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A simple GC–MS method for the determination of diphenylamine, tolylfluanid propargite and phosalone in liver fractions

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ABSTRACT

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

1. Introduction

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

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

However, Oliveira et al. and Kaczynski et al. have recently published multiresidues analytical protocols including the determination of propargite and phosalone [28] or the quantification of propargite and tolylfluanid [29] in fish liver. These methods were based on the use of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The study reported by Russo et al. [30] is, to the best of our knowledge, the only one dealing with real human liver samples.

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Authors carried out the development of a gas chromatography coupled to a negative chemical ionization mass spectrometry (GC-NCI-MS) method for the analysis of several organophosphorus pesticides including phosalone. Valente et al. [31] quantified this latter in human blood along with other organophosphates thanks to a GC–MS method. In the same matrix, Sharma et al. [32] successfully quantitated phosalone using gas chromatography coupled to a flame thermionic detector (GC-FTD).

For sample purification, works published by Valente et al. and Sharma et al. depicted a one-step phosalone extraction/clean-up from blood liquid samples using either silica based C18 reversed-phase [31] or florisil [32]. Robles-Molina et al. [33] extracted diphenylamine and phosalone from water with Oasis HLB sorbent. Lehotay et al. [34] validated automated mini-SPE with $MgSO_4$/primary secondary amine (PSA)/C18/CarbonX sorbent for the analysis of diphenylamine and propargite in food samples.

On the other hand, solvent extractions proved to be suitable for the analysis of phosalone, propargite and tolyfluanid in liver samples [28–30].

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

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

2. Experimental

2.1. Chemicals, materials and biological samples

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

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

All experiments on human tissues were carried out according to the ethical standards of the responsible committee on human experimentation and the Helsinki Declaration. Cellular (hepatocytes) fractions were obtained after mechanical decomposition of liver tissues. Here we decided to carry out the experiments with thermally (100 °C for 3 min) inactivated hepatocytes previously isolated following Berry and Friend procedure [35].

2.2. Sample treatment

A 400 µL volume of thermally inactivated liver cells (16 mg) in 100 mM phosphate potassium buffer (pH 7.4) was added to 1.8 mL Eppendorf® tubes. During the validation study, prior to a brief vortex mix of the samples, the required amounts of diphenylamine, tolylfluanid, propargite and phosalone were added. At the same time, and whatever the type of the sample, endrin was spiked as internal standard (IS) at 0.1 µM. The best choice would have been to use as internal standards a labeled compound for each pesticide of the mixture. However, due to the high cost of this option, we chose to use endrin as a global internal standard, especially due to the capacity of this labile compound to allow the control of inertness and cleanness of the GC injector and the column first centimeters [36]. Then, 100 µL of ice-cold trichloroacetic acid (20%) and 400 µL of hexane were added to the tubes. The samples were vigorously shaken for 3 min and centrifuged at 16,000*g* and 4 °C for 4 min, enabling the precipitation of the denatured proteins and full separation of the liquid phases. The supernatant was then transferred into a 200 µL GC vial insert for GC analysis.

2.3. GC–MS analysis

Separation and quantification of the pesticides mixture were performed using a TRACE™ ultra gas chromatograph coupled to a DSQ II single quadrupole mass spectrometer (Thermo Fisher Scientific, Courtaboeuf, France). The gas chromatograph was equipped with a programmable split/splitless injector, a capillary column and a programmable oven. A sample volume of 2 µL was injected at 271 °C, in splitless mode during 1.50 min, in a baffle Siltek-deactivated liner (2 mm × 2.75 mm × 120 mm) provided by Thermo Fisher Scientific. A surge pressure of 490 kPa was applied for a 1.25 min period right after injection. The separation of the compounds was achieved on a TRACE TR5-MS (Thermo Fisher Scientific) capillary column (30 m × 0.25 mm, *i.d.*, 0.25 µm film thickness). The electron impact ion source operated in the positive mode with an electron energy of 70 eV and the transfer line temperatures were set to 250 and 280 °C, respectively. The elution was carried out using a 1.0 mL/min constant flow rate of helium carrier gas and a temperature gradient. After optimization of the standards mixture separation, the oven temperature was programmed as follows: the initial 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 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 optimized run time was 17 min. The criteria for positivity used to identify the compounds were both retention times and characteristic *m/z* signals. Quantification was accomplished in the selected ion monitoring (SIM) mode and the monitored ions are displayed in Table 1. Quantification of each compound was performed through addition of both base peak and fragment peak areas. IS was used as a global quality control for validation of the sample analysis. When its recovery yield was lower than 80%, the sample needed to be reprocessed.

2.4. Optimization of splitless injection parameters

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

Design and analysis of the central composite experiment were carried out using Statistica 8.0 software (Statsoft, Maison Alfort, France). A

Table 1
Ions monitored under the SIM mode by GC–MS[a] and their relative 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)

[a] The compounds were quantified with the sum of both base peak and fragment peak signals.
[b] Ionized in the positive mode with an electron energy of 70 eV.

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

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

(B)	No.	X_1	X_2	X_3	
	1	225	1.25	0	Experiments
	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	
	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	
	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	

total of 48 assays described in Table 2B were carried out by automated injections of 2 µL of a 10 µM multi-compounds standard solution containing diphenylamine, tolylfluanid, propargite and phosalone.

2.5. Method validation

Validation was achieved according to the Food and Drug Administration (FDA) guidance for bioanalytical methods [37].

The LLOQ, established as the lowest concentration that can be measured with acceptable imprecision (< 20%) and accuracy (± 20%), was assessed with four serial dilutions of spiked sample extracts containing 0.400 mM of each compound (six replicates). To validate a specific LLOQ, the chromatographic peak area of the corresponding analyte should be at least five times higher than the noise background of blank samples (*i.e.* peak to peak signal-to-noise ratio, S/N = 5). The LLOQ were established as 422.5, 868.0, 876.2 and 919.5 µg/kg of liver for diphenylamine, tolylfluanid, propargite and phosalone, respectively.

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

In addition, the matrix effect criterion was studied: ten different blank matrices were extracted, spiked with the pesticides mixture at the predetermined LLOQ concentrations and quantified. Fig. 2 presents both a typical chromatogram at the LLOQ and a chromatogram of a blank sample extract. The results were compared with those obtained for fortified aqueous extract of the same concentration levels. In the absence of acceptable limits defined in the FDA guidance and, as previously reported by Kadar et al. [38], to be acceptable, the deviation between the calculated and the nominal values should be less than ± 5%.

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

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

The imprecision (intra- and inter-day) and accuracy of the method were studied at three different concentration levels: low (0.1 µM), medium (5.0 µM) and high (50.0 µM). For intra-day imprecision and accuracy, five replicate samples per concentration level were prepared and analyzed on the same day. For inter-day imprecision, six different days on a 15 days period were chosen to repeat the preparation and analysis of sample duplicates at the same spiking levels. Intra- and inter-day imprecision were considered acceptable if the relative standard deviations (RSD%) were below 15% [38]. On the other hand, accuracy, expressed as the mean percentage deviation (Intra-day: Ar% and Inter-day: Br%) from the spiked value, was in accordance with the guidance when the yields ranged between 85 and 115% of the nominal concentrations.

To check the stability of the compounds, degradation tests were carried out in triplicate using processed samples previously spiked at 5 µM with the pesticides mixture. We deliberately limited these tests to one level since we get used to analyze the targeted pesticides in food items from animal origin (ground meat, poultry…). Various storage conditions were experimented: 96 h in the autosampler tray at + 25 °C, after three freeze–thawing cycles from − 20 °C to room temperature during 15 h or, long term storage for either 1 month, 3 months or 9 months at − 20 °C. For this criterion, the FDA did not set acceptable limits. Then, after samples' analysis using freshly prepared calibration curves, imprecision and accuracy were respectively considered acceptable if below 15% and if between 85% and 115% of their nominal values as depicted by Gonzalez et al. [39]. In addition, after consecutive analysis of 8 samples, a blank sample and a standard at 0.1 µM were

Table 3
Pesticides recoveries of a 10 µM concentration spiked samples regarding the chemical treatment applied.

Treatment	Recovery, RSD (%, n = 2)			
	Diphenylamine	Tolylfluanide	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

[a] LLE.
[b] Proteins precipitation followed by LLE.
[c] Simultaneous proteins precipitation and LLE.

analyzed to check for any pollution or significant drift (> 10%) of the instrument signal intensity.

3. Results and discussions

3.1. Method development

3.1.1. Sample treatment

On the basis of previously published works [40–43], hexane and ethyl acetate seemed to be good solvents candidates for liquid/liquid extraction of the studied pesticides mixture from a 400 µL sample of thermally inactivated human liver cells. As presented in Table 3, the assay was optimized through a sequence of experiments aiming at establishing the best sample treatment prior to GC–MS analysis. First, the extraction efficiencies of the two hydrophobic solvents were compared on 400 µL of spiked phosphate potassium buffer samples. As displayed in Table 3, in the absence of matrix, hexane proved to be the best compromise for the whole pesticides mixture. Then, the experiment was repeated on a spiked liver cells sample. However, due to the formation of a very compact emulsion, no clear layers appeared, thus preventing GC–MS analysis. Partial solubilization of proteins, highly present, could explain the outbreak of this emulsion. Thanks to their combined hydrophobic and hydrophilic properties, these proteins are attracted to both the non-polar solvent and the aqueous medium. Afterwards, comparison of simultaneous protein precipitation and LLE *versus* sequential protein precipitation and LLE was carried out. For this purpose, two efficient protein precipitators were compared: TCA and ammonium sulfate [44]. Globally, the results in Table 3 show that regardless of the assay procedure (either simultaneous or sequential), recoveries obtained were higher with TCA compared to ammonium sulfate. In fact, contrarily to ammonium sulfate [45], TCA is known to help with the release of bound analytes into solution [46]. Indeed, at pH lower than the protein isoelectric point, TCA interacts with the positively charged amine group of proteins to form an insoluble salt [47]. In addition, as displayed in Table 3, simultaneous protein precipitation and LLE gave the best recoveries with TCA. This is probably because the vigorous shaking enabled a rapid solvent exchange between aqueous and organic solution while precipitation of protein was taking place simultaneously.

Highest recoveries were obtained using simultaneous proteins precipitation and LLE after TCA and hexane were added. Recoveries varied from 86% for tolylfluanide to 96% for propargite, with a maximum RSD value of 1.8%.

The final protocol was as follows: 100 µL of 20% TCA and 400 µL of hexane were added to the human hepatic preparation before 3 min vigorous shaking. Then, the sample was centrifuged for 4 min at 16,000*g* and 4 °C. Finally, the supernatant was transferred into a vial prior to GC–MS analysis.

3.1.2. Optimization of splitless time, temperature and surge pressure using response surface methodology (RSM)

The experimental results were analyzed by multiple linear regression to fit to the postulated model (Eq. (1)) where Y is the instrumental response, X_1, X_2 and X_3 are the three independent variables described above and β_i, β_{ii} and β_{ij} are the fitting coefficients.

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 \quad (1)$$

Calculated coefficients of the pesticides response model, corresponding determination coefficients (R^2) and adjusted determination coefficients (Adj. R^2) are reported in Table 4. ANOVA results presented in Table 5 demonstrated that the model was highly significant for each compound. The values of R^2 and Adj. R^2 (0.9180 and 0.8985 for diphenylamine; 0.8531 and 0.8183 for tolylfluanid; 0.8922 and 0.8667 for propargite; 0.9363 and 0.9212 for phosalone) indicated a

Table 4
Second polynomial equations obtained for the different pesticides.

Pesticides	Equations	R^2	Adj. R^2
Diphenylamine	$Y = 1.62E8 - 1.82E6X_1 + 5.87E8X_2 + 8.83E5X_3 - 2.99E5X_1X_2 + 7.03E3X_1X_3 - 3.87E5X_2X_3 + 1.10E03X_1^2 - 1.61E08X_2^2 - 2.37E03X_3^2$	0.9180	0.8985
Tolylfluanid	$Y = -9.11E8 + 9.66E6X_1 + 5.25E8X_2 + 2.89E5X_3 - 3.87E5X_1X_2 + 6.02EX_1X_3 - 2.00E05X_2X_3 - 2.64E4X_1^2 - 1.06E8X_2^2 - 1.34E3X_3^2$	0.8531	0.8183
Propargite	$Y = -1.72E9 + 1.26E7\,X_1 + 6.93E8X_2 + 1.29E6X_3 - 3.23E5X_1X_2 + 4.8E3X_1X_3 - 3.44E5X_2X_3 - 2.64E4X_1^2 - 1.46E8X_2^2 - 2.10E3X_3^2$	0.8922	0.8667
Phosalone	$Y = -1.65E9 + 1.17E7\,X_1 + 6.16E8X_2 + 1.45E6X_3 - 2.19E5X_1X_2 + 4.10E3X_1X_3 - 2.03E5X_2X_3 - 2.41E4X_1^2 - 1.46E8X_2^2 - 2.58E3X_3^2$	0.9363	0.9212

Table 5
ANOVA of central composite design for diphenylamine, tolylfluanid, 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_1X_2	3663E+15	1	3663E+15	0.951	0.335552
X_1X_3	7543E+16	1	7543E+16	19.590	0.000078
X_2^2	2249E+17	1	2249E+17	58.423	0.000000
X_2X_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
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					
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_1X_2	1956E+15	1	1956E+15	0.622	0.435201
X_1X_3	2570E+16	1	2570E+16	8174	0.006863
X_2^2	1845E+17	1	1845E+17	58.665	0.000000
X_2X_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			

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

satisfying degree of correlation between the observed and the predicted values.

Even if it was possible to find the optimal injector settings for a single response using surface response design, here the study aimed at finding a compromise for the simultaneous optimization of responses from diphenylamine, tolylfluanid, propargite and phosalone. Consequently, the multicriteria methodology established by Derringer and Suich [48] was implemented for the ensuing work. This methodology first involves the construction of an individual desirability function (di) for each compound. Then, as depicted by Eq. (2), the overall desirability function (D) is defined as the weighted geometric average of the individual desirability (di).

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

A value of D close to 1 means that each individual pesticide's response is maximized and that the corresponding levels of factors are globally optimum. As can be observed in Fig. 1, the desirability surfaces obtained by Eq. (2) show the effect of operating conditions on the overall pesticides chromatographic responses in the range of the investigated variables.

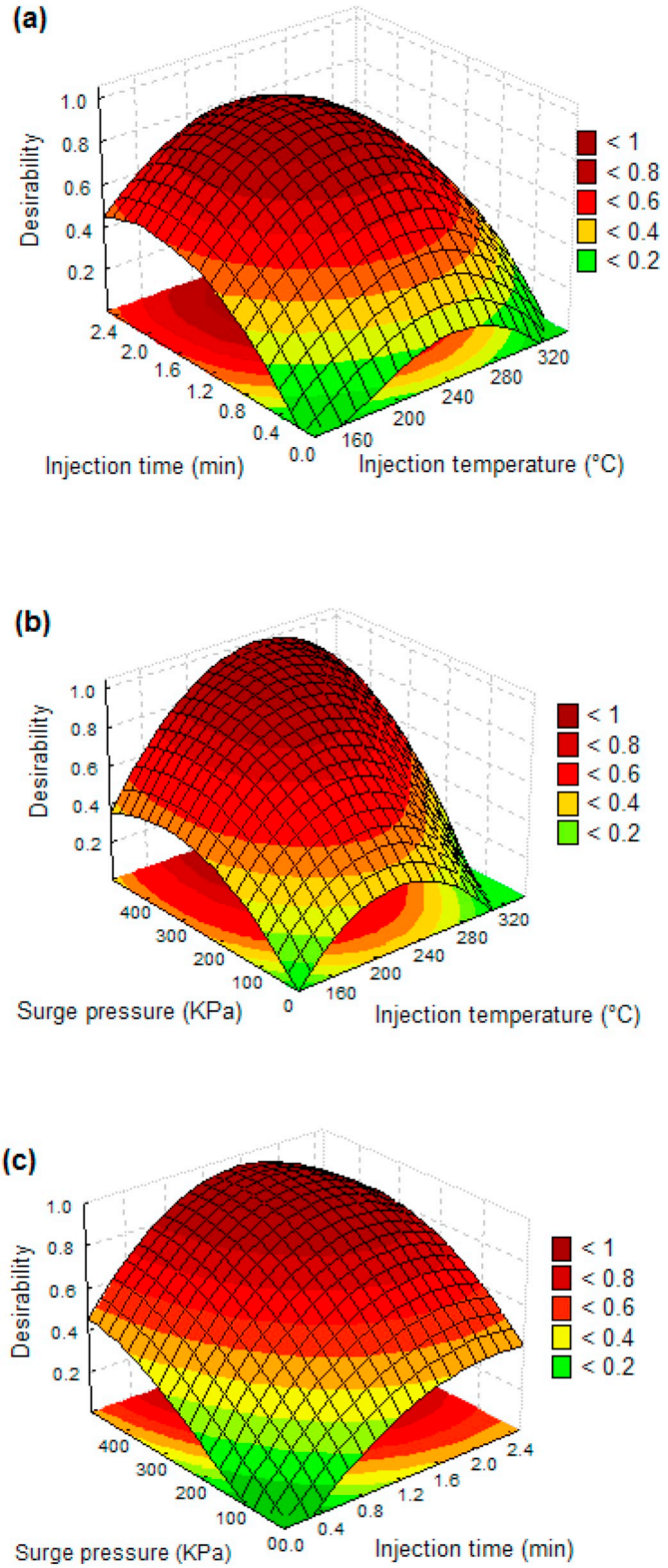

Fig. 1. Response surface and contour plots of (a) injection time and injection temperature, (b) surge pressure and injection temperature and (c) surge pressure and injection time on the overall desirability of diphenylamine, tolylfluanid, propargite and phosalone mixture response.

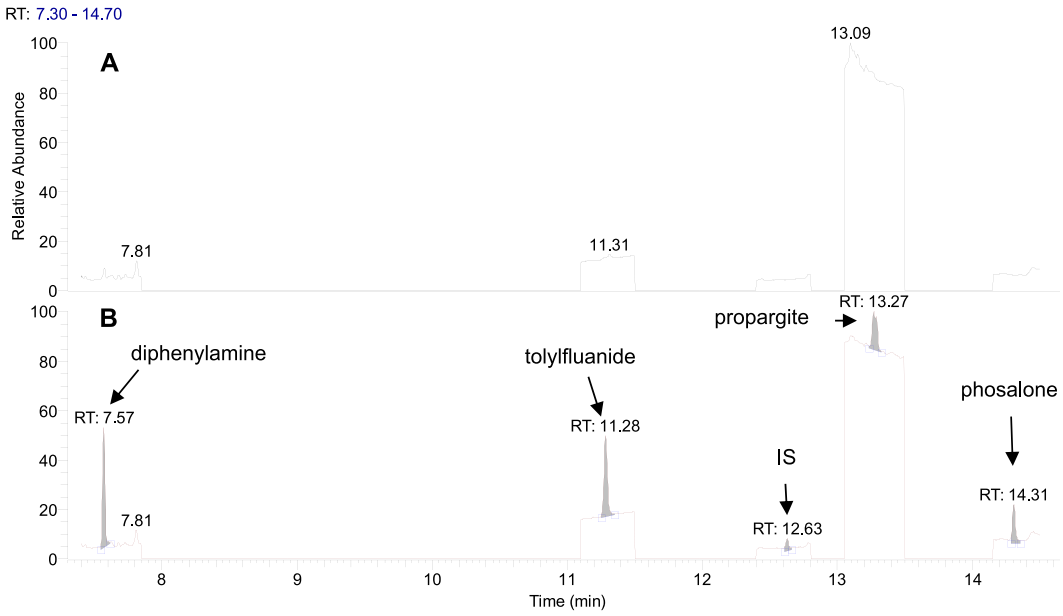

Fig. 2. Hepatocytes liver extract chromatograms, A: blank sample, B: sample at the LLOQ.

Therefore, applying the numerical optimization function from the Statistica software to the Derringer's desirability function allowed to find the following injection experimental conditions: X_1 (271.7 °C), X_2 (1.25 min) and X_3 (490 kPa); which simultaneously maximized the signals of all the compounds.

3.2. Performance of the analytical method

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

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

Table 6 lists the key elements of the method validation.

The determination coefficient values (R^2) were always > 0.999 indicating that there was a good correlation of linearity through the

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

Parameter	Diphenylamine		Tolylfluanid		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									
Intra-day	Ar%	RSD%							
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%
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%

R%: percent recovery; RSD%: percent relative standard deviation.
Ar%: intra-day percent accuracy rate; Br%: inter-day percent accuracy rate.
SCt%: cold-war percent stability; SLt%: long term percent stability; SA%: autosampler percent stability.

concentrations range used and a homoscedastic distribution of replicates at all levels. On the basis of recovery results ranging from 9560% to 99.6%, with a maximum RSD of 2.0%, the implemented sample treatment was considered satisfactory.

In addition, intra- and inter-day imprecision and accuracy exhibited acceptable values, ranging from 1.3% to 6.6% and from 92.4% to 102.9%.

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

The evaluated LLOQ for diphenylamine (422.5 µg/kg), tolylfluanid (868.0 µg/kg), propargite (876.2 µg/kg) and phosalone (919.5 µg/kg) are higher than the values published by other authors in the context of biomonitoring studies. Indeed, for diphenylamine and propargite, Marschner et al. [49] obtained an LLOQ of 166.5 µg/kg on animal liver. Moreover, the phosalone LLOQ published by Russo et al. [30] was 15 µg/kg of human liver.

4. Conclusion

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

Convenient optimization of simultaneous protein precipitation and LLE extraction conditions allowed to achieve very good recoveries and efficient sample purification using a user-friendly sample treatment. This one-step LLE and cleanup method followed by GC–MS analysis complied with current FDA requirements and showed satisfying selectivity, linearity, recovery, precision and accuracy. With the aim of applying the developed method to *in vitro* metabolism studies, the limits of quantification obtained in this work are satisfying. Considering previously published works related to human and animal liver samples, in case of using the present method for biomonitoring studies, some adaptations are needed (*i.e.* tandem mass spectrometry) in order to improve LLOQ.

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

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