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

3
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10

11 ABSTRACT

12 A generic method for the determination of linuron, chlorfenvinphos and ethion in liver samples by
13 liquid chromatography-tandem mass spectrometry (LC-MS/MS) is described. In vitro sample
14 treatment was performed by using solid phase extraction (SPE) after protein precipitation. The lowest
15 elution solvent volumes providing the highest recoveries were obtained with SolaTM polymeric
16 reversed-phase cartridges. Gradient elution using 10 mM ammonium formate in methanol (A) and
17 10 mM ammonium formate in water (B) was used for chromatographic separation of analytes on a
18 HypersilTM end-capped Gold PFP column (100 mm×2.1 mm, 3 μm). All analytes were quantified
19 without interference, in positive mode using multiple reaction monitoring (MRM) with
20 chlorfenvinphos-d10 as internal standard. The whole procedure was successfully validated according
21 to the Food and Drugs Administration (FDA) guidelines for bioanalytical methods. The calibration
22 curves for all compounds were linear over the concentration range of 0.005 – 2 μM, with
23 coefficients of determination higher than 0.998. A Lower limit of quantification of 0.005 μM was
24 achieved for all analytes. Compounds extraction recovery rates ranged from 92.9 to 99.5% with a
25 maximum relative standard deviation (RSD) of 2.3%. Intra-- and inter--day accuracies were within
26 90.9% and 100%, and imprecision varied from 0.8 to 8.2%. Stability tests proved all analytes were
27 stable in liver extracts during instrumental analysis (+12°C in autosampler tray for 72 h) at the end of

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

30 *Keywords:*

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

32

33 **1. Introduction**

34 For many years, pesticides have been used on a broad scale for pest control in agriculture. Despite
35 their outstanding positive influence on farm productivity, these active ingredients are harmful for the
36 environment. Owing to their physicochemical properties and their wide use, many of the pesticide
37 residues end-up in water resources and in agricultural products. Consequently, the entire food chain is
38 exposed to such toxic molecules, which may ultimately reach human beings through bioaccumulation
39 or directly by the consumption of contaminated water or foodstuffs (Cao et al., 2011; Damalas et al.,
40 2011; Ding, 2014). Most of studies aiming to estimate dietary exposure of the general population
41 highlighted that the consumers were simultaneously exposed to different residues (Iñigo-Nuñez et al.,
42 2010, Claeys et al., 2011, Chen et al., 2011, Nougadère et al., 2012, Bakırcı et al., 2014, Betsy et al.,
43 2014, Lozowicka, 2015, Szyrka et al., 2015 and Lemos et al., 2016). In France, Crepet et al. (2013)
44 established that the general population was mainly exposed to 7 different pesticide mixtures consisting
45 of 2 to 6 compounds. Among them, a mixture including chlorfenvinphos, linuron and ethion was
46 significantly correlated to basic food items such as carrots and potatoes. After the consumption of
47 these potentially contaminated vegetables, and once these xenobiotics have passed into the body, the
48 blood flow delivers them to the liver for degradation and subsequent elimination.

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

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

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

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

67 **2. Experimental**

68 *2.1. Chemicals, materials and biological samples*

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

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

87 *2.5. Sample treatment*

88 400 µL of thermally inactivated liver cells at a total protein concentration of 0.5 mg/mL in 100 mM
89 phosphate potassium buffer (pH 7.4) were pipetted into 1.8 mL Eppendorf® tubes. The samples were
90 spiked with the required amounts of chlorfenvinphos, ethion, linuron and ITSD before being briefly
91 vortex-mixed. Then, 400 µL of glacial acetonitrile was added to the tubes. Centrifugation performed at
92 16000g for 5 min allowed the denatured proteins to precipitate. The supernatant was purified
93 according to the optimized following SPE protocol. The samples were diluted in 6 mL borosilicate
94 glass tubes by addition of purified water in order to obtain a ratio of acetonitrile–water (25:75, v/v) in
95 the mixture. The samples were then loaded onto Thermo Sola™ extraction cartridges, which had been
96 pre-cleaned using 1 mL of methanol, followed by 1 mL of acetonitrile and finally conditioned using
97 1 mL of acetonitrile–water (25:75, v/v). Compounds of interest were trapped on the cartridges while
98 interferences were successively eluted with 1 mL of acetonitrile–water (25:75, v/v) and 1 mL of purified
99 water. After 5 min SPE manifold vacuum drying (-10 PSI) of the cartridges, compounds of interest
100 were eluted under vacuum with 2×0.2 mL of pure acetonitrile. Finally, the eluates were diluted 1:1 in
101 purified water prior to analysis.

102 *2.6. LC-MS/MS analysis*

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

108 France) equipped the Surveyor module. LC separation was achieved at a flow rate of 280 $\mu\text{L}\cdot\text{min}^{-1}$
109 using a mobile phase composed of 10 mM ammonium formate in methanol (solvent A) and 10 mM
110 ammonium formate in water (solvent B). The gradient program was run as follows: maintain 35% A
111 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
112 the initial conditions from 12 to 17 min and stabilization during 3 min before the next injection.
113 The detection and quantification were performed using a TSQ Quantum triple quadrupole mass
114 spectrometer equipped with electrospray ionization (ESI) source. To prevent a contamination of the
115 ESI source, from 0 to 6 min, the column effluent was systematically diverted to the waste by means of
116 a motorized Divert/Inject valve.
117 The mass spectrometer was operated in multiple reaction monitoring (MRM) positive acquisition
118 mode. The ion capillary temperature was heated to 350 °C and the ESI needle voltage was set at
119 4000 V. The sheath and auxiliary gas (N_2) pressures were respectively tuned at 40 and 30 (arbitrary
120 units). A 4 V source collision induced dissociation (CID) offset and a 1.5 mTorr collision gas (Ar)
121 pressure were applied at the collision cell. For each compound, sensitive quantitative determination
122 was performed using an addition of the MRM transitions displayed in [Table 1](#).
123 All MS parameters were optimized by direct infusion and the source parameters were subsequently
124 adjusted by flow injection. Data analysis was accomplished using Xcalibur™ 2.1 software.

125 *2.7. Analytical method validation*

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

130 *2.7.1. Limit of quantification*

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

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

138 2.7.2. Selectivity and matrix effects

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

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

147 2.7.3. Linearity

148 For each compound, the calibration curve range varied from the validated LLOQ (0.005 μM) to
149 2.0 μM . Calibration curve standard samples were prepared in replicates (n=6) in a mixture of purified
150 water and acetonitrile (50:50, v/v), and then analyzed. Data was reprocessed and validity of the
151 linearity was checked through ANOVA statistical analyses (Microsoft Excel). The goodness of fit
152 (GoF) and lack of fit (LoF) were determined and correlated with the corresponding Fisher theoretical
153 table value. The fitting of the calibration curves was obtained with a 1/x weighted least squares linear
154 regression.

155 2.7.4. Recovery

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

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

163 *2.7.5. Precision and accuracy*

164 Thanks to freshly prepared calibration curves, imprecision (intra- and inter-day) and accuracy were
165 back calculated for the mixture respectively at four concentration levels: low (0.05 μM), medium 1
166 (2.0 μM), medium 2 (5.0 μM) and high (50.0 μM).

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

174 *2.7.6. Stability of pesticides in the matrix extract*

175 Stability tests were conducted in triplicate with processed samples that had been previously spiked at a
176 concentration level of 5 μM . Different storage conditions were tested: 72 h in the autosampler tray at
177 +12 $^{\circ}\text{C}$, after 15 h of three cycles of freezing (-20°C) and thawing (room temperature) for either
178 1 month, 3 months or 9 months at -20°C . The results were calculated using freshly prepared
179 calibration curves. The imprecision and accuracy calculated for samples' stability should be below
180 15% and between 85 and 115% of their nominal levels, respectively.

181 **3. Results and discussion**

182 *3.1. Method development*

183 *3.1.2. Mass spectrometry optimization*

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

195 3.1.3. Chromatographic conditions

196 After optimization of the mass spectrometry parameters, different liquid chromatography columns
197 were tested. The first evaluations were achieved on two core-shell LC columns from Phenomenex (Le
198 Pecq, France): a Kinetex[®] C₁₈ and a Kinetex[®] PFP (100 mm × 2.1 mm, 3 μm). Both columns enabled a
199 satisfactory separation of linuron, chlorfenvinphos, ethion and IS. Less peak tailing was nevertheless
200 obtained with the Kinetex PFP, therefore contributing to a significant improvement on sensitivity,
201 especially for the chlorinated molecules such as chlorfenvinphos and linuron. This column was
202 initially selected, but the drawback of using this specific stationary phase appeared later during the
203 study. In fact, a high back pressure was observed only after a short time use. As a consequence, a fully
204 porous Thermo Hypersil[™] Gold PFP column was used instead. A new elution gradient was therefore
205 optimized for this column and enabled to achieve optimum resolution and compound detection, as
206 already depicted in “Experimental - LC-MS/MS analysis”. Finally, the analytes’ signals were
207 appropriately separated and chromatograms displayed good peak shapes as presented on [Fig. 1](#). The
208 carry-over in the chromatographic system was measured by injecting three blank solvents after the
209 highest calibration standard.

210 3.1.4. Sample purification

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

218 SPE sorbent types such as polymeric and silica based reversed-phase sorbents seemed appropriate for
219 the simultaneous extraction of organophosphorus and neutral phenylurea pesticides from biological
220 matrices (Cazorla-Reyes et al., 2011). The few studies dedicated to their purification from human body
221 fluids indicated that both polymeric reversed-phase cartridges (Nguyen et al., 2007; Raposo et al.,
222 2010) and silica based reversed-phase sorbent cartridges (Pitarch et al., 2003) could be used. In this
223 study, the aim was to minimize elution volumes in order to simplify sample treatment by avoiding the
224 concentration step after the elution of the analytes. As a result, the polymeric reversed-phase (PRP)
225 sorbents were chosen for their higher loading capacities which allowed the use of lower sorbent
226 amounts, associated with reduced elution volumes. In this work, three different commercial PRP
227 cartridges were compared (OasisTM HLB, Strata X[®] and SolaTM) with the goal to choose the one which
228 could give the maximum recoveries with the minimum elution volume. Aiming at obtaining
229 comparable results, a common SPE methodology was applied for all the cartridges. First, after being
230 successively rinsed using 1 mL of methanol and 1 mL acetonitrile, the cartridges were conditioned
231 with 1 mL of purified water. Then, a blank liver cells sample containing all the test compounds at
232 20 µM was loaded on the cartridge. Then, a washing step consisting of 1 mL of purified water,
233 followed by a 5 min drying step and an elution of the target compounds with 2×0.2 mL of pure
234 acetonitrile under vacuum (-5 PSI), were applied. Acetonitrile was chosen for its LC-MS compatibility
235 and its higher elution strength in comparison with methanol. Indeed, the latter did not allow for
236 satisfactory elution of ethion even if a larger volume was used (up to 2 mL). After dilution (50:50, v/v)

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

240 As incubation of both hepatocytes and microsomes samples was stopped by adding cold-acetonitrile
241 (50/50, v/v), further optimization was performed to determine the maximum acceptable amount of
242 acetonitrile in the liver extracts to be purified by SPE. To this goal, as described above, blank samples
243 were prepared and loaded onto Sola™ cartridges. Then, an elution/retention profile of the analytes was
244 established with the successive addition of mixtures of water and acetonitrile, as shown in [Table 3](#).
245 The experiments were performed in duplicate. All the analytes were retained with acetonitrile
246 proportion below 30%. Linuron, as the most polar compound, was the first one to be desorbed. As a
247 consequence, we considered that the liver extracts from kinetic experiments should be diluted with the
248 appropriate amount of water (25:75, v/v) prior to the solid phase extraction.

249 *3.2. Performance of the analytical method*

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

255 The results of the linearity, the intra- and inter-day precision and accuracy, as well as the stability of
256 pesticides in the matrix extracts are summarized in [Table 4](#).

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

261 Recovery data collected on three replicates of three wide range covering levels ranged from 92.9% to
262 99.5%, with a maximum RSD of 2.3%, demonstrating the efficiency of the SPE purification process.

263 Moreover, intra- and inter-day imprecision and accuracy were all within the established ranges of

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

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

268 **4. Conclusions**

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

280

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288 **References**

- 289 Berry, M.N., Friend, D.S, 1969. High-yield preparation of isolated rat liver parenchymal cells: a
290 biochemical and fine structural study. *J. Cell Biol.* 43, 506–20.
- 291 Betsy, A., Vemula, S.R., Sinha, S., Mendu, V.V., Polasa, K. 2014. Assessment of dietary intakes of
292 nineteen pesticide residues among five socioeconomic sections of Hyderabad—a total diet study
293 approach. *Environ. Monit. Assess.* 186, 217-28.
- 294 Cao, L.L., Yan, C.H., Yu, X.D., Tian, Y., Zhao, L., Liu, J.X., Shen, X.M., 2011. Relationship between
295 serum concentrations of polychlorinated biphenyls and organochlorine pesticides and dietary habits of
296 pregnant women in Shanghai. *Sci. Total Environ.* 409, 2997–3002.
- 297 Cazorla-Reyes, R., Fernández-Moreno, J.L., Romero-González, R., Frenich, A.G., Vidal, J.L., 2011.
298 Single solid phase extraction method for the simultaneous analysis of polar and non-polar pesticides in
299 urine samples by gas chromatography and ultra high pressure liquid chromatography coupled to
300 tandem mass spectrometry. *Talanta.* 85, 183–196.
- 301 Damalas, C.A., Eleftherohorinos, I.G., 2011. Pesticide Exposure, Safety Issues, and Risk Assessment
302 Indicators. *Int. J. Environ. Res. Public Health.* 8, 1402–1419.
- 303 Deme, P., Azmeera, T., Kanjilal, S., Jonnalagadda, P., Upadhyayula, V.S., 2012. LC-MS/MS
304 determination of organophosphorus pesticide residues in coconut water. *Food Anal. Met.* 6, 1162–
305 1169.
- 306 Ding, G., 2014. Revisiting pesticide exposure and children's health: focus on China. *Sci. Total*
307 *Environ.* 472, 289–295.
- 308 Lemos, J., Sampedro, M. C., de Ariñod, A., Ortiza, A., Barrio, R.J., 2016. Risk assessment of
309 exposure to pesticides through dietary intake of vegetables typical of the Mediterranean diet in the
310 Basque Country. *J. Food Compos. Anal.* 49, 35–41.

311 Liska, I., Slobodnik, J., 1996. Comparison of gas and liquid chromatography for analysing polar
312 pesticides in water samples. *J. Chromatogr. A* 733, 235–258.

313 Nguyen, J.V., Olsson, A.O., Bravo, R., Needham, L.L., Barr, D.B., 2007. Quantification of atrazine,
314 phenylurea, and sulfonylurea herbicide metabolites in urine by high-performance liquid
315 chromatography-tandem mass spectrometry. *J Anal. Toxicol.* 31, 181–186.

316 Pitarch, E., Serrano, R., López, F.J., Hernández, F., 2003. Rapid multiresidue determination of
317 organochlorine and organophosphorus compounds in human serum by solid-phase extraction and gas
318 chromatography coupled to tandem mass spectrometry. *Anal. Bioanal. Chem.* 376, 189–197.

319 Raposo, R., Barroso, M., Fonseca, S., Costa, S., Queiroz, J.A., Gallardo, E., Dias, M., 2010.
320 Determination of eight selected organophosphorus insecticides in postmortem blood samples using
321 solid-phase extraction and gas chromatography/mass spectrometry. *Rapid Commun. Mass. Spectrom.*
322 24, 3187–3194.

323 Sinha, S.N., Rao, M.V.V., Vasudev, K., Odetokun, M., 2012. A liquid chromatography mass
324 spectrometry-based method to measure organophosphorous insecticide, herbicide and non-
325 organophosphorous pesticide in grape and apple samples. *Food Control* 25, 636–646.

326 Szyrka, E., Kurdziel, A., Matyaszek, A., Podbielska, M., Rupa, J., Słowik-Borowiec, M., 2015.
327 Evaluation of pesticide residues in fruits and vegetables from the region of south-eastern Poland. *Food*
328 *Control.* 48, 137–142.

329 US Food and drug Administration (2013) Guidance for industry: bioanalytical method validation.
330 Rockville, MD: US Department of Health and Human Services, Food and Drug Administration,
331 Center for Drug Evaluation and Research. <<http://www.fda.gov>> (Accessed 20 Jun 2016).

332 Weaver, R., Riley, R.J., 2006. Identification and reduction of ion suppression effects on
333 pharmacokinetic parameters by polyethylene glycol 400. *Rapid Commun. Mass Spectrom.* 20, 2559–
334 2564.

- 335 Yaroshenko, D.V., Kartsova, L.A., 2014. Matrix Effect and Methods for Its Elimination in
336 Bioanalytical Methods Using Chromatography–Mass Spectrometry. *J. Anal. Chem.* 69, 311–317.

Table 1Ions 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 TM HLB	Strata X [®]	Thermo Sola TM
Linuron	92 (4)	87 (11)	100 (2)
chlorfenvinphos	90 (8)	83 (12)	97 (2)
Ethion	85 (9)	43 (3)	98 (5)

Table 3Elution of the pesticides mixture from SolaTM 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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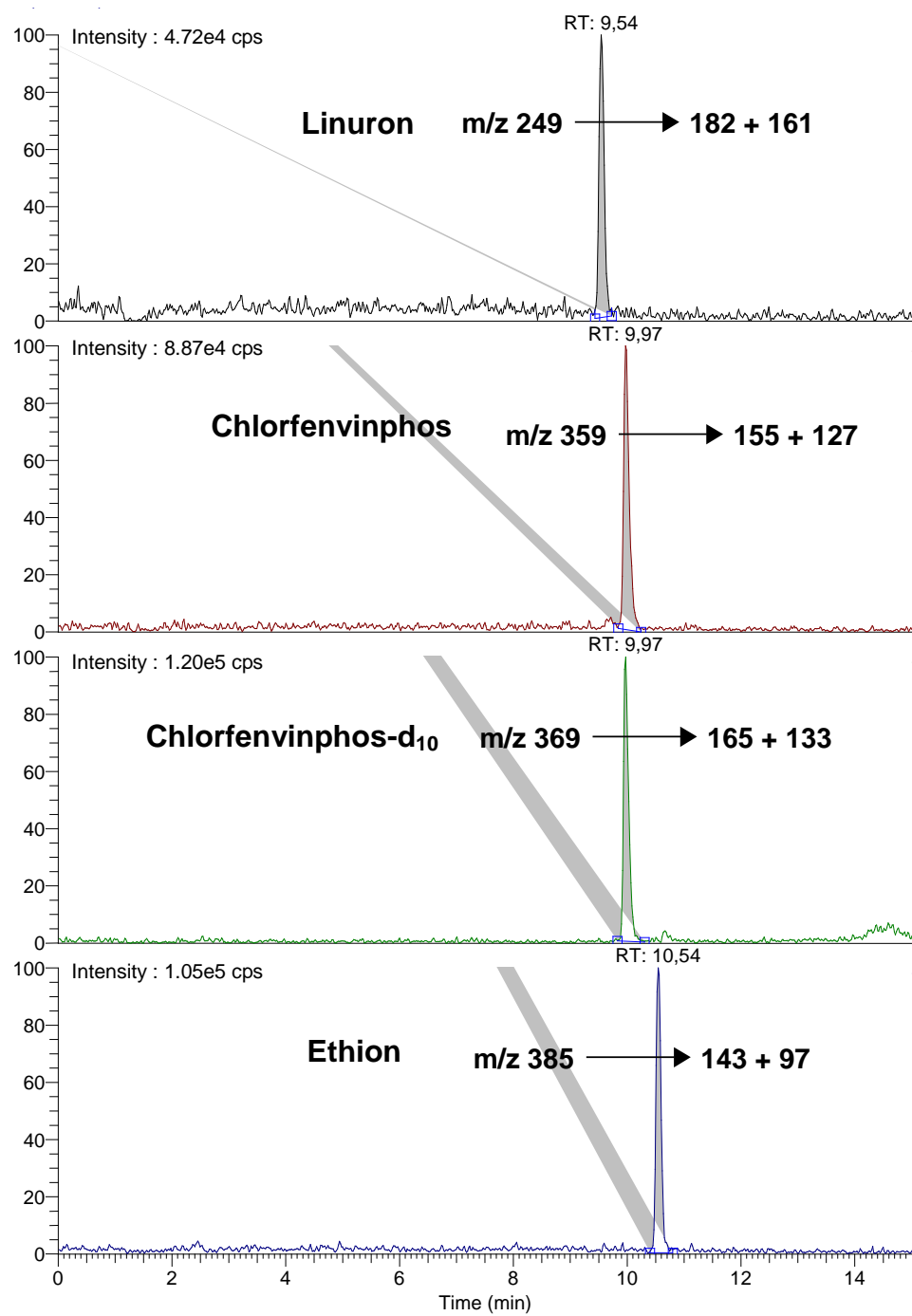


Fig. 1. LC-MS/MS chromatograms obtained from fortified inactivated hepatocytes extract (LLOQ)

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 SolaTM 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 HypersilTM 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.