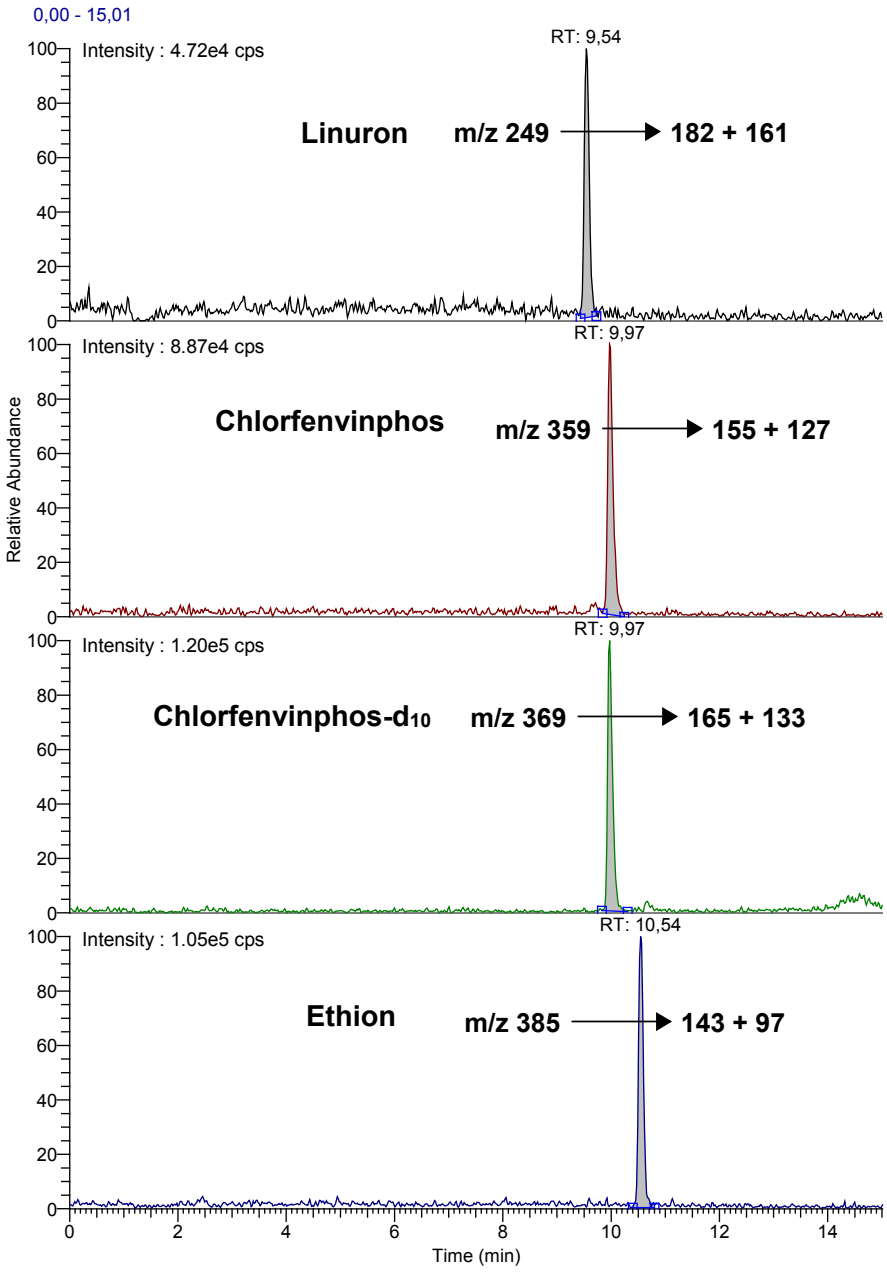

Fig. 1. LC-MS/MS chromatograms obtained from fortified inactivated hepatocytes extract (LLOQ).

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)

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 5.

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

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

Elution mixture acetonitrile/water (1/0)	Elution rate from (%)		
	linuron	chlorfenvinphos	ethion
First 200 μL	89	80	77
Second 200 μL	11	20	23
Third 200 μL	0	0	0
Fourth 200 μL	0	0	0
Fifth 200 μL	0	0	0

Table 5
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; R%: Percent recovery; RSD%: Percent relative standard deviation. Ar%: Intra-day percent accuracy rate; Br%: Inter-day percent accuracy rate. SFt%: Freeze-thaw percent stability; SLt%: Long term percent stability; SA%: Autosampler percent stability.

with FDA recommendations, making this method reliable and rugged for future studies. The evaluated LLOQ for chlorfenvinphos (5.76 μg/kg), ethion (6.08 μg/kg) and linuron (3.84 μg/kg) are comparable to the values shown in some previous studies. Indeed, for chlorfenvinphos, Kaczyński et al. (2017) obtained 3.3 μg/kg of animal liver. On the other hand, this LLOQ was far below the one published for this compound by García de Llasera and Reyes-Reyes, (2009) and Gutiérrez Valencia and García de Llasera, (2011), i.e. 200 μg/kg. In the case of ethion, Souza et al. (2016) obtained a LLOQ of 10 μg/kg of bovine liver, which is comparable to the one achieved here but higher than the one published by Kaczyński et al. (2017) on fish liver (0.80 μg/kg). Finally, the same authors presented a LLOQ of 0.33 μg/kg of fish liver for linuron 10 times lower than the LLOQ obtained in the present work in human liver samples.

The present method was applied successfully to authentic human liver samples (hepatocytes and microsomes) as part of an in vitro metabolism study (Kadar et al., 2017).

4. Conclusions

To our knowledge, this is the first reported analytical method for determination of chlorfenvinphos, ethion and linuron in human liver samples. This one-step extraction cleanup LC-MS/MS method developed with one stable isotope-labeled internal standard exhibited satisfactory performance in terms of selectivity, linearity, recovery, precision and accuracy, in compliance with current FDA requirements. The limit of quantifications obtained in human liver samples are globally in the core of the previous published LLOQ obtained from animal liver samples. A user-friendly sample treatment process providing excellent recoveries and high sample purification 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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