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1 **A simple GC-MS method for the determination of diphenylamine,**
2 **tolyfluanid propargite and phosalone in liver fractions**

3 Ali Kadar ^{a*}, Ludovic Peyre ^{b*}, Henri Wortham ^a, Pierre Doumenq ^a

4 ^a Aix Marseille Univ, CNRS, LCE, Marseille, France,

5 ^b Cabinet conseil Santé-Environnement, 43 Rue Antoine Brun, 06150 Cannes, France

6 *Corresponding authors. *E-mail addresses:* ali.kadar@etu.univ-amu.fr (A. Kadar) and ludovic.peyre-
7 teisseire@hotmail.fr (L. Peyre)

8

9 **ABSTRACT**

10 In this study, an accurate and robust gas chromatography/mass spectrometry method was developed for
11 quantitative analysis of diphenylamine, tolyfluanid, propargite and phosalone in liver fractions.
12 Different injector parameters were optimized by an experimental design of experiment technique
13 (central composite design). An optimal combination of injector temperature (°C), splitless time (min)
14 and overpressure (KPa) values enabled to maximize the chromatographic responses. Sample preparation
15 was based on protein precipitation using trichloroacetic acid followed by liquid-liquid extraction (LLE)
16 of the pesticides with hexane. All compounds were quantified without interference in selected ion
17 monitoring mode using endrin as internal standard. The calibration curves for diphenylamine,
18 tolyfluanid, propargite and phosalone compounds were linear over the concentration range of
19 0.1 to 25 µM (*i.e.* 0.1, 0.5, 2.0, 5.0, 15.0 and 25.0 µM) with determination coefficients (R^2) higher than
20 0.999. A lower limit of quantification of 0.1 µM was obtained for all analytes, *i.e.* 422.5, 868.0, 876.2
21 and 919.5 µg/kg of liver fraction (hepatocytes) for diphenylamine, tolyfluanid, propargite and
22 phosalone, respectively. All compounds showed extraction recoveries higher than 93%, with a
23 maximum RSD of 3.4%. Intra- and inter-day accuracies varied from 88.4 to 102.9% and, imprecision
24 varied from 1.1 to 6.7%. Stability tests demonstrated that all pesticides were stable in liver extracts
25 during instrumental analysis (20°C in the autosampler tray for 72 h) following three successive freeze-
26 thaw cycles and, at -20°C for up to 12 months. This simple and efficient analytical procedure is thus
27 suitable for assessing mammals liver contamination or metabolism studies.

28 **Keywords:** Bioanalytical method, Pesticides mixture, Experimental design, GC-MS, Liver fractions,
29 Protein precipitation.

30

31 1. Introduction

32 For more than half a century, agricultural practices have involved the use of a large amounts of
33 pesticides. These pesticides provide better agricultural yields and allow extending the shelf life of
34 perishable fruits and vegetables to fulfill the need of the growing worldwide population [1].
35 Unfortunately, these helpful active compounds may at the same time constitute a significant risk to
36 animal and human health [2]. Indeed, pesticide residues are widespread in the whole environment and
37 are likely to be present in water resources and agricultural products [3-5]. Water and food crop
38 consumption is the predominant pathway of exposure for the general population [6-12].

39 On this population scale, several authors evaluated the dietary exposure to pesticides residues and
40 pointed out that the consumers may be simultaneously exposed to different residues [13-22]. In their
41 work [23], Crepet et al. concluded that depending on the foodstuff consumed the French general
42 population was mainly and most heavily exposed to 7 different pesticide mixtures composed of 2 to 6
43 compounds. Diphenylamine, tolylfluanid, propargite and phosalone belong to a mixture that was
44 significantly correlated to common fruits such as apples and pears. Once these potentially contaminated
45 fruits have been consumed and the compounds have passed into the body, these latter are transported by
46 the blood flow to the liver for metabolization [24]. **Therefore, in the scope of [metabolism \[24\]](#) or
47 [biomonitoring \[25-27\]](#) studies, a sensitive and reliable analytical method is needed to assess the
48 [pesticides level in liver fractions](#).** Until now, as far as we know, bibliographic research shows that no
49 work has been published on the simultaneous determination of diphenylamine, phosalone, propargite
50 and tolylfluanid in human biological samples.

51 However, Oliveira et al. and Kaczynski et al. have recently published multiresidues analytical protocols
52 including the determination of propargite and phosalone [24] or the quantification of propargite and
53 tolylfluanid [25] in fish liver. These methods were based on the use of liquid chromatography coupled
54 to tandem mass spectrometry (LC-MS/MS). The study reported by Russo et al. [26] is, to the best of our
55 knowledge, the only one dealing with real human liver samples. Authors carried out the development of
56 a gas chromatography coupled to a negative chemical ionization mass spectrometry (GC-NCI-MS)
57 method for the analysis of several organophosphorus pesticides including phosalone. Valente et al. [27]

58 quantified this latter in human blood along with other organophosphates thanks to a GC-MS method. In
59 the same matrix, Sharma et al. [28] successfully quantitated phosalone using gas chromatography
60 coupled to a flame thermionic detector (GC-FTD).

61 For sample purification, works published by Valente et al. [27] and Sharma et al. [28] depicted a one-
62 step phosalone extraction/clean-up from blood liquid samples using either silica based C 18 reversed-
63 phase [27] or florisil [28]. Robles-Molina et al. [x] extracted diphenylamine and phosalone from water
64 with Oasis HLB sorbent. Lehotay et al. [y] validated automated mini-SPE with MgSO₄/primary
65 secondary amine (PSA)/C18/CarbonX sorbent for the analysis of diphenylamine and propargite in food
66 samples.

67 On the other hand, solvent extractions proved to be suitable for the analysis of phosalone, propargite
68 and tolyfluanid in liver samples [24-26].

69 An analytical protocol using solvent extraction followed by a GC separation and MS detection seemed
70 convenient for assessing liver contamination by the present pesticide mixture.

71 Thus, this work aimed at developing and validating a simple, accurate and robust analytical method for
72 the analysis of diphenylamine, tolyfluanid, propargite and phosalone in human liver fractions
73 (hepatocytes).

74 **2. Experimental**

75 *2.1. Chemicals, materials and biological samples*

76 Certified standards of diphenylamine, tolyfluanid, propargite, phosalone and endrin with purities higher
77 than 99.5 %; ammonium sulfate salts and trichloroacetic acid (TCA) of research grade purity were
78 purchased from Sigma-Aldrich (St Quentin Fallavier, France). *n*-Hexane, ethyl acetate and acetonitrile
79 of ultra-trace analysis grade were purchased from Carlo Erba (Val de Reuil, France). Standard stock
80 solutions were prepared from pure compounds and appropriately diluted in acetonitrile.

81 A high-throughput tissue grinder (MM 300) produced by Retsch (Haan, Germany) was used as a
82 powerful transversal shaker for the LLE experiments. Phase separation was achieved using a Thermo
83 IEC Micromax RF benchtop centrifuge from Thermo Fisher Scientific (Illkirch, France).

84 All experiments on human tissues were carried out according to the ethical standards of the responsible
85 committee on human experimentation and the Helsinki Declaration. Cellular (hepatocytes) fractions
86 were obtained after mechanical decomposition of liver tissues. Here we decided to carry out the
87 experiments with thermally (100°C for 3 min) inactivated hepatocytes previously isolated following
88 Berry and Friend procedure [29].

89 *2.2 Sample treatment*

90 A 400 µL volume of thermally inactivated liver cells (16 mg) in 100 mM phosphate potassium buffer
91 (pH 7.4) was added to 1.8 mL Eppendorf® tubes. Prior to a brief vortex mix of the samples, the required
92 amounts of diphenylamine, tolylfluanid, propargite, phosalone and endrin were added as internal
93 standard (IS). Then, 100 µL of ice-cold trichloroacetic acid (20%) and 400 µL of Hexane were added to
94 the tubes. The samples were vigorously shaken for 3 minutes and centrifuged at 16000g and 4°C for 4
95 min, enabling the precipitation of the denatured proteins and full separation of the liquid phases. The
96 supernatant was then transferred into a 200 µL GC vial insert for GC analysis.

97 *2.3. GC-MS Analysis*

98 Separation and quantification of the pesticides mixture were performed using a TRACE™ ultra gas
99 chromatograph coupled to a DSQ II single quadrupole mass spectrometer (Thermo Fisher Scientific,
100 Courtaboeuf, France). The gas chromatograph was equipped with a programmable split/splitless
101 injector, a capillary column and a programmable oven. A sample volume of 2 µL was injected at 271°C,
102 in splitless mode, in a baffle Siltek-deactivated liner (2 mm × 2.75 mm × 120 mm) provided by Thermo
103 Fisher Scientific . A surge pressure of 490 kpa was applied for a 1.25 min period right after injection.
104 The separation of the four compounds was achieved on a TRACE TR5-MS (Thermo Fisher Scientific)
105 capillary column (30 m × 0.25 mm, *i.d.*, 0.25 µm film thickness). The electron impact ion source and
106 transfer line temperatures were set at 250 and 280°C, respectively. The elution was carried out using a
107 1.0 ml/min constant flow rate of helium carrier gas and a temperature gradient. After optimization of
108 the standards mixture separation, the oven temperature was programmed as follows: the initial
109 temperature set at 50 °C for 2.0 min followed by a first ramp of 35 °C .min⁻¹ up to 210 °C and held for
110 4.0 min; then a second ramp of 35 °C min⁻¹ up to 280 °C was set and maintained for 4.1 min. The total

111 optimized run time was 17 min. The criteria for positivity used to identify the compounds were both
112 retention times and characteristic m/z signals. Quantification was accomplished in the selected ion
113 monitoring (SIM) mode and the monitored ions are displayed in **Table 1**.

114

115 Table 1. Ions monitored under the SIM mode by GC-MS^a and their relative
116 intensities (%).

Compound	Molecular ion (m/z)	Base peak ion ^b (m/z)	Fragment ion 1 (m/z)
Diphenylamine	169	169(100)	168(50)
Endrin (IS)	380	263(100)	281(65)
Phosalone	367	182(100)	184(33)
Propargite	350	135(100)	173(15)
Tolylfluanid	346	137(100)	238(48)

117 ^a The compounds were quantified with the sum of both base peak and fragment peak signals.

118 ^b Ionized in the positive mode with an electron energy of 70 eV.

119

120 2.4. Optimization of splitless injection parameters

121 To achieve the lowest instrumental detection limits, in addition to preliminary optimization of
122 chromatographic and ionization source settings, the splitless injection was optimized. For that purpose,
123 a central composite design (CCD) with three independent variables (X_1 , Temperature; X_2 , Surge
124 pressure; X_3 , Splitless Time) was performed. Preliminary experiments with one factor at a time allowed
125 to determine the appropriated ranges of these independent variables: X_1 : 133–318 °C; X_2 : 0–490 kPa;
126 X_3 : 0–2.50 min. As shown in **Table 2A**, five levels were used for optimization of the three factors.

127 Design and analysis of the central composite experiment were carried out using Statistica 8.0 software
128 (Statsoft, Maison Alfort, France). A total of 48 assays described in **Table 2B** were carried out by
129 automated injections of 2 μ L of a 10 μ M multi-compounds standard solution containing diphenylamine,
130 tolylfluanid, propargite and phosalone.

131

132 Table 2. Values of the factors at the five levels examined (A) and experiments
 133 undertaken for the central composite design (B).

(A)	Temperature (°C)	Time (min)	Surge pressure (kPa)	
Lowest	133	0.00	0	
Low	170	0.50	100	
Center	225	1.25	245	Factor levels
High	280	2.00	390	
Highest	318	2.50	490	

(B)	No.	X ₁	X ₂	X ₃	
	1	225	1.25	0	
	2	170	2	100	
	3	225	1.25	245	
	4	225	2.51	245	
	5	170	2	390	
	6	318	1.25	245	
	7	225	0	245	
	8	225	1.25	490	
	9	280	2	100	
	10	280	0.5	390	
	11	280	0.5	100	
	12	170	0.5	390	
	13	225	1.25	245	
	14	170	0.5	100	Experiments
	15	133	1.25	245	
	16	280	2	390	
	17	225	1.25	0	
	18	170	2	100	
	19	225	1.25	245	
	20	225	2.51	245	
	21	170	2	390	
	22	318	1.25	245	
	23	225	0	245	
	24	225	1.25	490	
	25	280	2	100	
	26	280	0.5	390	

(B)	No.	X ₁	X ₂	X ₃
	27	280	0.5	100
	28	170	0.5	390
	29	225	1.25	245
	30	170	0.5	100
	31	133	1.25	245
	32	280	2	390
	33	225	1.25	0
	34	170	2	100
	35	225	1.25	245
	36	225	2.51	245
	37	170	2	390
	38	318	1.25	245
	39	225	0	245
	36	225	1.25	490
	37	280	2	100
	38	280	0.5	390
	39	280	0.5	100
	40	170	0.5	390
	41	225	1.25	245
	42	170	0.5	100
	43	133	1.25	245
	44	280	2	390
	45	225	1.25	0
	46	170	2	100
	47	225	1.25	245
	48	225	2.51	245

135

136

137 *2.5. Method validation*

138 Validation was achieved according to the Food and Drug Administration (FDA) guidance for
 139 bioanalytical methods [30].

140 The LLOQ, established as the lowest concentration that can be measured with acceptable imprecision

141 (<20%) and accuracy ($\pm 20\%$), was assessed with four serial dilutions of spiked sample extract

Commenté [ZZ1]: Si plusieurs échantillons spikés mettre sample au pluriel

142 containing 0.400 mM of each compound (six replicates). To validate a specific LLOQ, the
143 chromatographic peak area of the corresponding analyte should be at least five times higher than the
144 noise background of blank samples (*i.e.* peak to peak signal-to-noise ratio, S/N=5). The LLOQ were
145 established as 422.5, 868.0, 876.2 and 919.5 µg/kg of liver for diphenylamine, tolylfluanid, propargite
146 and phosalone, respectively.

147 Selectivity was evaluated on ten different blank human cells extracts by checking that chromatographic
148 peaks of pesticides and endrin (IS) did not co-elute with any endogenous compound. The evaluation
149 consisted in verifying that specific retention times and SIM responses of pesticides were well
150 discriminated from potential interfering signals.

151 In addition, the matrix effect criterion was studied: extracting ten different blank matrices were extracted
152 and spiked with the pesticides mixture at the predetermined LLOQ concentrations and quantified. Figure
153 2. presents both a typical chromatogram at the LLOQ and a chromatogram of a blank sample extract.
154 The results were compared with those obtained for fortified aqueous extract of the same concentration
155 levels. In the absence of acceptable limits defined in the FDA guidance and, as previously reported by
156 Kadar et al. [31], to be acceptable, the deviation between the calculated and the nominal values should
157 be less than $\pm 5\%$.

158 To study the linearity for diphenylamine, tolylfluanid, propargite and phosalone, 6 replicates of
159 calibration standard samples from the validated LLOQ (0.1 µM) to 25 µM were prepared in hexane and
160 analyzed by GC-MS.

161 Recovery rates of analytes were evaluated at low (0.1 µM), medium (5.0 µM) and high (50.0 µM)
162 concentration levels. Despite the detector linearity being validated up to 25 µM only, a validation of the
163 last concentration allowed expanding the concentration range of applicability of the method. Three
164 replicates were prepared by fortifying thermally inactivated human hepatocytes samples before
165 extraction and analysis. The ratio between mean peak areas from these samples and the one from post-
166 extracted spiked samples allowed determining each pesticide percentage recovery.

167 The imprecision (intra- and inter-day) and accuracy of the method were studied at four different
168 concentration levels: low (0.1 µM), medium (5.0 µM) and high (50.0 µM). For intra-day imprecision
169 and accuracy, five replicate samples per concentration level were prepared and analyzed on the same

170 day. For inter-day imprecision, six different days on a 15 days period were chosen to repeat the
171 preparation and analysis of sample duplicates at the same spiking levels. Intra- and inter-day imprecision
172 were considered acceptable if the relative standard deviations (RSD%) were below 15% [31]. On the
173 other hand, accuracy, expressed as the mean percentage deviation (Intra-day: Ar% and Inter-day: Br%)
174 from the spiked value, was in accordance with the guidance when the yields ranged between 85 and
175 115% of the nominal concentrations.

176 To check the stability of the compounds, degradation tests were carried out in triplicate using processed
177 samples previously spiked at 5 μM with the pesticides mixture. Various storage conditions were
178 experimented: 96 h in the autosampler tray at +25°C, after three freeze – thawing cycles from -20°C to
179 room temperature during 15h or, long term storage for either 1 month, 3 months or 9 months at -20°C.
180 For this criterion, the FDA did not set acceptable limits. Then, after samples' analysis using freshly
181 prepared calibration curves, imprecision and accuracy were respectively considered acceptable if below
182 15% and if between 85% and 115% of their nominal values as depicted by Gonzalez et al. [3]. In
183 addition, after consecutive analysis of 8 samples, a blank sample and a standard at 0.1 μM were analyzed
184 to check for any pollution or significant drift (> 10%) of the instrument signal.

185 **3. Results and discussions**

186 *3.1. Method development*

187 *3.1.1. Sample treatment*

188 On the basis of previously published works [32-35], hexane and ethyl acetate seemed to be good solvents
189 candidates for liquid/liquid extraction of the studied pesticides mixture from a 400 μL sample of
190 thermally inactivated human liver cells. As presented in **Table 3**, the assay was optimized through a
191 sequence of experiments aiming at establishing the best sample treatment prior to GC-MS analysis. First,
192 the extraction efficiencies of the two hydrophobic solvents were compared on 400 μL spiked phosphate
193 potassium buffer samples. As displayed in **Table 3**, in the absence of matrix, hexane proved to be the
194 best compromise for the whole pesticides mixture. Then, the experiment was repeated on a sample
195 spiked liver cells sample. However, due to the formation of a very compact emulsion, no clear layers
196 appeared, thus preventing GC-MS analysis. Partial solubilization of proteins, highly present, could

197 explain the outbreak of this emulsion. Thanks to their combined hydrophobic and hydrophilic properties,
 198 these proteins are attracted to both the non-polar solvent and the aqueous medium. Afterwards,
 199 comparison of simultaneous protein precipitation and LLE versus sequential protein precipitation and
 200 LLE was carried out. For this purpose, two efficient protein precipitators were compared: TCA and
 201 ammonium sulfate [36]. Globally, the results in **Table 3** show that regardless of the assay procedure
 202 (either simultaneous or sequential), recoveries obtained were higher with TCA compared to ammonium
 203 sulfate. In fact, contrarily to ammonium sulfate [37], TCA is known to help with the release of bound
 204 analytes into solution [38]. Indeed, at pH lower than the protein isoelectric point, TCA interacts with the
 205 positively charged amine group of proteins to form an insoluble salt [39]. In addition, as displayed in
 206 **Table 3**, simultaneous protein precipitation and LLE gave the best recoveries with TCA. This is
 207 probably because the vigorous shaking enabled a rapid solvent exchange between aqueous and organic
 208 solution while precipitation of protein was taking place simultaneously.

209

210 Table 3. Pesticides recoveries of a 10 μ M concentration spiked samples
 211 regarding the chemical treatment applied.

Treatment	Recovery, RSD (% , n = 2)			
	Diphenylamine	Tolyfluanide	Propargite	Phosalone
Buffer/hexane ^a	95, 1.6	100, 2.8	99, 5.0	99, 4.7
Buffer/ethyl acetate ^a	98, 2.6	99, 3.3	96, 6.1	93, 4.3
Medium/hexane ^a	n/a	n/a	n/a	n/a
Medium + SO ₄ (NH ₄) ₂ /hexane ^b	84, 4.8	70, 13.9	71, 24.2	81, 6.4
Medium + TCA/hexane ^b	87, 4.3	81, 3.9	96, 2.3	88, 4.6
Medium/SO ₄ (NH ₄) ₂ + hexane ^c	87, 4.0	75, 2.4	91, 2.5	89, 3.6
Medium/TCA + hexane ^c	90, 1.5	86, 0.9	96, 1.8	93, 0.6

212 ^a LLE.

213 ^b Proteins precipitation followed by LLE.

214 ^c Simultaneous proteins precipitation and LLE.

215 Highest recoveries were obtained using simultaneous proteins precipitation and LLE after TCA and
 216 hexane were added. Recoveries varied from 86 % for tolylfluanide to 98 % for endrin, with a maximum
 217 RSD value of 1.8 %.

218 The final protocol was as follows: 100 µL of 20 % TCA and 400 µL of hexane were added to the human
 219 hepatic preparation before 3 minutes vigorous shaking. Then, the sample was centrifuged for 4 minutes
 220 at 16000 g and 4°C. Finally, the supernatant was transferred into a vial prior to GC-MS analysis.

221 *3.1.3 Optimization of splitless time, temperature and surge pressure using response surface*
 222 *methodology*

223 The experimental results were analyzed by multiple linear regression to fit to the postulated model (1)
 224 where Y is the instrumental response, X₁, X₂ and X₃ are the three independent variables described above
 225 and β_i, β_{ii} and β_{ij} are the fitting coefficients.

226
$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

227

228 Table 4. Second polynomial equations obtained for the different pesticides.

Pesticides	Equations	R ²	Adj. R ²
Diphenylamine	Y=1.62E8-1.82E6X1+5.87E8X2+8.83E5X3-2.99E5X1X 2+7.03E3X1X3-3.87E5X2X3+1.10E03X12-1.61E08X22 -2.37E03X32	0.9180	0.8985
Tolyfluanid	Y=-9.11E8+9.66E6X1+5.25E8X2+2.89E5X3-3.87E5X1 X2+6.02E3X1X3-2.00E05X2X3-2.64E4X12-1.06E8X22 -1.34E3X32	0.8531	0.8183
Propargite	Y=-1.72E9+1.26E7X1+6.93E8X2+1.29E6X3-3.23E5X1 X2+4.8E3X1X3-3.44E5X2X3-2.64E4X12-1.46E8X22- 2.10E3X32	0.8922	0.8667
Phosalone	Y=-1.65E9+1.17E7X1+6.16E8X2+1.45E6X3-2.19E5X1 X2+4.10E3X1X3-2.03E5X2X3-2.41E4X12-1.46E8X22 -2.58E3X32	0.9363	0.9212

229

230 Calculated coefficients of the pesticides response model, corresponding determination coefficients (R²)
 231 and adjusted determination coefficients (Adj. R²) are reported in **Table 4**. ANOVA results presented in
 232 **Table 5** demonstrated that the model was highly significant for each compound. The values of R² and
 233 Adj. R² (0.9180 and 0.8985 for diphenylamine; 0.8531 and 0.8183 for tolylfluanid; 0.8922 and 0.8667

234 for propargite; 0.9363 and 0.9212 for phosalone:) indicated a satisfying degree of correlation between
 235 the observed and the predicted values.

236

237 Table 5. ANOVA of central composite design for [diphenylamine](#), tolylfluanid,
 238 propargite and phosalone.

Parameter	SS	df	MS	F	p
Diphenylamine					
X ₁	7327E+16	1	7327E+16	19.029	0.000095
X ₂	5603E+17	1	5603E+17	145.522	0.000000
X ₃	5806E+17	1	5806E+17	150.802	0.000000
X ₁ ²	3056E+14	1	3056E+14	0.079	0.779694
X ₁ X ₂	3663E+15	1	3663E+15	0.951	0.335552
X ₁ X ₃	7543E+16	1	7543E+16	19.590	0.000078
X ₂ ²	2249E+17	1	2249E+17	58.423	0.000000
X ₂ X ₃	4241E+16	1	4241E+16	11.015	0.002001
X ₃ ²	6971E+16	1	6971E+16	18.104	0.000132
Error	1463E+17	38	3850E+15		
Total SS	1782E+18	47			
Tolylfluanid					
X ₁	1853E+17	1	1853E+17	31.71080	0.000002
X ₂	3570E+17	1	3570E+17	61.10285	0.000000
X ₃	4680E+17	1	4680E+17	80.08768	0.000000
X ₁ ²	1771E+17	1	1771E+17	30.31713	0.000003

Parameter	SS	df	MS	F	p
X_1X_2	6119E+15	1	6119E+15	104,716	0.312633
X_1X_3	5527E+16	1	5527E+16	945,954	0.003881
X_2^2	9680E+16	1	9680E+16	16.56632	0.000229
X_2X_3	1136E+16	1	1136E+16	194,441	0.171290
X_3^2	2231E+16	1	2231E+16	381,869	0.058073
Error	2220E+17	38	5843E+15		
Total SS	1512E+18	47			

Propargite

X_1	2897E+17	1	2897E+17	45.7029	0.000000
X_2	6701E+17	1	6701E+17	105.7338	0.000000
X_3	7132E+17	1	7132E+17	112.5357	0.000000
X_1^2	1770E+17	1	1770E+17	27.9339	0.000005
X_1X_2	4257E+15	1	4257E+15	0.6717	0.417570
X_1X_3	3514E+16	1	3514E+16	55,450	0.023801
X_2^2	1840E+17	1	1840E+17	29.0286	0.000004
X_2X_3	3368E+16	1	3368E+16	53,143	0.026706
X_3^2	5496E+16	1	5496E+16	86,716	0.005490
Error	2408E+17	38	6338E+15		
Total SS	2233E+18	47			

Phosalone

Parameter	SS	df	MS	F	p
X ₁	3225E+17	1	3225E+17	102.556	0.000000
X ₂	5291E+17	1	5291E+17	168.278	0.000000
X ₃	6332E+17	1	6332E+17	201.362	0.000000
X ₁ ²	1477E+17	1	1477E+17	46.986	0.000000
X ₁ X ₂	1956E+15	1	1956E+15	0.622	0.435201
X ₁ X ₃	2570E+16	1	2570E+16	8174	0.006863
X ₂ ²	1845E+17	1	1845E+17	58.665	0.000000
X ₂ X ₃	1166E+16	1	1166E+16	3708	0.061661
X ₃ ²	8276E+16	1	8276E+16	26.321	0.000009
Error	1195E+17	38	3144E+15		
Total SS	1874E+18	47			

239 SS = sum of squares, ddl = degree of freedom, MS = mean square, F = ratio and p = probability
240 level.

241

242 Even if it was possible to find the optimal injector settings for a single response using surface response
243 design, here the study aimed at finding a compromise for the simultaneous optimization of responses
244 from diphenylamine, tolylfluanid, propargite and phosalone. Consequently, the multicriteria
245 methodology established by Derringer and Suich [40] was implemented for the ensuing work. This
246 methodology first involves the construction of an individual desirability function (di) for each
247 compound. Then, as depicted by Eq. (2), the overall desirability function (D) is defined as the weighted
248 geometric average of the individual desirability (di).

$$249 \quad D = \sqrt[4]{d_1 d_2 d_3 d_4} \quad (2)$$

250 A value of D close to 1 means that each individual pesticide's response is maximized and that the
251 corresponding levels of factors are globally optimum. As can be observed in **Fig. 1**, the desirability

252 surfaces obtained by Eq. (2) show the effect of operating conditions on the overall pesticides
253 chromatographic responses in the range of the investigated variables.

254 Therefore, applying the numerical optimization function from the Statistica software to the Derringer's
255 desirability function allowed to find the following injection experimental conditions: X1 (271.7°C), X2
256 (1.25 min) and X3 (490 KPa); which simultaneously maximized the signals of all the compounds.

257

258 *3.2 Performance of the analytical method*

259 No instrumental carry-over or significant drift were noticed during all the validation process.

260 The absence of interfering peaks on chromatograms from ten different blank human hepatocyte samples,
261 compared chromatograms acquired from reference standard in neat solvents led to the full validation of
262 the method selectivity. Indeed, chromatographic signals of pesticides were always satisfactorily
263 discriminated on the basis of their specific retention times and SIM responses. In addition, no matrix
264 effects were observed at the LLOQ level for all pesticides.

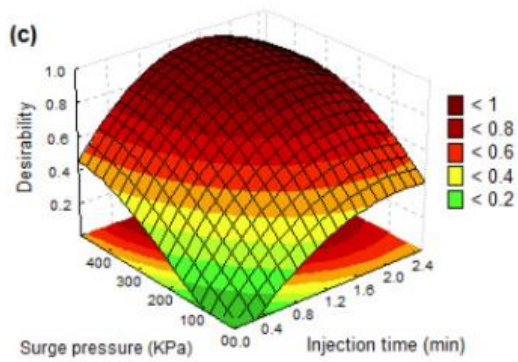
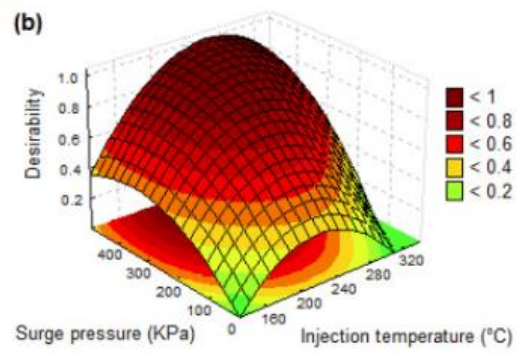
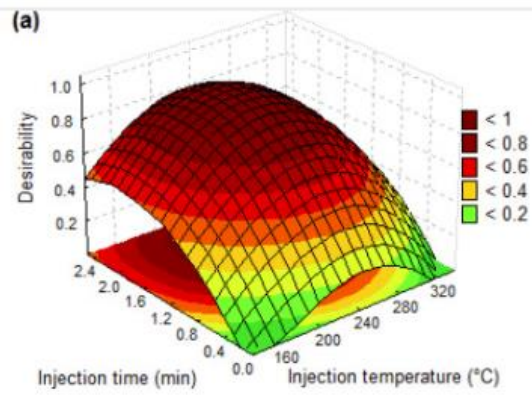
265 **Table 6** lists the key elements of the method validation.

266 The determination coefficient values (R^2) were always greater than 0.999 indicating that there was a
267 good correlation of linearity through the concentrations range used and a homoscedastic distribution of
268 replicates at all levels. On the basis of recovery results ranging from 96.5% to 100.0%, with a maximum
269 RSD of 3.4%, the implemented sample treatment was considered satisfactory.

270 In addition, intra- and inter-day imprecision and accuracy exhibited acceptable values, ranging from
271 1.1% to 6.7%.

272 Moreover, stability tests demonstrated that all the analytes did not degrade in the final extract while
273 stored at -20°C or manipulated at room temperature. Finally, all the data met the validation criteria set
274 by the FDA guidelines, rendering this method suitable for future researches.

275 The evaluated LLOQ for diphenylamine (422.5 µg/kg), tolylfluanid (868.0 µg/kg), propargite (976.2
276 µg/kg) and phosalone (919.5 µg/kg) are higher than the values published by other authors in the context
277 of biomonitoring studies. Indeed, for diphenylamine and propargite, Marschner et al. [41] obtained an
278 LLOQ of 166.5 µg/kg on animal liver. Moreover, the phosalone LLOQ published by Russo et al. [26]
279 was 15 µg/kg of human liver.

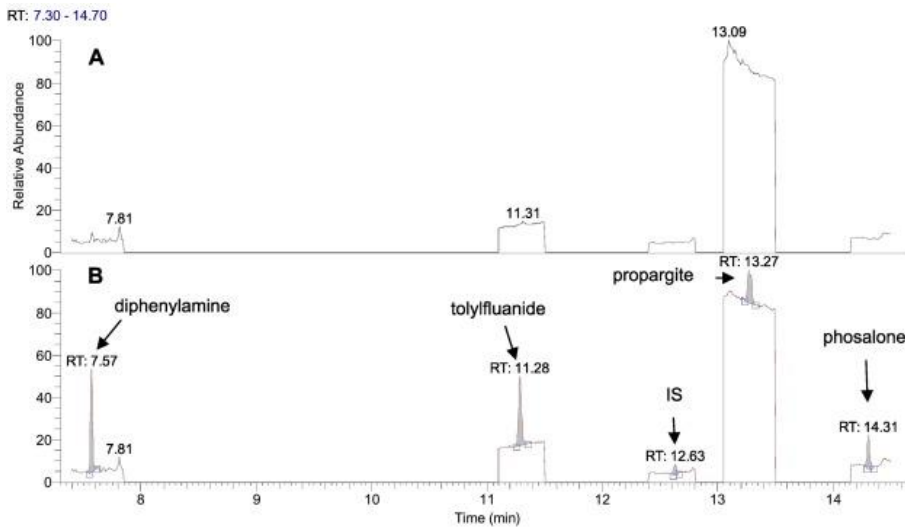


280

281 Fig. 1. Response surface and contour plots of (a) injection time and injection temperature, (b)

282 surge pressure and injection temperature and (c) surge pressure and injection time on the

283 overall desirability of [diphenylamine](#), tolylfluaniid, propargite and phosalone mixture
 284 response.
 285



286
 287 Fig. 2. Hepatocytes liver extract chromatograms, A: blank sample, B: sample at the LLOQ.
 288

289 Table 6. Results of the analytical method validation: linearity (n = 6), recovery
 290 (n = 3), within-run accuracy (n = 5), between-run accuracy (n = 2, 6 days).

Parameter	Diphenylamine	Tolylfluaniid	Propargite	Phosalone	Limits
Linearity					
Slope	62,715 ±285	274,740 ±790	62,186 ±295	40,608 ±258	
Intercept	-6447 ±2672	-18,292 ±7398	5862 ±2767	-5546 ±2420	
R ²	0.999 ±0.001	1.000 ±0.000	±1.000 ±0.000	0.999 ±0.001	
Recovery					
	R%	RSD%			
Low	99.0	1.2	96.5	1.9	98.1 1.1 99.3 2.0
Medium	95.0	1.1	97.8	2.0	97.2 1.2 97.2 1.5 n.a
High	99.1	1.0	99.6	1.1	98.4 1.2 97.8 1.5
Accuracy					
	Ar%	RSD%			
Intra-day					
Low	98.2	3.9	93.7	3.5	84.4 2.1 99.7 3.2 ±20%, ≤20%
Medium	93	1.5	99.1	2.8	96.3 1.8 95.7 2.2 ±15%, ≤15%

Parameter	Diphenylamine		Tolylfluaniid		Propargite		Phosalone		Limits
High	99.6	1.3	100.3	1.3	92.6	1.6	98.3	2.1	
Inter-day	Br%	RSD%							
Low	96	6.2	99.5	5.8	95.6	6.5	102.9	6.6	±20%, ≤20%
Medium	92.4	4.9	93.2	4.6	94.4	5.2	93.2	5.8	±15%, ≤15%
High	99.9	3.0	100.4	2.7	101.2	2.5	100.8	4.9	
Stability									
Cold-warm	SCt%								
-20/20 °C-15 h	100.2	1.1	99.8	1.2	100.0	1.3	99.8	1.0	±15%, ≤15%
Long term	SLt%								
1 month	100.3	1.4	100.1	1.3	99.7	1.5	100.5	1.2	±15%, ≤15%
6°months	99.8	1.2	99.6	1.1	99.8	1.3	100.1	1.4	
12 months	100.4	1.8	100.0	1.5	100.2	1.4	99.7	1.7	
Autosampler	SA%								
72 h	100.0	0.7	99.9	0.9	100.1	0.5	100.3	1.0	±15%, ≤15%

291 R%: percent recovery; RSD%: percent relative standard deviation.

292 Ar%: intra-day percent accuracy rate; Br%: inter-day percent accuracy rate.

293 SCt%: cold-war percent stability; SLt%: long term percent stability; SA%: autosampler
294 percent stability.

295

296 4. Conclusion

297 In this paper, a simple and selective GC-MS method was developed to simultaneously determine the
298 amounts of diphenylamine, tolylfluaniid, propargite and phosalone in human liver samples. The
299 instrumental settings were optimized to obtain the highest chromatographic responses for the above-
300 mentioned compounds. The optimum splitless injection conditions obtained through RSM and global
301 Derringer's desirability function were 271.7°C, 1.25 min and 490 KPa for injection temperature,
302 splitless time and surge pressure, respectively.

303 Convenient optimization of simultaneous protein precipitation and LLE extraction conditions allowed
304 to achieve very good recoveries and efficient sample purification using a user-friendly sample treatment.

305 This one-step LLE and cleanup method followed by GC-MS analysis complied with current FDA
306 requirements and showed satisfying selectivity, linearity, recovery, precision and accuracy. The limits

307 of quantification obtained in this work are satisfying, considering previously published works related to
308 human and animal liver samples.

309 This procedure is thus appropriate for the monitoring of the parent compound loss during *in vitro* human
310 liver metabolism studies. Finally, upgrading it to a more sensitive technology (MS/MS) it may also be
311 useful for conducting human or mammalian biomonitoring studies.

312

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318

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