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Extraction and determination of 2-pyrazoline derivatives using liquid phase microextraction based on solidification of floating organic drop

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ABSTRACT

A simple, rapid and efficient microextraction method for the extraction and determination of some 2-pyrazoline derivative compounds in aqueous samples was developed. Microliter volumes of 1-undecanol were delivered to the surface of the aqueous sample and the sample was agitated for a desire time. The sample vial was cooled by inserting it into an ice bath for 5 min. The solidified solvent was transferred into a suitable vial and immediately melted. One μL of the organic solvent was injected into a gas chromatography (GC) for analysis. Several factors affecting the microextraction efficiency such as sampling temperature, stirring rate, pH, nature and volume of the organic solvent and extraction time were investigated and optimized values were obtained as 70 °C, 1250 rpm, 5.0, 8.0 μL (1-undecanol) and 30 min, respectively. Under the optimal conditions, detection limits of the method for determination of the compounds were in the range of 5–10 $\mu g L^{-1}$. The relative standard deviations (RSDs%) for the extraction and determination of the analytes at the concentration level of 250 $\mu g L^{-1}$ were in the range of 3.0–11.4. Dynamic linear ranges of 25–800 $\mu g L^{-1}$ with correlation coefficients in the range of $0.9857 < r^2 < 0.9968$ were observed. After 30 min of extraction duration, the enrichment factors varied from 183 to 538. Finally, the study was applied to the determination of the compounds in several real samples including serum and urine and satisfactory results were obtained.

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1. Introduction

Compounds containing pyrazole ring are extensively used in pharmaceutical, agrochemical, food and cosmetic industries as well as being used as complexing agents for the synthesis of hydrogenation catalysts and UV stabilizers [1–5]. Moreover, pyrazolines and pyrazoles play a crucial role in the development of theory in heterocyclic chemistry and also are extensively used as useful synthons in organic synthesis [6]. 2-Pyrazolines and the corresponding pyrazoles have been shown to possess antitumor, anesthetic, analgesic, tranquilizer, muscle relaxant, antidepressant, neuroleptic, hypnotic, anticonvulsant, anti-inflammatory, antipyretic, antidiabetic, antiarrhythmic, antibacterial, antifungal, antiparasitic, antituberculosis, and insecticidal activities [7–9].

Sample pretreatment is usually necessary in order to extract, isolate and concentrate analytes of interest from complicated matrices to obtain samples compatible for instrumental analysis. Conventional liquid–liquid extraction (LLE), offering high reproducibility and high sample capacity, is the most widely used sample pretreatment method for liquid samples [10]. LLE requires large amounts of expensive and toxic solvents, resulting in the production of hazardous laboratory waste [11].

In the last decade, some interests have been focused on the miniaturizing of analytical LLE, termed by liquid-phase microextraction (LPME) [12–18]. LPME was first introduced in 1996, based on a microdroplet of an organic solvent hanging at the end of a microsyringe needle. The organic microdroplet was immersed in the aqueous sample and the analytes were extracted into the organic droplet driven by diffusion and distribution coefficient of the analytes as well. Subsequently, the organic droplet was withdrawn into the microsyringe and transferred into GC injection port for final analysis. Using LPME, high pre-concentration factors may be achieved for the analytes with high partition coefficients, because the sample volume is much larger than the microdroplet volume. The present study reports a novel LPME of some 2-pyrazoline derivatives namely as 1,3,5-triphenyl-4,5-dihydro-1H-pyrazole (**A**), 5-(2-pyridyl)-1,3-diphenyl-4,5-dihydro-1H-pyrazole (**B**), 5-(3-methyl phenyl)-1,3-diphenyl-4,5-dihydro-1H-pyrazole (**C**) and 5-(4-methyl phenyl)-1,3-diphenyl-4,5-dihydro-1H-pyrazole (**D**) [19]. To the best of our knowledge, LPME based on solidification of floating organic drop have not been employed previously for the extraction and

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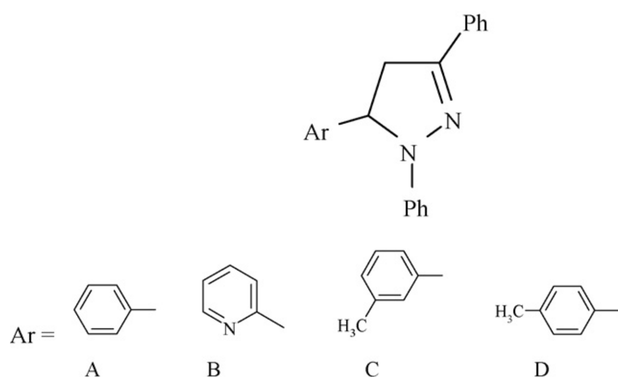

Fig. 1. The chemical structures and their corresponding log *P* values of 2-pyrazoline derivatives ((**A**, log *P* = 3.8 ± 0.6; **B**, log *P* = 2.3 ± 0.6; **C**, log *P* = 4.3 ± 0.6; **D**, log *P* = 4.3 ± 0.6; calculated by ACD lab software Copy right 1994–1997).

concentration of the mentioned compounds from aqueous samples.

2. Experimental

2.1. Chemicals

The 2-pyrazoline derivatives were synthesized and then purified (the purities were greater than 97%). The chemical structures and their corresponding log *P* values (octanol–water partitioning coefficient) are shown in Fig. 1. Stock standard solutions (1000 mg L^{-1}) were prepared in acetone. All of the standard solutions were kept in the fridge at 4 °C. 1-Undecanol (98%, bp: 248–250 °C), 1-dodecanol (98%, bp: 261–263 °C), 2-dodecanol (95%, bp: 249–250 °C), *n*-hexadecane (98%, bp: 283–286 °C), reagent grade acetone and sodium chloride were purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland) Companies. Double distilled water was used for preparing the working solution.

2.2. Apparatus

Injections of the solutions into gas chromatograph were carried out using a 5-μL SGE microsyringe (Code: 5B-7, Switzerland). Stirring of the solution was carried out using a Heidolph MR 3001 K magnetic heater–stirrer (Kelheim, Germany) and an 8 mm × 4 mm stirring bar. A simple water bath was placed on the heater–stirrer to control the temperature of the samples. Separation and identification of the compounds were performed using a Shimadzu 17-A GC (Tokyo, Japan) equipped with a flame ionization detector (FID) and CPB-5 fused-silica capillary column (25 m × 0.22 mm I.D. and 1.5 μm film thicknesses). The injector and detector temperatures both were set at 270 °C. The GC split ratio was 1:10 and helium (purity 99.999%) was used as carrier gas to give the column flow of 1 mL min^{-1}. The column temperature was held at 200 °C for 1 min, then raised to 250 °C at 5 °C min^{-1} and held for 30 min. The analytical signal was taken as the relative peak area ratio of the compounds to the internal standard and the overall response was shown in each diagram.

2.3. Extraction procedure

A 300 μg L^{-1} solution of the compounds, prepared in double distilled water, was used in the extraction studies. Twenty mL of an aqueous solution containing the compounds was transferred into a 21 mL vial and the desired volume of 1-undecanol placed on the surface of solution using a microliter syringe. Then, the vial was sealed and the stirrer turned on. Once the desired extraction time reached, the sample vial was transferred into an ice beaker and the organic solvent was solidified after 5 min. The solidified solvent was then transferred into the conical vial by a simple spatula where, it started to melt [20]. Finally, 1.0 μL of the extractant was injected into GC for quantification.

A detailed description of the equipment and operating procedures has been given previously [20].

3. Results and discussion

The equations describing the effects of several parameters on the efficiency of the proposed LPME method are similar to those of liquid–liquid extraction equations. Thermodynamic and kinetic equations of liquid–liquid extraction are as follows:

$$C_{o.f} = KC_{aq.f} = \frac{KC_{aq.i}}{(1 + KV_o/V_{aq})} \quad (1)$$

$$\frac{dC_o}{dt} = \frac{A_i\beta}{V_o(KC_{aq} - C_o)} \quad (2)$$

where $C_{o.f}$ is the final concentration of the analyte in the organic phase; $C_{aq.f}$ and $C_{aq.i}$ are the final and initial analyte concentrations in the aqueous phase, respectively; V_o and V_{aq} are the organic and aqueous phase volumes, respectively; K is the distribution coefficient; C_o and C_{aq} are the analyte concentrations in the organic and aqueous phases at the time t, respectively; A_i is the interfacial area and β is the overall mass transfer coefficient with respect to the organic phase [14,21].

3.1. Selection of extracting solvent

The organic solvent used as the extracting solvent in this method has to satisfy several criteria:

(1) It should be immiscible with water; (2) it should be low volatile to prevent loss of the solvent during the extraction process; (3) it should provide an appropriate extraction efficiency to provide high extraction recoveries and thus high enrichment factor; (4) its chromatographic peaks should be separated from the compounds peaks; and (5) its melting point should be near room temperature (10–30 °C).

Accordingly, several extracting solvents such as 1-undecanol (melting point, mp = 13–15 °C), 1-dodecanol (mp = 22–24 °C), 2-dodecanol (mp = 17–18 °C) and *n*-hexadecane (mp = 18 °C) were investigated. Based on the obtained results, 1-undecanol was found to get the best extraction efficiency, while its chromatographic peak was easily separated from the analyte peaks. Also because of its low vapor pressure at the extraction conditions, the extractant was stable at the extraction period. Therefore, 1-undecanol was selected as the extraction solvent. When it comes to compare the polarity of the selected solvent with the analytes, it seems compound **B** has more similarity than the others, possibly due to the formation of inter molecular hydrogen bonding. It means that the less log *P* of the compounds were; the more they would be extracted. One of the peaks resulting from the impurities of the extracting solvent was used as the internal standard [20].

3.2. Effect of organic solvent volume

The effect of the organic solvent volume on the analytical signal was studied in the range of 6.0–14.0 μL. Fig. 2 shows that the analytical signals of the compounds increased slowly by increasing of the solvent volume in the range of 6–8 μL. Then, it decreased when solvent volume increased to 14.0 μL. Based on LLE equations; the rate of the analytes transport into microdrop is directly related to the interfacial area between the two liquid phases and inversely related

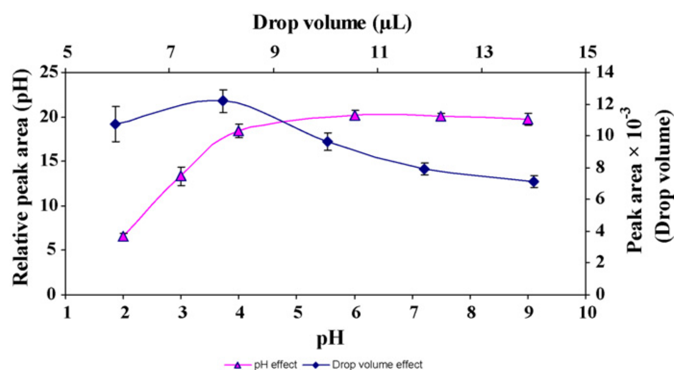

Fig. 2. The effect of organic solvent volume and pH on the extraction efficiency. *Conditions*: sample solution temperature, 70 °C; stirring rate, 1250 rpm; sample volume, 20 mL; extraction time, 30 min and without salt addition.

to the organic-phase volume. Thus, by increasing the drop volume, the effect of the interfacial area predominates and the analytical signals increase. By further increasing of the microdrop volume, the effect of the solvent volume predominates and the analytical signals decrease. Thus, in further experiments 8 μL of the extracting solvent was floated on surface of the aqueous solution.

3.3. Effects of pH and salt addition

The effect of pH of the sample solution on the extraction efficiency was tested in the range of 2–9. As shown in Fig. 2, the extraction efficiency decreased at low pH values (pH < 4) because of protonation of the compounds. So, the pH of the solutions was adjusted at pH > 6 in the subsequent studies.

To study the effect of salt addition on the extraction efficiency, the concentrations of NaCl were changed in the range of 0.0–2.0 M while the concentrations of the compounds were kept at the level of 300 μg L^{-1}. The results showed that extraction efficiency of the compounds decreased with an increase in the ionic strength as shown in Fig. 3. In the presence of salt, diffusion of the analytes towards the organic solvent becomes more and more difficult [22]. Also NaCl dissolved in water might have changed physical properties of the Nernst diffusion film and reduced the rate of diffusion of the target analytes into the drop [17]. Hence, further extractions were performed without adding NaCl to the solutions.

3.4. Extraction time

To increase the precision and sensitivity of the LPME method, it is necessary to select an exposure time that guarantees the equilibrium between the aqueous and organic phases. A series of experiments were performed and the extraction time profile was obtained by plotting the relative peak area against the extraction time evaluated in the range of 20–40 min. As Fig. 3 shows, the relative peak areas increased by increasing of the exposure time up to 30 min and then remained constant. Thus, the exposure time of 30 min was selected for the subsequent experiments.

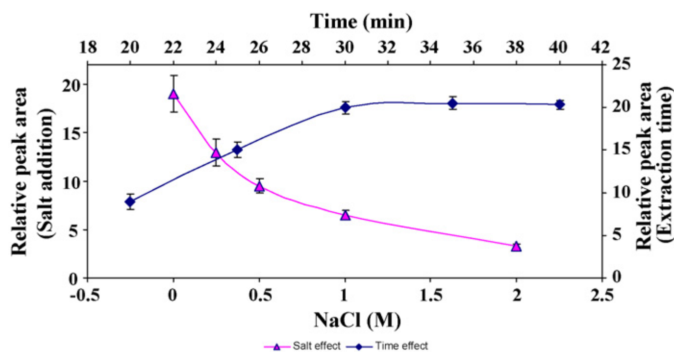

Fig. 3. The effect of salt addition and extraction time on the extraction efficiency. *Conditions*: sample solution temperature, 70 °C; organic solvent volume, 8 μL; sample volume, 20 mL; stirring rate: 1250 rpm.

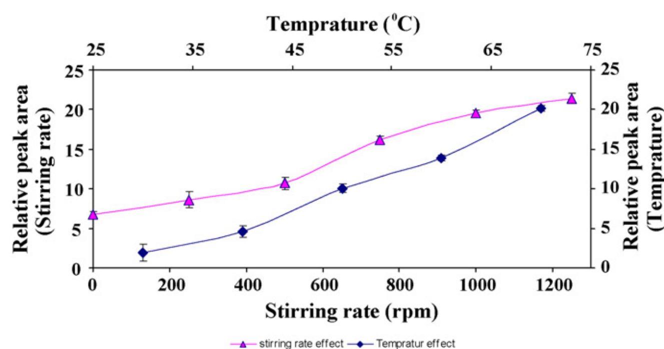

Fig. 4. The effect of stirring rate and aqueous sample solution temperature on the extraction efficiency. *Conditions*: organic solvent volume, 8 μL; sample volume, 20 mL; extraction time, 30 min and without salt addition.

3.5. Effect of stirring rate

Agitation of the sample solution enhances the rate of extraction. The stirring speed has a direct influence on extraction efficiency in limited times due to increasing mass transfer into the organic drop. In this work, the samples with a volume of 20 mL were stirred at different stirring rates (0, 250, 500, 750, 1000, 1250 rpm) on a stirrer plate. According to Fig. 4, the relative peak area increases with increasing of the stirring rate up to 1250 rpm which is the highest stirring rate attainable with the stirrer. Hence, a stirring rate of 1250 rpm was chosen for further studies.

3.6. Effect of sample solution temperature

Generally, by increasing temperature, higher enrichment factors can be obtained in LPME experiments [17]. As the sample solution temperature increases the viscosity of the organic drop decreases. As a result, mass transfer of the analytes from the surface of the aqueous sample into the bulk of organic drop, which is very crucial in the kinetic of the extraction, increases so, does the extraction efficiency. The effect of sample solution temperature on the extraction efficiency was studied in the range of 30–70 °C by floating 10 μL drops of 1-undecanol for 30 min on the surface of the aqueous samples containing 300 μg L^{-1} of the compounds. Fig. 4 shows that by increasing the sample solution temperature, the extraction efficiency increased. At high temperatures (>70 °C), over-pressurization of the sample vial made the extraction system unstable. Thus, in the subsequent experiments, the sample vial temperature was held at 70 °C.

3.7. Method performance

The preconcentration factor (PF) can be calculated based on the following equation:

$$PF = \frac{C_{o.f}}{C_{aq.i}} \tag{3}$$

where, $C_{aq.i}$ was selected as 150 μg L^{-1} and $C_{o.f}$ calculated from a suitable calibration curve which was obtained from the direct

Table 1
Figures of merit of the proposed method in determination of the compounds

Drug	DLR (μg L^{-1})	Regression equation	r^2	LOD (μg L^{-1})	PF	Percent extracted
A	25–600	$A = 0.0042C + 0.2857$	0.9968	5	234	9.4
B	25–800	$A = 0.0127C - 0.1395$	0.9922	10	538	21.5
C	25–300	$A = 0.0038C + 0.2087$	0.9922	5	183	7.3
D	25–300	$A = 0.0064C + 0.2070$	0.9857	5	230	9.2

injection of the solutions of standards in 1-undecanol into GC. Preconcentration factors in the range of 183–538-fold were achieved for the compounds (Table 1). Also percent extractions of the compounds were calculated based on the following equation:

$$\text{Percent extraction} = 100 \times \frac{C_{\text{o.f}} \times V_{\text{o}}}{C_{\text{aq.i}} \times V_{\text{aq}}} \tag{4}$$

The percent extractions were obtained in the range of 7.3–21.5%, which is also shown in Table 1. The dynamic linearity of the proposed method was investigated in the concentration range of 25–800 μg L^{-1} and good linearities with correlation coefficients (r^2) in the range of 0.9857–0.9968 were observed. The corresponding regression equations, correlation coefficients, dynamic linear ranges (DLRs) and the limit of detections (LODs) were calculated and summarized in Table 1. The limit of detections, defined as the analytical signal which is larger than the blank by multiple three of the variation in the blank, were in the range of 5–10 μg L^{-1} (Table 1).

Applicability of the extraction method was investigated in four different spiked aqueous samples. Tap water sample was collected freshly from our laboratory (University of Tehran, Tehran, Iran) and human urine samples obtained from two healthy males. Iranian Blood Transfusion Organization (Tehran, Iran) was the supplier of the serum sample as well. It is noteworthy that the strategy of two and twenty time dilution was performed for the urines and serum samples, respectively. All the mentioned samples were filtered through 0.45 μm-pore-size cellulose acetate membrane filters prior to the extraction.

The results of relative standard deviations (RSDs) for LPME of the compounds from the real samples based on four replicate measurements are shown in Table 2. The data demonstrated a good recovery in the range between 89 and 115%. Finally, the relative standard deviations for the compounds determination in the examined real samples were located in the range of 3.0–11.4% (Table 2).

Table 2
Results obtained from analysis of some real samples

Sample[a]	A	B	C	D
Tap water				
Concentration[b]	282	245	248	245
Recovery (%)	113	98	99	98
R.S.D. (%)	6.2	3.0	7.2	5.7
Urine 1				
Concentration	222	280	235	230
Recovery (%)	89	112	94	92
R.S.D. (%)	3.2	3.3	8.0	11.4
Urine 2				
Concentration	252	245	265	277
Recovery (%)	101	98	106	111
R.S.D. (%)	8.0	7.5	8.0	6.4
Serum				
Concentration	287	257	242	240
Recovery (%)	115	103	97	96
R.S.D. (%)	3.8	6.2	9.0	11.3

[a] To each sample the concentration level of 250 μg L^{-1} was added.
[b] μg L^{-1}.

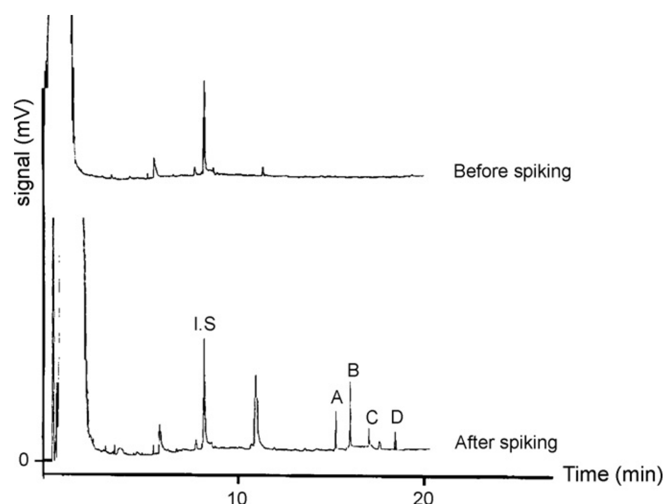

Fig. 5. The LPME–GC–FID chromatograms resulted from the extraction of the compounds at the optimum conditions before and after spiking of the urine sample with 50 μg L^{-1} of the compounds. Column temperature programming was set at 200 °C for 1 min, then raised to 250 °C at 5 °C min^{-1}, held for 30 min. **A**: 1,3,5 triphenyl-4,5-dihydro-1H-pyrazole; **B**: 5-(2-pyridyl)-1,3 diphenyl-4,5-dihydro-1H-pyrazole; **C**: 5-(3-methyl phenyl)-1,3 diphenyl-4,5-dihydro-1H-pyrazole; **D**: 5-(4-methyl phenyl)-1,3-diphenyl-4,5-dihydro-1H-pyrazole and **I.S.**: the internal standard.

Fig. 5 depicts chromatograms of the compounds at the spiked concentration level of 50 μg L^{-1} in the urine sample before and after spiking.

4. Conclusions

A modified, simple and flexible method of liquid-phase microextraction referred to as solidification of floating organic drop was applied in the present work. Since fresh organic solvent was used for each extraction, there was no memory effect, and since the volume of organic phase was only 8.0 μL, large preconcentration factors were achieved. Furthermore, because no special apparatus was required for holding the organic solvent, it was safe to stir the sample solution at the highest stirring speed attainable with the stirrer (about 1250 rpm) and several extraction vials can be stirred simultaneously.

The major advantages of the present method in comparison with LLE and other similar methods are as follows:

(1) LLE requires large volumes of organic solvents to perform. Also, recent concerns about the hazardous nature of many commonly used solvents, the costs and environmental dangers of waste solvent disposal are serious problems. While, the proposed method consumes only 8.0 μL of the organic solvent for each extraction allowing using different variety of expensive solvents which is not logically practical due to high cost of the operation in LLE.
(2) LLE results in a dilute extract leading to the sharp rise in cost and time of the analysis. On the other hand, the analysts are much more subjected to toxic and hazardous organic solvents, while in the proposed method the above mentioned set backs are significantly minimized.
(3) Most of similar methods are time consuming and labor intensive, while the proposed method is very simple, fast and easy to operate using least utilities.

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