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Determination of Phenol and Carvacrol in Honey Samples Using Dispersive Liquid-Liquid Microextraction and Experimental Design for Optimization

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A very simple, rapid and sensitive dispersive liquid-liquid microextraction (DLLME) followed by gas chromatography and flame ionization detection (GC-FID) was developed for the determination of phenol and carvacrol in honey samples. A mixture of 100 μl dichloromethane (extraction solvent) and 0.5 ml acetonitrile (disperser solvent) was rapidly injected into sample solution. Thereby a cloudy solution was formed. After centrifuging, the fine droplets of extraction solvent were sedimented in the bottom of the conical test tube. Sedimented phase (0.6 μl) was injected into the GC-FID system. Experimental parameters which control the performance of DLLME, such as type and volumes of extraction and disperser solvents, pH, salt effect and extraction time were investigated. Under optimum conditions obtained by the response surface methodology, the method was found to be linear in the range of 10-200 mg l^{-1} . The limits of detection for phenol and carvacrol were 4.15 and 3.9, respectively, and the extraction recovery ranged from 67-97.3%.

Keywords: Phenol, Carvacrol, Dispersive liquid-liquid microextraction, GC-FID

INTRODUCTION

Phenolic compounds are a group of substances that are important in food hygiene due to their bactericidal effects. It is generally accepted that a natural product is healthier than that of synthetic origin. However, this question is still open for discussions, and many studies have been carried on this subject [1,2]. Nevertheless, some natural products have been used in folk medicine since ancient times, and it is important to ascertain the origin of biological activity to apply products for human wellbeing.

Honey was selected for the analysis because it has been produced in many countries since olden times. It is the most popular and easiest available bee product highly appreciated by people. Honey consists of carbohydrates, amino acids, proteins, organic acids, vitamins, minerals and various phytochemicals [3]. It is rich in phenolic acids and flavonoids, which exhibit a wide range of biological effects and act as natural antioxidants [4]. Honey was found to exhibit radical scavenging capacity [5], anticancer and

wound healing properties [6]. The composition of honey phenolic compounds depends on the floral source used to collect nectar, seasonal and environmental factors, geographic origin, storage conditions [7,8]. The variation of the botanical composition of honey is very wide; some honeys have a stronger biological activity than others [9].

The main phytochemicals reported in honey are phenolic compounds. The total content of phenolic compounds depends on botanical source of honey and collection region [10]. Spanish plan for residue control and healthy food (Plan CREHA) of 2004 has established a maximum limit for phenol in honey at 300 ng g^{-1} [11]. Different chromatographic methods have been used for the determination of thymol and carvacrol in various matrixes. Abu-Lafi *et al.* reported the determination of thymol and carvacrol from the leaves of wild Palestinian *Majorana syriaca* [12]. The method of quantitative thin-layer chromatography (TLC) combined with densitometry has been used to determine thymol and carvacrol content of *Oleum thyme* essential oil [13]. Vinas and co-workers have determined phenol, thymol and carvacrol in honey using high performance liquid chromatography with fluorimetric

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detection [14]. These compounds have been also determined using gas chromatographs equipped with flame ionization [15,16] and mass spectrometric detection [17].

In this research factorial design was chosen to determine the most significant factors (volume of disperser and extraction solvent and pH). The chemometric approach relies on a rational experimental design, which allows the simultaneous variation of all experimental factors, saving time and material, with minimum number of test runs. With factorial design it is possible to determine the main effect as well the interactive effects of the selected factor.

The analysis of the response surfaces can be done in several ways. The most immediate way of concluding the optimum conditions is the graphical inspection of the surfaces, since the 3D pictures give the complete overview of the systems. The aim of the present work was optimizing a DLLME method followed by GC-FID for the extraction and determination of phenol and carvacrol in honey samples. Response surface methodology (RSM) is a collection of statistical and mathematical techniques successfully used for developing, improving and optimizing processes. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions. Therefore, it is less laborious and time-consuming than other approaches required to optimize a process. Unlike the conventional empirical method, RSM can generate a mathematical model, and take into account the possible inter relationship among the test variables while minimizing the number of experiments [18].

EXPERIMENTAL

Reagents and Standards

Phenol, carvacrol (2-isopropyl-2methylphenol), methanol (HPLC grade), Ethanol (HPLC grade), acetone (HPLC grade), acetonitrile (ACN) (HPLC grade), sodium chloride were supplied by Merck (Darmstadt, Germany).

Instrumentation

The analysis was performed with a gas chromatograph (GC-17 Shimadzu, Japan) equipped with a flame-ionization detector (GC-FID) and a BP5 capillary column (25 m × 0.32 mm I.D., film thickness 0.5 μm). Helium (99.999%) was

used as the carrier gas. The inlet was operated in split mode with a split ratio of 20:1. The oven temperature was programmed as follows: initial 90 °C (held for 1.0 min) ramped at 15 °C min⁻¹ to 180 °C (held for 6.0 min) and then was raised to 270 °C at 80 °C min⁻¹ (held for 5 min). The temperatures of injector and detector were set at 280 °C and 300 °C, respectively.

GC-MS analyses were performed using an Agilent gas chromatograph 7890A (Agilent, Little Falls, DE, USA) coupled with an electronically controlled split/split less injection port and interfaced to a MSD-5975C mass selective detector. The gas chromatograph was equipped with a DB-5MS fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) purchased from J & W Scientific (Folsom, CA, USA). Helium (99.9999%) was used as carrier gas, with a flow rate of 1 ml min⁻¹. The oven temperature was programmed as follows: initial temperature 40 °C (held for 1 min), ramped at 50 °C min⁻¹ to 250 °C (held for 10 min). The injection was performed at 280 °C in the split mode (ratio 20:1). The transfer line and ion source were set at 280, 250 and 200 °C, respectively. The mass spectra were taken in total-ion-scanning (TIC) mode and the electron impact energy was set at 70 eV. Mass range was from m/z 50-600 amu. The identification of analytes was performed by matching its retention time against that of the standards and GC-MS.

The pH measurements were made with a 780 pH meter (Metrohm, Switzerland) equipped with a combined Ag/AgCl glass electrode. The centurion scientific centrifuge (model K280R, UK) was used for centrifuging.

DLLME Procedure

5.0 ml of sample solution containing 2 mg l⁻¹ phenol and carvacrol was poured in a screw cap test tube with conic bottom. Acetonitrile (0.5 ml) as disperser solvent containing 100 μl of dichloromethane (as extraction solvent) was injected rapidly into the sample solution using a 1.00 ml microsyringe (100 μl, F-LC, SGE, Australia). The mixture was gently shaken, a cloudy solution was formed, then the mixture was centrifuged for 5 min at 2000 rpm which resulted in sedimentation of the fine dichloromethane droplets in the bottom of the conical test tube. Sedimented phase (0.6 μl) was injected into the GC injection port for further analysis.

Preparation of Standard Solutions and Samples

Stock standard solution of two analytes (100 mg l^{-1}) was prepared in CH_2Cl_2 . Working solutions were prepared by appropriate dilution of the stock standard solution. All solutions were stored in the dark. 30 g of honey were diluted with 200 ml of double distilled water in a beaker. The solution was homogenized over 30 min using a magnetic stirring bar, then filtered and subjected to the above mentioned DLLME-GC-FID.

Experimental Design and Statistical Analysis

Response surface methodology was applied to determine the optimized extraction conditions for phenol and carvacrol from honey samples. Statistical analysis was performed using the Minitab 16 (Minitab Inc., State College, PA, USA) software and fitted to a second-order polynomial regression model containing the coefficient of linear, quadratic and interaction terms. Central composite design (CCD) was used to investigate the effects of three independent variables (pH, volume of extraction solvent (μl) and volume of disperser solvent (ml)) at three levels on phenol and carvacrol response. Each variable was coded at three levels, -1, 0 and +1. The quadratic model for each response was as follows:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^k \beta_{ij} X_i X_j \quad (1)$$

where β_0 , β_i , β_{ii} , β_{ij} are regression coefficients for intercept, linear, quadratic and interaction terms, respectively. X_i and X_j are coded value of the independent variables while k equals to the number of the tested factors ($k = 3$).

An analysis of variance (ANOVA) with 95% confidence level was then carried out for each response variable to test the model significance and suitability. The significances of all terms in the polynomial were statistically analyzed by computing the F-value at probabilities (p) of 0.001, 0.01 and 0.05. The absence of any lack of fit ($p > 0.05$) also strengthened the reliability of all models. The models were used for the construction of three dimensional response surface plots to predict the relationship between independent and dependent variables.

RESULTS AND DISCUSSION

In this research, DLLME combined with GC-FID was developed for determination of phenol and carvacrol in honey samples. The effect of different parameters such as kind of extraction and disperser solvents and volume of them, extraction time, pH and salt addition were examined and optimal conditions were selected. In this research factorial design was chosen to determine the most significant factors (volume of disperser and extraction solvent and pH). Chromatographic peak area of analytes was used to assess the extraction efficiency under experimental conditions tested.

Selection of Extraction Solvent

Selection of an appropriate solvent is a major parameter for a DLLME process. Organic solvent is selected based on higher density rather than water, extraction capability of interested compounds and good gas chromatography behavior. Dichloromethane, chloroform and tetrachloride carbon were selected as extraction phase and compared for extraction of phenol and carvacrol from aqueous solution containing 2 mg l^{-1} of two analytes. According to Fig. 1, dichloromethane provides the higher extraction efficiencies for both phenol and carvacrol. Thus the dichloromethane was selected as extraction solvent.

Selection of Disperser Solvent

Miscibility of disperser solvent in organic phase (extraction solvent) and aqueous phase (sample solution) is the most important point for selection of disperser solvent. Thereby acetonitrile, acetone, methanol and ethanol which have this ability, were selected for this purpose. A series of sample solutions were studied by using 1 ml of each disperser solvent containing 150 μl dichloromethane. According to Fig. 1 the acetonitrile provides the better extraction efficiency. Thus acetonitrile was selected as a disperser solvent.

Effect of Extraction Time

In DLLME extraction time is defined as interval time between injection the mixture of disperser solvent and extraction solvent before starting to centrifuge. The effect of extraction time was examined in the range of 0-30 min with

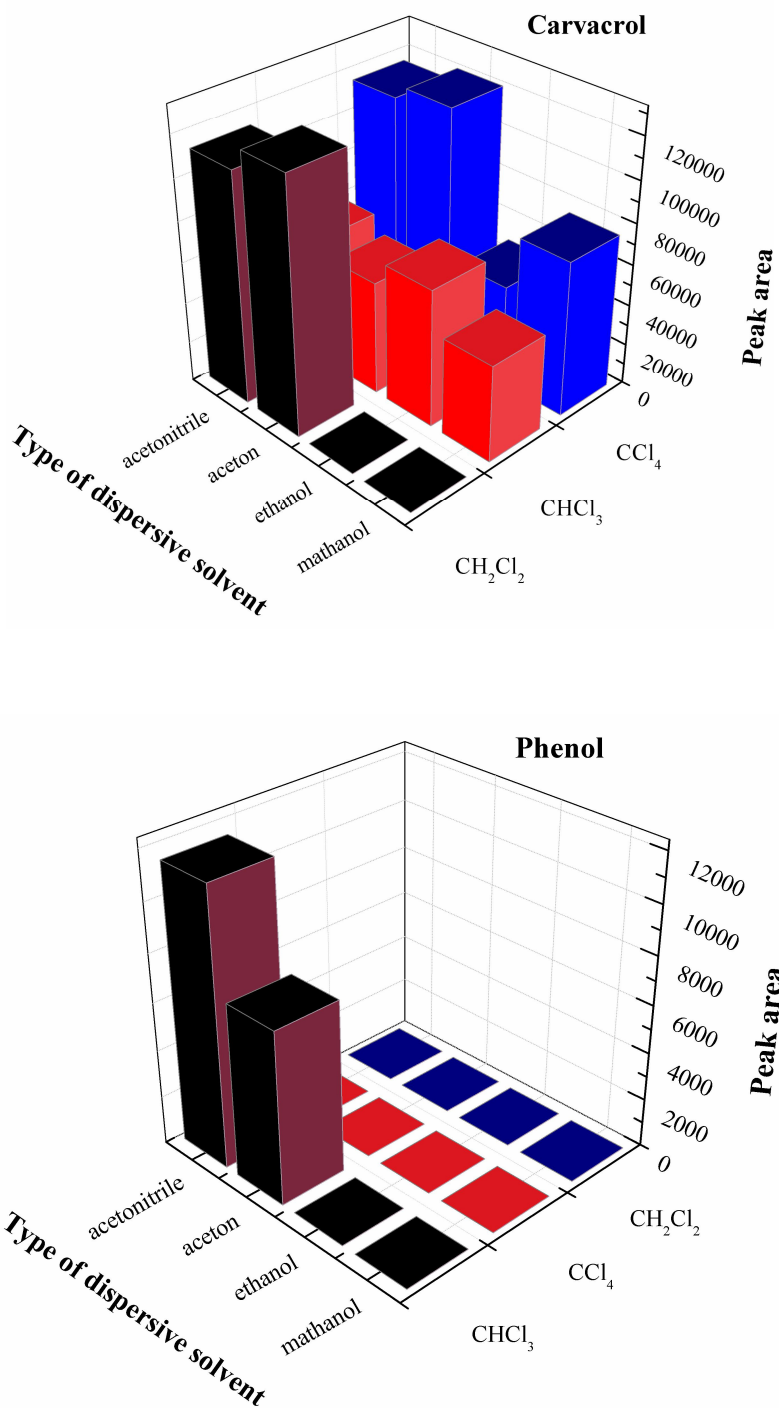


Fig. 1. Selection of extraction and dispersive solvents for the determination of phenol and carvacrol using GC-FID. Extraction conditions: volume of disperser solvent, 1 ml; volume of extraction solvent, 100 μ l.

constant experimental condition. Figure 2 shows the peak area of analytes versus extraction time. According to Fig. 2, the variations of peak area versus extraction time were not remarkable. Indicating that time has no impact on extraction efficiency. It is revealed that after formation of cloudy solution, the surface area between extraction solvent and aqueous phase (sample) becomes infinitely large. Thereby transition of analytes from aqueous phase to extraction solvent is fast. Subsequently equilibrium state is achieved quickly, so that the extraction time is very short. Low extraction time is an advantage of DLLME technique.

Effect of Salt Addition

The effect of increasing the ionic strength of the aqueous solutions was evaluated by adding NaCl (0-10, w/v) into the aqueous solution containing 2 mg l⁻¹ of phenol and carvacrol. Plots of average peak area as a function of the percentage of NaCl are shown in Fig. 3. According to this figure, analytical signals of the analytes, are increased with addition of NaCl up to 3% (w/v) and then decreased with further increasing NaCl concentration. At the beginning of extraction, salting out effect played a predominant role, resulting in a decrease of dissolubility of analytes in aqueous phase and therefore an improvement of extraction. With increasing the NaCl concentration, another effect caused by the presence of salt changed the physical properties of the solution. Hence, salt concentration of 3/100 was chosen for further experiments [19].

Selection of Extraction and Disperser Solvent Volume and pH by Factorial Design

A three-level factorial design requires more experimental runs to evaluate all possible combinations of the three levels of each considered. The following factors were evaluated: extraction and disperser solvent volume and pH. Table 1 lists the factors, corresponding symbols and levels and Table 2 shows the experimental design matrix and the results derived from each run. According to Fig. 4, optimum condition for extraction solvent, disperser solvent and pH were respectively: 100 µl, 0.5 ml, 3.

Modeling of the Extraction Process

The responses (total phenolic content, antioxidant activities and yield) of each run of the experimental design

are presented in Table 2. Regression analysis was performed on the experimental data and the coefficients of model were evaluated for the model significance.

Equations (2-3) show the relationship between X₁ (pH), X₂ (volume of extraction solvent (µl)) and X₃ (volume of disperser solvent (ml)) on the peak area of phenol (Y₁) and carvacrol (Y₂), respectively:

$$Y_1 = 118530 - 23094 X_1^* - 21 X_2 - 9405 X_3 + 1813 X_1^2 - X_2^2 - 9504 X_3^2 + 14 X_1 X_2 + 1710 X_1 X_3 + 105 X_2 X_3^* \\ SE = 3361; R^2 = 0.915 \quad (2)$$

$$Y_2 = 345914 + 42019 X_1 - 2082 X_2^* - 319522 X_3^* - 3584 X_1^2 + 3 X_2^2 + 58110 X_3^2 - 26X_1 X_2 + 1115 X_1 X_3 + 656 X_2 X_3^* \\ SE = 13320; R^2 = 0.971 \quad (3)$$

An analysis of variance (ANOVA) with 95% confidence level was then carried out for each response variable to test the model significance and suitability. As shown, the linear, quadratic and interaction terms have significant effects (star means significant variables at p ≤ 0.05). The large values of R² indicated that the models adequately represent the experimental results. The models were used for the construction of three dimensional response surface plots to predict the relationship between independent and dependent variables (Fig. 4).

Analysis of the experimental results showed that pH has the greatest effect on phenol area. The linear term (pH), quadratic term (V_{ex}²) and interaction terms (pH × V_{ex} and V_{ex} × V_{dis}) were important. The effect of pH and volume of extraction solvent or volume of disperser solvent on phenol area appeared as a saddled shape (Fig. 4).

Carvacrol area was highly affected by volumes of extraction solvent and disperser solvent. The linear terms (V_{ex} and V_{dis}), quadratic term (V_{ex}²) and interaction term (V_{ex} × V_{dis}) were important. Quadratic term (V_{ex}²) and interaction term (V_{ex} × V_{dis}) were appeared in two RSM models and showed that interaction is significant between V_{ex} and V_{dis}. These types of interaction cannot detect in on-at-a-time optimization approach. As shown in Fig. 4, peak area of phenol and carvacrol were maximum when both V_{ex} and V_{dis} were 300 µl and 0.5 ml, respectively.

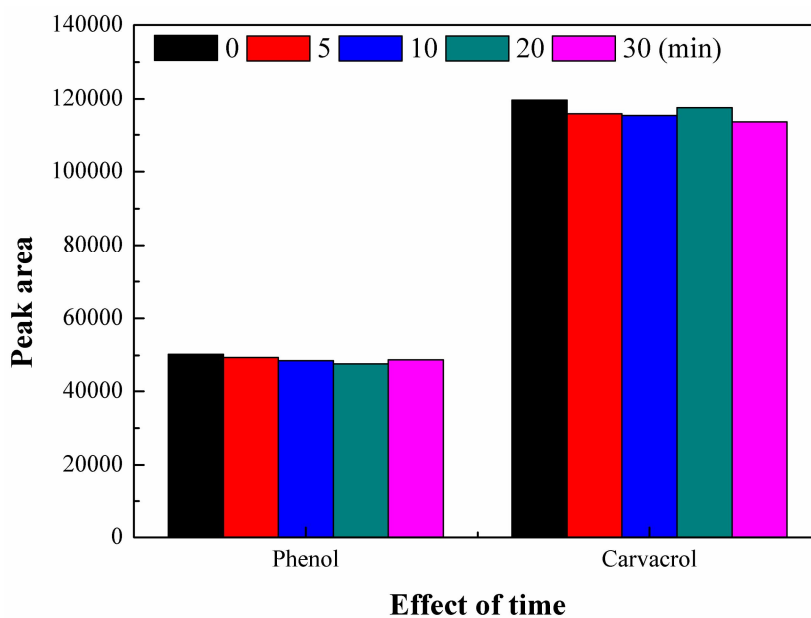


Fig. 2. Effect of time on the extraction efficiency of phenol and carvacrol. Experimental conditions: disperser solvent: (acetonitrile, 1 ml), extraction solvent: (dichloromethane, 100 μ l).

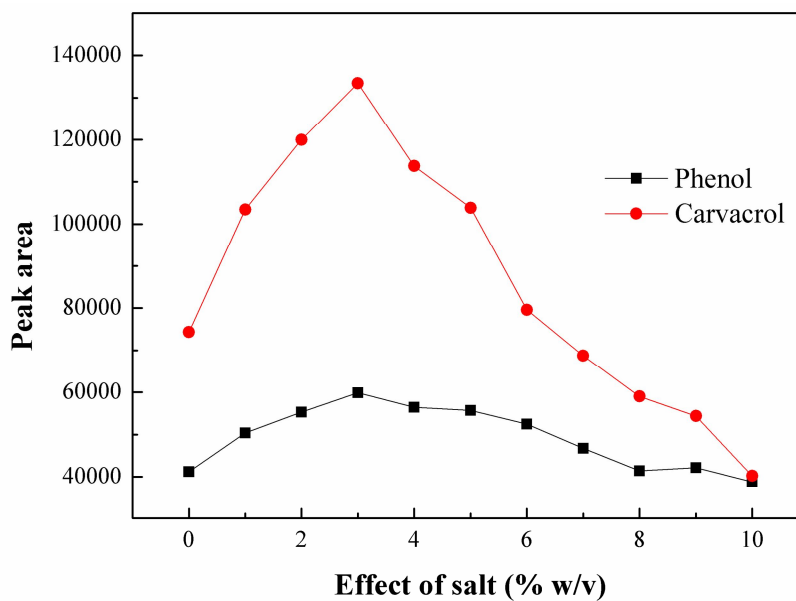


Fig. 3. Effect of salt on the extraction efficiency. Experimental conditions: disperser solvent: (acetonitrile, 1 ml), extraction solvent: (dichloromethane, 100 μ l).

Table 1. Factors and Their Levels in Factorial Design

Factors	-1	0	1
(X ₁) pH	3.0	5.0	7.0
(X ₂) Volume of extraction solvent (μl)	100	200	300
(X ₃) Volume of disperser solvent (ml)	0.5	1.0	1.5

Table 2. Design Matrix and Responses for Factorial Design

Run	X ₁	X ₂	X ₃	Phenol response	Carvacrol response
1	1	1	1	49213	3082
2	1	-1	1	48716	27419
3	-1	-1	-1	65332	135978
4	0	-1	0	44718	100765
5	0	0	0	48743	2157
6	-1	1	1	44153	1528
7	1	-1	-1	52273	153765
8	0	0	-1	41512	74293
9	0	0	1	44800	1064
10	-1	1	-1	33598	1088
11	-1	0	0	55055	10618
12	1	1	-1	37739	1874
13	1	0	0	50512	7014
14	-1	-1	1	49015	1483
15	0	0	0	50406	2212
16	0	1	0	35642	2835
17	0	0	0	51163	2278

The multiple response optimization was also performed. The multi-response optimization for extraction was carried out by desirability function approach. The maximization of these two responses is of practical importance since they might conflict with each other. Therefore, it is necessary to find out the optimal point as a compromise for the maximal phenol and carvacrol extraction. The individual desirability (di) for Y₁ and Y₂ were calculated by one side transformation and then used to calculate overall desirability (D) of the optimization. The scale in the range of 0.0

(undesirable) to 1.0 (very desirable) is used to obtain a global function (D) that should be maximized according to efficient selection and optimization of designed variables. The overall desirability of optimization was found to be D = 0.94. At this D value, optimum values of the selected variables were as follows: pH 3, volume of extraction solvent 100μl and volume of disperser solvent 0.5 ml which responded for Y₁ = 63079 and Y₂ = 141670, respectively. At optimal conditions, experimental area were Y₁ = 65332 and Y₂ = 135978, respectively.

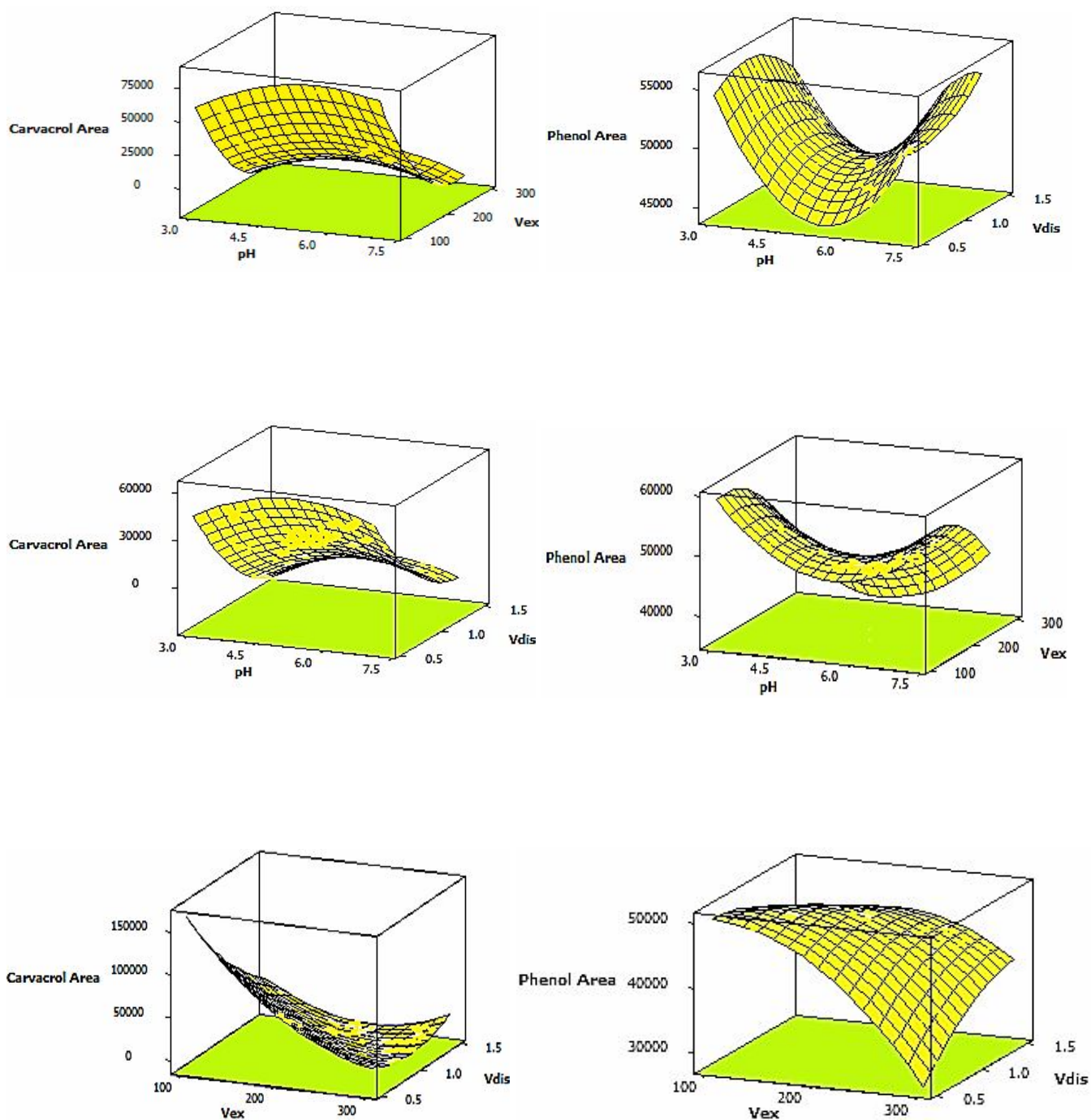


Fig. 4. Selection of the volume of extraction and disperser solvents and pH by surface analysis. Extraction conditions: disperser solvent: (acetonitrile, 1 ml), extraction solvent: (dichloromethane, 100 μ l).

Calibration Curves

Calibration graphs were performed using the external standard technique. The limits of detection, linear ranges and regression coefficient obtained from the calibration graphs are shown in Table 3.

Real Sample Analysis and Recovery

The extraction of phenol and carvacrol from honey is very easy because of its dissolution in water. Recovery of

two compounds was determined by adding the standards at three different concentrations to honey samples prior to applying the extraction procedure. The analytes were extracted from four kinds of honey samples in optimized condition with DLLME method. Figure 5 shows the GC-FID chromatogram of analytes and Fig. 6 GC-MS shows chromatogram of analytes in honey sample. Results of the relative recovery of honey samples using the optimized DLLME method are given in Table 4.

Table 3. Linear Range (L.R.), Coefficient of Determination (R^2) and Limit of Detection (LOD) for Phenol and Carvacrol

Analyte	R^2	LOD (mg l^{-1})	L.R. (mg l^{-1})
Phenol	0.9978	4.146	10-200
Carvacrol	0.9989	3.8499	10-200

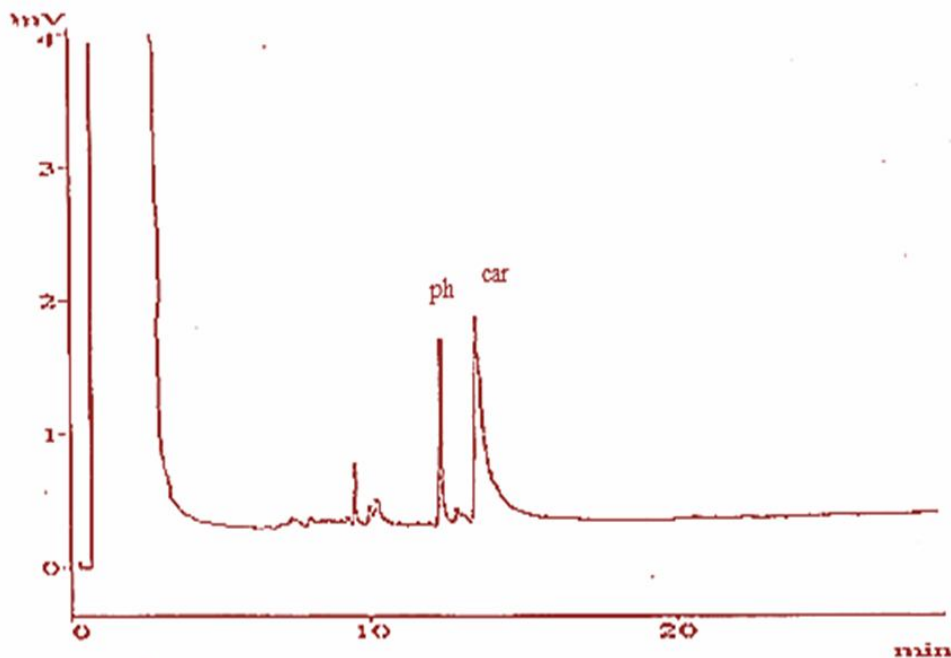


Fig. 5. GC-FID chromatogram of phenol and carvacrol obtained by DLLME under optimized conditions.

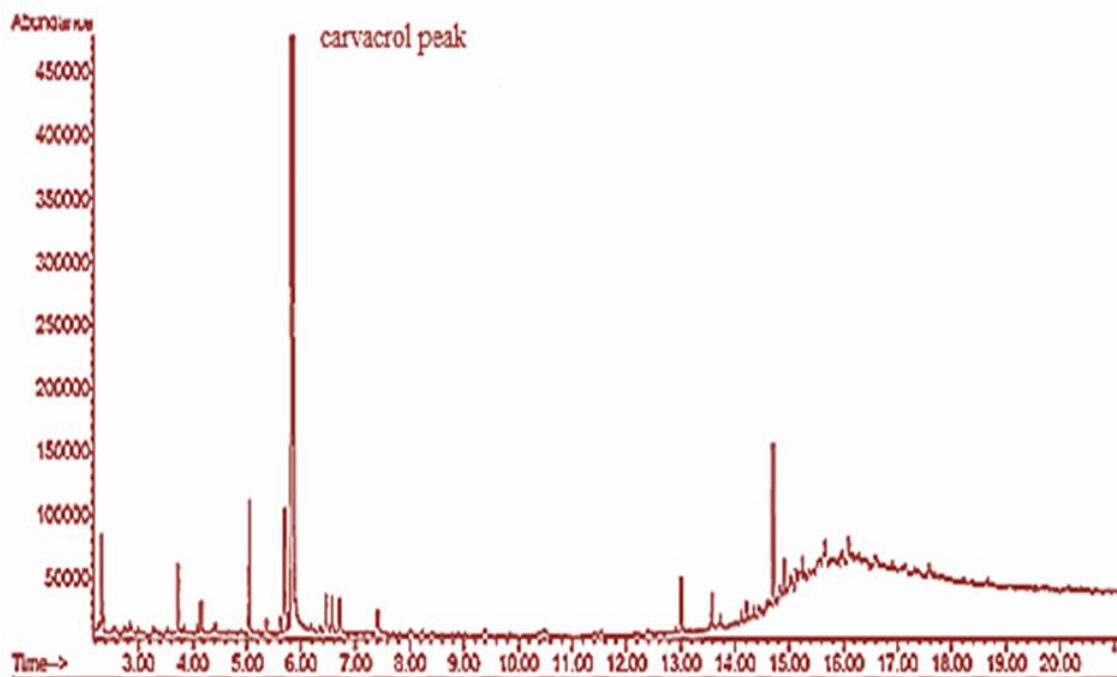


Fig. 6. GC-MS chromatogram of honey sample obtained by DLLME under optimized conditions.

Table 4. Recovery Results of Phenol and Carvacrol

Sample honey	Analyte	Found (mg l ⁻¹)	Added (mg l ⁻¹)		
			15	50	100
			Recovery (%)		
Evergreenoak (<i>Quercus Brantiikink</i>)	Phenol	0.575	93	92	97
	Carvacrol	7.121	96	89	83
Milkvetch (<i>Astragalus adscendens</i> Boiss)	Phenol	1.587	91	78	89
	Carvacrol	3.148	93	96	89
Algerian cedar (<i>Cedrus Atlantica</i>)	Phenol	4.083	83	94	86
	Carvacrol	4.99	97	89	81
Black cumin (<i>Nigella sativa-sibth</i>)	Phenol	6.123	80	71	85
	Carvacrol	2.738	88	82	67

CONCLUSIONS

In this study, DLLME combined with GC-FID has been used for determination of phenol and carvacrol in honey samples. DLLME provides the good repeatability, high recovery and a much short time. The comparison of this method with other extraction methods demonstrated that DLLME is very fast, simple, accurate and inexpensive.

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