

# Experimental central composite design-based dispersive liquid-liquid microextraction for HPLC-DAD determination of diazinon in human urine samples: method development and validation

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Diazinon poisoning is an important issue in occupational, clinical, and forensic toxicology. While sensitive and specific enough to analyse diazinon in biological samples, current methods are time-consuming and too expensive for routine analysis. The aim of this study was therefore to design and validate a simple dispersive liquid-liquid microextraction (DLLME) for the preparation of urine samples to be analysed for diazinon with high performance liquid chromatography with diode-array detector (HPLC-DAD) to establish diazinon exposure and poisoning. To do that, we first identified critical parameters (type and volume of extraction and disperser solvents, pH, surfactant, and salt concentrations) in preliminary experiments and then used central composite design to determine the best experimental conditions for DLLME-HPLC-DAD. For DLLME they were 800  $\mu$ L of methanol (disperser solvent) and 310  $\mu$ L of toluene (extraction solvent) injected to the urine sample rapidly via a syringe. The sample was injected into a HPLC-DAD (C<sub>18</sub> column, 250 $\times$ 4.6 mm, 5  $\mu$ m), and the mobile phase was a mixture of acetonitrile and buffer (63:37 v/v, pH 3.2; flow rate: 1 mL/min). Standard calibration curves for diazinon were linear with the concentration range of 0.5–4  $\mu$ g/mL, yielding a regression equation  $Y=0.254X+0.006$  with a correlation coefficient of 0.993. The limit of detection and limit of quantification for diazinon were 0.15  $\mu$ g/mL and 0.45  $\mu$ g/mL, respectively. The proposed method was accurate, precise, sensitive, and linear over a wide range of diazinon concentrations in urine samples. This method can be employed for diazinon analysis in routine clinical and forensic toxicology settings.

**KEY WORDS:** disperser solvent; extraction solvent; high performance liquid chromatography; liquid phase microextraction; Taguchi orthogonal array

Diazinon (O,O-diethyl O-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate) is one of the most common causes of occupational, clinical, and forensic organophosphate (OP) poisoning in the world (1–14). In biological samples it can be determined with several analytical methods for experimental, clinical, and forensic purposes (15–18), such as solid-phase extraction (SPE) followed by gas chromatography/mass spectrometry (GC/MS) in postmortem blood samples (15), high performance liquid chromatography with diode-array detector (HPLC-DAD) in serum and urine of patients with acute poisoning (16), or liquid chromatography with tandem MS in gastric content and blood for forensic toxicology (18).

Although these methods are sensitive, and specific enough to analyse diazinon in biological samples, they are too time-consuming and expensive for routine analysis. This issue has called for the development of simple, fast, low-cost, user- and environment-friendly sample preparation methods such as liquid phase microextraction (LPME), which requires a small volume of a water-immiscible solvent (19).

One of the LPME methods is the so called dispersive liquid-liquid microextraction (DLLME). It is rapid, simple, inexpensive, efficient, and requires minimal (microlitre) volumes of low- and high-density solvents for the extraction of many water-based samples (20, 21).

However, to obtain optimal efficiency (20–22), this method has to be fine-tuned through trial and error, which is time-consuming, or through statistical models and experimental designs, such as the Taguchi orthogonal array design and central composite design (CCD) (23, 24).

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The aim of this study was to make use of experimental design and develop a fast, simple, inexpensive and specific DLLME-HPLC-DAD method for the determination of diazinon in human urine samples for routine analysis in clinical and forensic toxicology laboratories.

## MATERIALS AND METHODS

### Chemicals

HPLC-grade methanol, acetonitrile, water, toluene and dichloromethane were purchased from Merck Chemical Co. (Darmstadt, Germany). HPLC-grade standards for diazinon, pirimiphos-methyl, azinphos-ethyl, and chlorpyrifos were purchased from Dr. Ehrenstofer GmbH (Augsburg, Germany). All other chemicals and reagents were of analytical grade, purchased from Merck Chemical Co.

### Instrumentation and chromatographic conditions

Separation, identification, and quantification were carried out on a Knauer HPLC system (Smartline Series 1200, Berlin, Germany). Chromatography was run isocratically on a Nucleosil® C<sub>18</sub> analytical column (250×4.6 mm, 5 µm particle size, Perfectsil® Target). An RP-18 guard column was fitted upstream of the analytical column. The mobile phase was a mixture of acetonitrile and buffer, optimised (63:37 v/v, pH 3.2) and delivered by a Knauer 1050 HPLC pump at a flow rate of 1 mL/min. A diode array detector (K-2800, Knauer) with a wavelength range of 190–740 nm was used for detection. The system was equipped with ChromGate® software (version 3.3.2., Knauer).

### Sample preparation

We used diazinon-free urine samples provided by healthy volunteers in our laboratory. They were kept frozen at -20 °C until analysis and then thawed to room temperature. Each sample was added 10 µL of pirimiphos-methyl (internal standard, IS) (2.5 µg/mL) and vortexed at 1250xg for 10 min. Then we added 100 µL of sodium lauryl sulphate (SLS) (3 % w/v) and 100 µL of sodium chloride (NaCl) (1 %,w/v) to the glass tubes containing 1000 µL of

urine. The final solution was then prepared following the DLLME procedure.

### DLLME procedure

A mixture of 800 µL of methanol (disperser solvent) plus 310 µL of toluene (extraction solvent) was quickly injected to the samples with a syringe (Hamilton, NV, USA), which dispersed fine droplets of toluene to form a cloudy solution. Over just a few seconds, the analytes were extracted on toluene droplets and after centrifugation at 1250xg for 15 min, these droplets became a supernatant on the surface of the conical test tube. The supernatant phase was then completely transferred into another conical test tube and the residue dried by evaporation with nitrogen in a water bath, dissolved to a mobile phase, and then 20 µL of the sample injected into the HPLC.

### DLLME optimisation with experimental design

To achieve maximum recovery, the selection of extraction efficiency variables was based on preliminary experiments that yielded distinct responses of eight variables on three levels of experimental designs (Table 1). Optimisation was performed on spiked samples.

### Preparation of standard solutions

Standard solutions were prepared by serially diluting the diazinon stock solution (100 µg/mL) with HPLC-grade water to 0.5, 1.0, 1.5, 2, 2.5, 3, and 4 µg/mL. The stock solution (2.5 µg/mL) of pirimiphos-methyl (IS) in methanol was prepared and stored at -20 °C. The stock and standard solutions were prepared on a daily basis and stored in the dark at 4 °C. All solutions were used on the day they were prepared.

### Experimental design

To obtain optimal conditions, we relied on the four-factor-two-level central composite design (CCD), which is used in response surface methodology. Briefly, each numeric factor is varied over five levels: plus and minus alpha (axial points), plus and minus 1 (factorial points), and the centre point. If categorical factors are added, CCD will be duplicated for every combination of the categorical factor

**Table 1** Variables and their levels for experimental design

Symbol	Level 3	Level 2	Level 1	Factor
A		methanol	acetonitril	type of disperser solvent
B		10	0	sonication duration (minute)
C	dichloromethane	chloroform	toluene	type of extraction solvent
D	600	300	100	volume of extraction solvent (µL)
E	1000	500	0	volume of disperser solvent (µL)
F	5	3	1	surfactant concentration (% w/v)
G	5	3	1	salt concentration (% w/v)
H	10	7	4	pH

levels. It was also used to investigate parabolic interactions between the following parameters: volume of extraction solvent (toluene), salt percentage (NaCl), surfactant percentage (SLS), and the volume of disperser solvent (methanol). This CCD design allowed modelling the response surface by fitting a second-order polynomial with the number of experiments equal to 21 for four factorial designs at five levels and five replicated points. Table 2 shows the range of independent variables used in this study in terms of actual and coded values.

#### HPLC method validation

Validation included the following parameters: linearity, precision, accuracy, limits of detection and quantification, and selectivity (25). For calibration we used seven concentrations ranging from 0.5 to 4 µg/mL of diazinon. Each concentration was prepared in triplicate and analysed three times. Calibration curves were constructed by plotting the concentration of compounds versus peak area response. The linearity was evaluated with the least square regression method.

The limit of quantification (LOQ) was determined during the evaluation of the linear range of calibration curve.

**Table 2** Analysis of variance for the proposed model

Source	Sum of Squares	df	Mean square	F value	p-value* Prob.> F
Model	6195.89	14	442.56	41.69	0.0053
A	2223.78	1	2223.77	206.49	0.0007
B	72.231	1	72.31	6.81	0.0797
C	726.08	2	363.04	34.2	0.0086
D	862	2	431	40.6	0.0067
E	229.05	2	114.52	10.79	0.0426
F	1029.91	2	514.95	48.51	0.0052
G	915.54	2	457.77	43.12	0.0062
H	137.22	2	68.61	6.46	0.0817
Residual	31.85	3	10.62		
Correction Total	6227.774	17			

\* all p-values are statistically significant

The limit of detection (LOD) and LOQ were calculated as follows:

$$\text{LOD}=3.3\sigma/S, \text{ and}$$

$$\text{LOQ}=10\sigma/S,$$

where  $\sigma$  is the standard deviation of response, and S is the slope of the calibration curve.

The method's precision was determined by repeatability (intra-day) and intermediate precision (inter-day) and was expressed as relative standard deviation (RSD). Five replicate injections of diazinon standard solutions were prepared at concentrations ranging from 0.5 to 4 µg/mL. The intra-day variation was assessed on the same day, while inter-day precision encompassed three consecutive days. Both assessments were carried out by the same analyst.

The accuracy of the method was tested with five replicates of three samples containing different diazinon concentrations, and the measurements were compared with

its actual concentration. The accuracy was expressed with recovery percentage.

Selectivity was evaluated by comparing chromatograms of different batches of urine spiked with diazinon, IS, tramadol, azinphos-ethyl, pirimiphos-methyl, and chlorpyrifos.

#### Data analysis

For regression analysis and diagram plotting for the experimental results we used the Design Expert v. 7.01 software (Stat-Ease Inc., Minneapolis, MN, USA).

#### Ethical approval

This project was approved by the Ethics Committee of the Legal Medicine Research Centre.

## RESULTS AND DISCUSSION

#### Results of DLLME optimisation

Sonication and pH had negative effects on maximum recovery ( $p>0.05$ ). Other parameters had a positive effect ( $p<0.05$ ) and were selected for further optimisation

(Table 3). Methanol (as disperser solvent) and toluene (as extraction solvent) had positive effects on all variables and were used in the experiments.

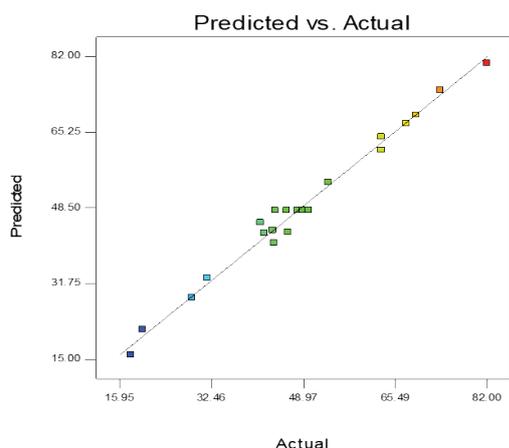
#### Response surfacing based on CCD

The actual and statistically predicted diazinon recoveries for experiments are shown in Table 4, while Figure 1 shows

**Table 3** Experimental ranges and levels of independent variables for the central composite design

			$\alpha^-$	1-	0	1+	$\alpha^+$
Methanol	µL	A	200	400	600	800	1000
NaCl	%	B	0	1	2	3	4
SLS	%	C	0	1	2	3	4
Toluene	µL	D	225	300	375	450	525

SLS – sodium lauryl sulphate



**Figure 1** Probability plot of the effects

the relationship between the two. The mathematical model was as follows:

$$(\text{Recovery})^{0.81} = +22.87 - 1.01 * A - 3.76 * B + 2.98 * C + 2.97 * D - 2.64 * AB + 2.58 * AC - 3.35 * AD - 1.56 * BC + 1.09 * A^2 - 1.79 * C^2 + 1.01 * D^2$$

This equation represents the relationship that diazinon recovery (R) has with the volume of the disperser solvent (A), salt concentration (B), surfactant concentration (C), the volume of extraction solvent (D), and their combinations (AB, AC, AD, and BC). Table 5 shows the results of the

**Table 4** Experimental conditions according to the central composite design and observed response values

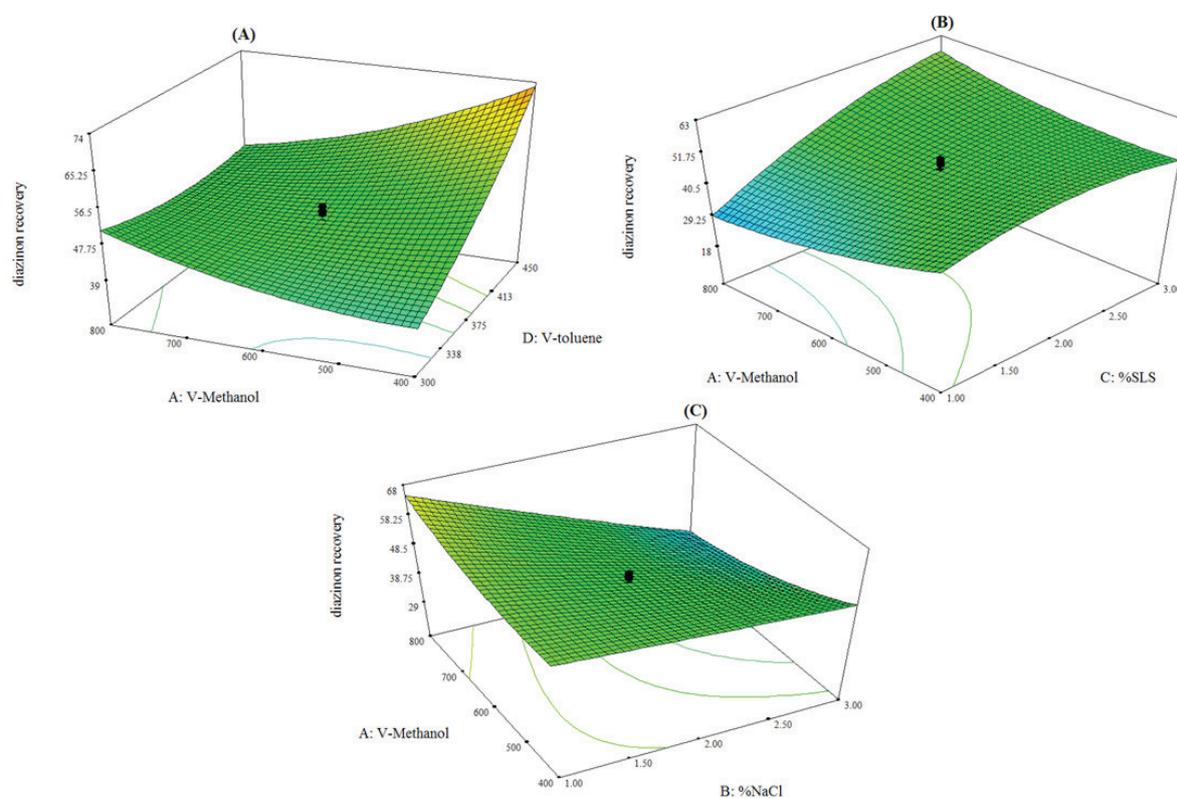
Experiment No.	Methanol volume (µL)	NaCl conc. (%w/v)	SLS conc. (%w/v)	Toluene volume (µL)	Actual recovery	Predicted recovery
1	800	3.00	3.00	300.00	43.70	40.72
2	800	3.00	1.00	300.00	20.10	21.55
3	800	1.00	3.00	450.00	82.00	80.49
4	400	3.00	1.00	450.00	69.27	68.99
5	800	1.00	1.00	450.00	43.52	43.50
6	400	1.00	3.00	300.00	46.3	43.06
7	400	3.00	3.00	450.00	63.00	61.23
8	400	1.00	1.00	300.00	31.80	32.99
9	200	2.00	2.00	375.00	63.00	64.14
10	1000	2.00	2.00	375.00	53.50	54.13
11	600	0.00	2.00	375.00	67.50	67.14
12	600	4.00	2.00	375.00	29.00	28.64
13	600	2.00	0.00	375.00	18.02	15.94
14	600	2.00	4.00	375.00	41.33	45.18
15	600	2.00	2.00	225.00	42.00	42.88
16	600	2.00	2.00	525.00	73.60	74.48
17	600	2.00	2.00	375.00	48.00	47.89
18	600	2.00	2.00	375.00	46.00	47.89
19	600	2.00	2.00	375.00	49.00	47.89
20	600	2.00	2.00	375.00	50.00	47.89
21	600	2.00	2.00	375.00	44.00	47.89

SLS – sodium lauryl sulphate

analysis of variance (ANOVA) for the CCD model and the significance of each coefficient determined by F-values (variation of data about mean value) and P-values (probability). The model turned out to be highly predictive of the experimental results. Extraction solvent volume had a high linear and quadratic effect on response. In addition, the interaction effects of combined variables were significant. The correctness of the model was also ensured by multiple correlation coefficient (R<sup>2</sup>), which was 0.9872 and showed high prediction of the actual value and excellent response, with 0.85 % of the total variation. The predicted R<sup>2</sup> (0.8624) was in reasonable agreement with the adjusted R<sup>2</sup> (0.9716). Furthermore, the coefficient of variance (CV=4.76 %) was low, which indicates significant precision and reliability of the experimental data.

For precision to be adequate, the signal-to-noise ratio should be >4. With our model it was 30.73, indicating that it could be used to evaluate experiments.

Figure 2 shows a 3D response surface diagram of the effects of two factors on diazinon recovery. Figure 2A shows significant interactions of extraction and disperser solvent volume with diazinon recovery (p<0.0002). Diazinon recovery increased with the increase in extraction solvent volume (from 300 to 450 µL) and disperser solvent volume (from 400 to 800 µL). Significant increase in diazinon



**Figure 2** Surface plots showing the effects of variables with the highest impact on the recovery of the method (A) The effect of the volume of toluene and methanol; (B) the effect of the volume of methanol and the sodium lauryl sulphate (SLS) concentration; (C) the effect of methanol volume and the sodium chloride (NaCl) concentration

**Table 5** Analysis of variance for central composite design

Source	Sum of squares	df	Mean square	F value	p-value* Prob. > F
Model	840.2	11	76.37	63.10	<0.0001
A	16.28	1	16.28	13.45	0.0052
B	112.89	1	112.89	93.28	<0.0001
C	142.35	1	142.35	117.63	<0.0001
D	70.52	1	70.52	58.27	<0.0001
AB	27.88	1	27.88	23.04	0.0010
AC	53.21	1	53.21	43.97	<0.0001
AD	44.91	1	44.91	37.11	0.0002
BC	19.36	1	19.36	16.00	0.0031
A <sup>2</sup>	31.00	1	31.00	25.62	0.0007
C <sup>2</sup>	84.41	1	84.41	69.75	<0.0001
D <sup>2</sup>	26.72	1	26.72	22.08	0.0011
Residual	10.89	9	1.21		
Lack of Fit	7.37	5	1.47	1.67	0.3196
Pure Error	3.53	4	0.88		
Cor Total	850.91	20			

\* all p-values are statistically significant

recovery was noted when the extraction solvent volume reached 310 µL and disperser solvent 800 µL.

Figure 2B shows that the interactions between diazinon recovery and disperser solvent volume and surfactant concentrations were significant ( $p < 0.0001$ ) at maximum surfactant concentration of 3 % and maximum disperser solvent volume.

Figure 2C, in turn, shows that diazinon recovery also had significant interactions with disperser solvent volume when it reached its maximum volume of 800 µL and when salt percentage was at its lowest ( $p < 0.001$ ).

*Results of method validation*

Standard calibration curves for diazinon were linear with the concentration range of 0.5–4 µg/mL, yielding a regression equation of  $Y = 0.254X + 0.006$  with a correlation coefficient of 0.993. This is generally considered evidence of an acceptable fit and good linearity over the concentration range.

The method yielded LOD and LOQ of 0.15 µg/mL and 0.45 µg/mL, respectively, and its precision met the acceptance criteria (Table 6). The intra- and inter- day RSD values did not exceed 5 % (bias interval between 3.0 and 5.0 %), which indicates that the method is accurate, reliable, and reproducible.

Table 6 also shows that the recovery percentages comply with the acceptance criteria (25).

The specificity of the method was tested with peak purity on blank and spiked urine samples. Blank samples showed no interference when diazinon and IS were added. Under optimised conditions, the separation of diazinon and pirimiphos-methyl was complete (Figure 3).

*Method application in real conditions*

The applicability of the proposed DLLME-HPLC-DAD method was evaluated in undiluted urine samples collected from patients poisoned with diazinon who were receiving hospital treatment (Sanandaj, Iran). Relative diazinon recoveries were determined at the spiking level of 0.5, 1, and 3 µg/mL. The results of six replicate experiments of each sample were in the range of 75–95.6 %. Therefore, the proposed method can be applied for determining diazinon in human urine samples.

*Comparison of the DLLME-HPLC-DAD with other methods*

Table 7 summarises a comparison of the proposed DLLME-HPLC-DAD method with other methods and shows that its LOD,  $R^2$ , and recovery are well within acceptable ranges.

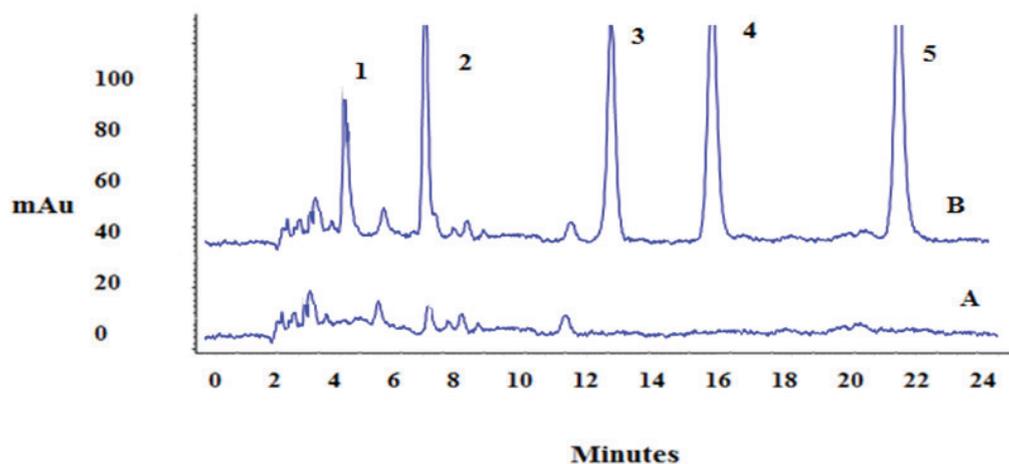
**Table 6** Method precision and accuracy (intra-day: n=5; inter-day: n=5 series per day, 3 days).

Diazinon concentration (µg/mL)	Intra-day (n=5)			Inter-day (n=5)		
	Mean±SD	CV (%)	Recovery±SD (%)	Mean±SD	CV (%)	Recovery±SD (%)
0.5	0.46±0.04	7.4	92.1±1.0	0.48±0.03	6.9	95.6±1.0
1	0.76±0.02	3.3	76.0±2.0	0.75±0.01	1.2	75.0±1.1
3	2.33±0.09	4.0	77.4±0.9	2.31±0.11	4.9	77.1±1.4

**Table 7** Comparison of the proposed DLLME-HPLC-DAD with other analytical methods for determination of diazinon in biological samples

Method	Matrix	LOD (µg/mL)	Correlation coefficient ( $R^2$ )	Recovery (%)	Ref. No.
SPE-GC-MS	whole blood	0.15	0.9981	78–87	15
SPE-HPLC-DAD	plasma	0.15	0.998	77.7–86.3	17
LLE-HPLC-DAD	whole blood, serum, urine	0.78	0.9996	97.4–99.01 (for blood and serum) 101.1–101.4 (for urine)	16
mini-QuEChERS-LC-MS-MS	whole blood, gastric content	0.1	0.95	80–100	18
MEPS-GC-MS-MS	whole blood	0.5	0.99	61–77	26
DBS-GC-MS-MS	whole blood	0.05	0.998	4.56–5.11	27
DLLME-HPLC-DAD	urine	0.15	0.993	75.0–95.6	this study

*SPE-GC-MS – solid-phase extraction and gas chromatography/mass spectrometry; SPE-HPLC-DAD – solid-phase extraction and high-performance liquid chromatography (HPLC) with diode array detector (DAD); LLE-HPLC-DAD – liquid-liquid extraction and high-performance liquid chromatography (HPLC) with diode array detector; mini-QuEChERS-LC-MS-MS – modified quick, easy, cheap, effective, rugged and safe (QuEChERS) method – liquid chromatography with tandem mass spectrometry; MEPS-GC-MS-MS – microextraction by packed sorbent (MEPS) – gas chromatography-tandem mass spectrometry; DBS-GC-MS-MS – dried blood spot (DBS) – gas chromatography coupled to tandem mass spectrometry; DLLME-HPLC-DAD – dispersive liquid- liquid phase microextraction-high performance liquid chromatography with diode array detector*



**Figure 3** Specificity of the proposed method for the analysis of diazinon in urine sample

Chromatogram A: blank urine; Chromatogram B: urine spiked with: 1 – tramadol, 2 – azinphos-ethyl, 3 – diazinon, 4 – pirimiphos-methyl, and 5 – chlorpyrifos

## CONCLUSION

Our findings evidence that our DLLME-HPLC-DAD is a rapid and simple extraction and determination method for diazinon in human urine samples. It overcomes the limitations of conventional sample preparation methods that involve the use of large volumes of expensive and toxic organic solvents. However, it is evident that further studies are necessary for different biological specimen in order to suppress matrix effects and enhance extraction recoveries. The proposed DLLME-HPLC-DAD method is simple, cheap, accurate, and sensitive enough to be applied in clinical and forensic toxicological analysis.

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### Conflicts of interest

None to declare.

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### Disperzivna tekućinsko-tekućinska mikroekstrakcija temeljena na eksperimentalnom centralnom kompozitnom dizajnu u svrhu određivanja diazinona u ljudskoj mokraći: razvoj i validacija metode

Trovanje diazinonom važan je problem za medicinu rada te kliničku i forenzičnu toksikološku praksu. Premda su postojeće metode njegova utvrđivanja u biološkim uzorcima dovoljno osjetljive i specifične, njihova je primjena za rutinske analize preskupa i dugotrajna. Zbog toga je cilj ovoga istraživanja bio razviti i validirati jednostavnu metodu disperzivne tekućinsko-tekućinske mikroekstrakcije (engl. *dispersive liquid-liquid microextraction*, krat. *DLLME*) kojom bi se uzorci ljudske mokraće pripremili za analizu diazinona tekućinskom kromatografijom visoke djelotvornosti s detektorom s nizom fotodioda (engl. *high performance liquid chromatography with diode-array detector*, krat. *HPLC-DAD*) radi utvrđivanja izloženosti i trovanja diazinonom. U tu smo svrhu u preliminarnim eksperimentima prvo odredili ključne parametre: vrstu i volumen ekstrakcijskoga i disperzijskoga otapala, pH, površinski aktivne tvari (surfaktanta) te koncentraciju soli. Zatim smo s pomoću eksperimentalnoga centralnoga kompozitnoga dizajna utvrdili optimalne eksperimentalne uvjete za *DLLME-HPLC-DAD*. Za *DLLME* oni su bili 800  $\mu$ L metanola (kao disperzijskoga otapala) te 310  $\mu$ L toluena (kao ekstrakcijskoga otapala) za brzu injekciju uzorka mokraće. Uzorak je injektiran u *HPLC-DAD* (5-mikrometarski analitički stupac C18, 250 $\times$ 4,6 mm), a mobilna je faza bila mješavina acetonitrila i pufera (63:37 v/v, pH 3.2; protok: 1 mL/min). Standardne su kalibracijske krivulje za diazinon bile linearne s rasponom koncentracija diazinona od 0,5 do 4  $\mu$ g/mL, a regresijska jednadžba  $Y=0,254X+0,006$  s koeficijentom korelacije 0,993. Granice detekcije odnosno kvantifikacije diazinona bile su 0,15  $\mu$ g/mL odnosno 0,45  $\mu$ g/mL. Ova se metoda pokazala točnom, preciznom, osjetljivom i linearnom u širokom rasponu koncentracija diazinona u uzorcima mokraće, stoga se može koristiti za njegovu rutinsku analizu u kliničkoj i forenzičnoj toksikološkoj praksi.

**KLJUČNE RIJEČI:** disperzijsko otapalo; ekstrakcijsko otapalo; mikroekstrakcija tekućinskom fazom; tekućinska kromatografija visoke djelotvornosti; Taguchijev ortogonalni niz