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Short communication

Liquid chromatographic determination of phenol, thymol and carvacrol in honey using fluorimetric detection

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Abstract

A comparison is made between the use of a silica-based monolithic column and a RP-AmideC_{16} column for the separation of phenol, thymol and carvacrol using reversed-phase liquid chromatography. The best results concerning total analysis time and sensitivity were obtained using the monolithic column. Detection was optimized using a fluorimetric detector which allowed better detection limits that those obtained with a photo-diode array spectrophotometer. Gradient elution with acetonitrile–water mixtures as mobile phases permitted good separation of the phenols. Identification of the peaks was based on their retention characteristics, varying the flow-rate, nature and composition of the mobile phase as well as the nature of the stationary phase, and using the fluorimetric detector to continuously measure the spectrum when the solute passed through the flow cell. Linearity, precision, recovery and sensitivity were satisfactory. The procedure was applied to the analysis of phenol, thymol and carvacrol in honey of different types. The extraction process was very simple, only involving dissolution of honey with water. Detection limits in the honey samples using the proposed procedure were between 1 and 4 ng g^{-1}.

Keywords: Liquid chromatography; Fluorimetric detection; Monolithic column; Phenol; Thymol; Carvacrol; Honey

1. Introduction

The global evaluation of honey requires the study of possible contamination by harmful substances. According to EC norm 2001/110/EC (2002), honey must be exempt of antibiotics, pesticides, atmospheric pollutants and heavy metals [1]. Phenol is present in honey at microgram level. The 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} [2], while in other countries, such as Germany, the maximum limit is only 50 ng g^{-1}. A recent study established that is difficult to find honey containing less than 40 ng g^{-1} of phenol [3]. The presence of phenol in honey is controversial because it was first used as a bee repellent. However, even beekeepers who did not follow this practice found phenol in honey, probably as a result of diffusion, as phenol is a volatile compound, from the varnish of barrels. An alternative theory [4] suggests that phenol is a natural component and, even for non-treated honeys, it is a natural constituent. Thymol is frequently used as disinfectant and for the control of varroasis in beekeeping. Ecological beekeepers use natural products, such as thyme oil (*Thymus vulgaris*), which is very rich in thymol and carvacrol. However, it was recommended to apply it only when there is no nectar flow, to reduce the appearance of residues in honey since, although not toxic, thymol residues can devalue honey quality.

Phenols can be successfully determined using liquid chromatography (LC) in the reversed-phase mode, but selection of the stationary phase is very important. For rapid chromatographic analysis, the use of monolithic columns [5,6] which are formed from a single piece of porous silica-gel that contains mesopores with diameters of approximately 13 nm and macropores with diameters of approximately 2 μm, could be used. In contrast with conventional LC columns, monolithic columns have a single silica skeleton, giving greater porosity and permeability, they can be operated at higher flow-rates maintaining a low back pressure and, because of their better mass transfer properties, they maintain high separation efficiency [5].

Chromatographic procedures for the determination of phenol in honey or beeswax are very scarce. LC has been used

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with UV detection after distillation of honey samples [7], with fluorimetric and colorimetric detectors with a distillation step prior to analysis [8,9] or with amperometric detection after distillation and solid-phase extraction [10]. In all these procedures, the sample treatment is very long and tedious. Recently, Gyorik et al. [11] proposed a LC-fluorimetric method which only involved dilution of the honey sample. Gas chromatography (GC) has also been used for phenol detection in honey after extraction with ether and using a flame ionization detector [12]. Thymol has been determined in honey by Martel and Zeggane [13], who analyzed several acaricides in honey by LC with UV diode-array detection. However, sample treatment is long because it involves a liquid–liquid extraction or a solid-phase extraction using cartridges. Thymol and other residues have also been determined in honey using GC with flame ionization [14,15] and mass spectrometric detection [16]. As far as we know, there are no studies in the literature concerning the chromatographic determination of carvacrol in honey.

In the present study, a new method for the determination of phenol, thymol and carvacrol was optimized using LC with fluorimetric detection. We compare the use of a stationary phase, involving a ligand with amide groups and the endcapping of trimethylsilyl (RP-AmideC_{16}), and a silica-based monolithic column. Sample preparation is minimal and the procedure is applied to the analysis of natural or residual phenols in different types of honey.

2. Experimental

2.1. Apparatus

The LC system consisted of a Shimadzu FCV-10ALvp (Shimadzu, Kyoto, Japan) liquid chromatograph operating at room temperature with a flow-rate of 1 mL min^{-1}. The solvents were degassed using a Shimadzu DGU-14A membrane system. The spectrophotometric detector was a photo-diode array Shimadzu SPD-M10Avp operating at wavelengths of 204 and 277 nm for all phenols. The software used was Class-LC10 (Shimadzu). The fluorescence detector was an Agilent FLD 1100 (Agilent Technologies, Waldbronn, Germany) operating at wavelengths of 274/590 nm (excitation and emission). Aliquots of 100 μL were injected manually using a Model 7125-075 Rheodyne injection valve (Rheodyne, Berkeley, CA, USA). The analytical columns used were a 150 mm × 4.6 mm Discovery RP-AmideC_{16}, with a particle size of 5 μm (Supelco, Bellefonte, PA, USA) and a guard column packed with the same stationary phase, and a 100 mm × 4.6 mm silica-based monolithic column Chromolith (Merck, KGaA, Darmstadt, Germany).

2.2. Reagents

Acetonitrile (ACN) and methanol (Lab Scan, Dublin, Ireland) were of liquid chromatographic grade. Doubly distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Stock solution (1000 μg mL^{-1}) of phenol (Sigma, St. Louis, MO, USA) was prepared by dissolving 10 mg of the commercial product, without previous purification, in 10 mL of water. Stock solutions (1000 μg mL^{-1}) of thymol and carvacrol (Aldrich, USA) were prepared by dissolving 10 mg of the commercial products, without previous purification, in 10 mL of ethanol. They were kept in dark bottles in the freezer at 4 °C and were stable for several months. Working standard solutions were prepared daily by dilution with water. Other reagents used were sodium phosphate, phosphoric acid and sodium hydroxide (Panreac, Barcelona).

2.3. Honey samples

Honey samples of different botanical origin (eucalyptus, rosemary, heather, citrus and biercol) were obtained from several beekeepers from Spain. All samples were kept sealed in the absence of light.

2.4. Analytical procedure

A sample of 2 g of honey was weighed into a 5-mL calibrated flask and dissolved with water. This mixture was homogenized and aliquots were filtered through 0.45 μm nylon Millipore chromatographic filters and injected into the chromatograph.

3. Results and discussion

3.1. Selection of the stationary phase

The separation of phenols by reversed-phase using an octadecylsilyl column provided poor results because phenols have an ionic character and they produced tailing peaks. Thus, we selected a stationary phase Discovery RP-AmideC_{16}, having a ligand with amide groups and endcapping of trimethylsilyl. Using a mobile phase of 40/60 (v/v) acetonitrile/water, phenols were separated but carvacrol and thymol took a long time to elute. With 60–70% acetonitrile, carvacrol and thymol eluted more quickly but phenol eluted at the void time. The main problem, then, with the amide column is that the total analysis time is too long. To improve resolution and analysis time, the separation conditions were modified by choosing a new stationary phase. A recent innovation for fast chromatographic analysis is to use monolithic columns and so we carried out a comparison of both the RP-AmideC_{16} and the Chromolith monolithic columns for analyzing phenols. The chromatographic profiles obtained using the same mobile phase of 40/60 (v/v) ACN/water are shown in Fig. 1. In both cases, the elution order was the same: 1, phenol; 2, carvacrol and 3, thymol. Table 1 shows a comparison of the main chromatographic parameters obtained when using both columns. As can be seen, the use of the monolithic column significantly reduced the total analysis time, which varied from 21 min with the amide column to 9 min with the monolithic column. Consequently, the monolithic column was selected for further study.

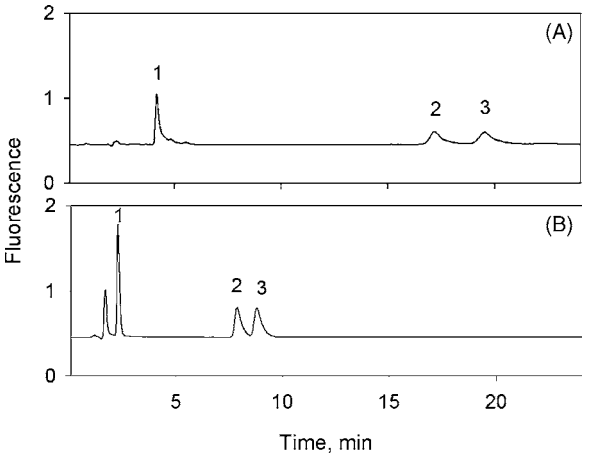

Fig. 1. Chromatograms for the phenols using the RP-AmideC$_{16}$ column (A) and the monolithic column (B) with a mobile phase of 40/60 (v/v) ACN/water. Flow-rate, 1 mL min^{-1}; injected sample (containing 50 ng mL^{-1} each), 100 μL. Peaks correspond to: 1, phenol; 2, carvacrol and 3, thymol.

3.2. Selection of the mobile phase

The optimal mobile phase was selected by varying the proportion of ACN and modifying the aqueous phase by adding phosphoric acid. When mixtures of ACN at percentages of between 20 and 50% (v/v) and water were used, all the compounds were eluted from the column and both retention and peak widths decreased as the proportion of organic solvent increased. A 40/60 (v/v) percentage was selected to decrease analysis time. The influence of pH was studied using a 40/60 (v/v) ACN/50 mM sodium phosphate mixture at pH values ranging between 2.5 and 7; phenol retention was not appreciably modified by the buffer pH. Finally, the influence of the phosphoric acid concentration (between 2 and 50 mM) was studied and, again, retention was not modified by this variable. Consequently, the addition of phosphoric acid was deemed not necessary, and phenol separation was optimized using ACN/water mixtures.

With the mobile phase selected as optimal, isocratic elution was not possible since the phenols presented a very different retention behavior. The optimal mobile phase for eluting phenol (containing 10–20%, v/v, ACN) did not elute the other compounds. For higher ACN percentages (40%, v/v, ACN), carvacrol and thymol were separated but phenol eluted at the void time. Consequently, a gradient elution technique was tried to achieve good peak resolution and to shorten the total analysis time. Several gradients with different profiles were assayed and the optimal gradient was found to be an initial isocratic step with a 20/80 (v/v) ACN/water mixture for 3 min, a gradient to 40/60 (v/v) ACN/water in 0.01 min and, finally, maintaining this mixture for 8 min. The initial conditions were re-established in 1 min and held for 15 min. The flow-rate was 1 mL min^{-1}. The chromatographic profile obtained using this gradient elution program shows that very good results, defined by total elution of the mixture components and the appearance of narrow and non-tailed peaks, were obtained using the monolithic column. Retention times were as follows: phenol, 3.92 min; carvacrol, 11.37 min and thymol, 12.24 min. Values for the separation factor (α) were between 1.1 and 4.2 and for resolution (R_s) between 2.3 and 23.

3.3. Calibration, detection limits and repeatability

Calibration graphs were performed using the external standard technique following linear regression analysis by plotting concentration (ng mL^{-1}) against peak area. Table 2 shows the equations obtained for the calibration graphs and the regression coefficients. The repeatability of the method was calculated using the average relative standard deviation (R.S.D.) for 10 replicate injections of the same sample at 10 ng mL^{-1}. The reproducibility was calculated using the R.S.D. for 10 injections of the same sample (10 ng mL^{-1}) on different days. The detection limits were calculated on the basis of 3σ (σ being the residual standard deviation of the intercept) and the quantitation limits on the basis of 10σ, using the regression lines for the standards. Values are also given in Table 2.

3.4. Extraction and recovery studies

The extraction of phenols from honey is very easy because they are water soluble. Thus, the procedure consisted on dissolving the honey with water and filtering the solution. A recovery study was carried out by the standard addition technique, spiking three samples of different types of honey with the phenol standards at levels between 70 and 140 ng g^{-1} prior to applying the extraction procedure. The mean recovery ± standard deviation for the three phenols in the analyzed samples (n = 6) were phe-

Table 1
Comparison of chromatographic factors for both the RP-AmideC$_{16}$ and the Chromolith monolithic columns

Parameter	Discovery RP-AmideC$_{16}$ column			Chromolith monolithic column		
	Phenol	Carvacrol	Thymol	Phenol	Carvacrol	Thymol
Back pressure (bar)		103			25	
Plate number		9920			7900	
Plate height (cm)		1.50×10^{-3}			1.27×10^{-3}	
Analysis time (min)		21			9	
Retention time (min)	4.20	17.20	19.54	2.30	7.90	8.81
Retention factor	1.62	9.80	11.27	0.34	3.60	4.11
Peak width (min)	0.20	0.67	0.78	0.13	0.35	0.40
Separation factor	6.05		1.15	10.59		1.14
Resolution	29.00		3.20	23.00		2.45

Table 2
Calibration characteristics of phenols

Compound	Intercept	Slope (mL ng^{-1})	Correlation coefficient	Linearity (ng mL^{-1})	DL (ng mL^{-1})	QL (ng mL^{-1})	Repeatability R.S.D. (%)	Reproducibility R.S.D. (%)
Phenol	−0.0234	0.0710	0.9999	2–100	0.43	1.43	1.7	2.3
Carvacrol	−0.0239	0.0426	0.9999	8–200	1.56	5.20	1.9	2.5
Thymol	−0.0151	0.0444	0.9999	8–200	1.70	5.62	2.5	2.9

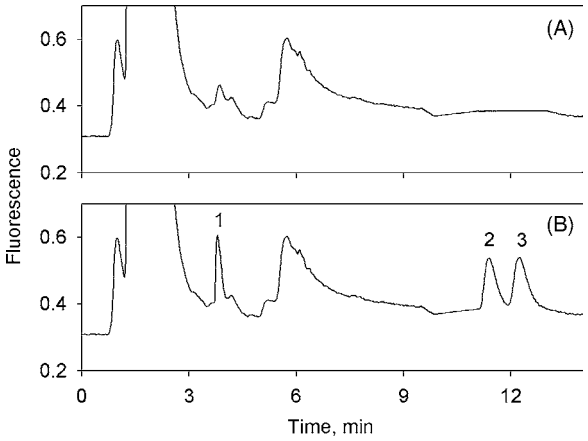

Fig. 2. Chromatograms for a citrus honey extract (A) and the same sample spiked with 140 ng g^{-1} of phenols (B) using the Chromolith column and gradient elution. Peaks correspond to: 1, phenol; 2, carvacrol and 3, thymol.

nol, 97 ± 1; carvacrol, 97 ± 1 and thymol, 98 ± 1. These results demonstrated that recoveries were quantitative.

3.5. Analysis of honey samples

The procedure was applied to the analysis of unifloral honey samples. Fig. 2A shows the chromatogram obtained for a citrus honey extract. The profile obtained for the same sample spiked with 140 ng g^{-1} of the phenols is shown in Fig. 2B. Similar chromatograms were obtained for other honey samples of different types.

The chromatographic peaks were identified by comparing the retention data obtained for the standards, the sample and the sample spiked with the standards under identical conditions and using the fluorescence detector to continuously measure the spectrum while the solute passed through the flow-cell. The following criteria were used to confirm the purity of the peaks: (A) retention times comparison. Table 3 shows that the average values (mean ± S.D.) for the retention times were as follows: phenol, 3.91 ± 0.05 min (n = 7); carvacrol, 11.28 ± 0.03 min (n = 4) and thymol, 12.10 ± 0.05 min (n = 6). These values indicate good agreement between the retention data of phenols in the different samples. (B) Comparison of fluorescence spectra. Good agreement was obtained for the spectra of standards and different samples when the solute passed through the flow cell, thus confirming the identity and the purity of the peaks. The peaks corresponding to the phenols added to the honey samples showed that no impurities coeluted with the analytical peaks and the purity of the peaks was satisfactory. Fig. 3 shows the concordance of the fluorescence spectra for a biercol honey sample. For the other types of honey, similar results were obtained. (C) Variation of the flow-rate. When the flow-rate of the mobile phase was increased to 2 and 3 mL min^{-1}, good concordance between the retention times of the phenol standards and the peaks corresponding in both the honey and the honey spiked samples was again obtained. (D) Variation of the mobile phase composition. When the ACN percentage was varied from 20 to 10% (v/v), the chromatograms for the different samples showed that the retention times of phenols were in good agreement. (E) Varia-

Table 3
Identification of phenols on a retention time database

Sample	Retention time (min)		
	Phenol	Carvacrol	Thymol
Standards	3.91	11.33	12.18
Citrus honey	3.81	–	–
Citrus honey, spiked	3.83	11.25	12.10
Heather honey	3.99	–	12.07
Heather honey, spiked	3.95	11.28	12.04
Biercol honey	3.93	–	12.17
Biercol honey, spiked	3.90	11.31	12.18

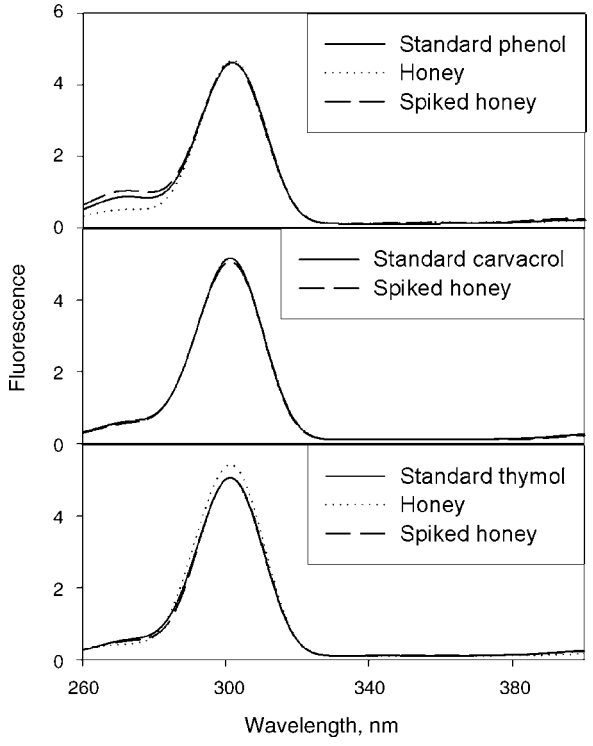

Fig. 3. Fluorescence spectra obtained for the standards, a biercol honey sample and the honey spiked with 140 ng g^{-1} of the standards.

Table 4
Contents of phenols in honey samples

Botanical origin	Pollinic characterization	Phenol (ng g^{-1})	Thymol (ng g^{-1})
	Eucalyptus	101 ± 5	ND
Eucalyptus	Multifloral	57 ± 4	ND
	Echium	33 ± 5	ND
	Echium	21 ± 3	ND
Rosemary	Rosemary	28 ± 5	ND
	Multifloral	22 ± 4	ND
	Citrus	20 ± 3	ND
Citrus	Citrus	24 ± 3	ND
	Citrus	20 ± 1	ND
	Multifloral	15 ± 4	ND
Heather	Heather	23 ± 5	142 ± 3
	Heather	46 ± 4	ND
	Heather	248 ± 2	310 ± 3
Biercol	Biercol, Heather	318 ± 3	283 ± 3
	Biercol	83 ± 2	346 ± 2
	Mixture	27 ± 5	ND
Mixture	Citrus	21 ± 4	ND
	Mixture	60 ± 5	ND
Forest	Heather	44 ± 5	ND

Mean ± standard deviation, $n = 3$; ND means no detected.

tion of the mobile phase selectivity. The replacement of ACN by methanol at 20% (v/v) leads to a similar retention behavior for standards and samples. Thus, the average values (mean ± S.D.) for the retention times of phenol in different samples ($n = 5$) was 5.55 ± 0.07 min, which indicates a good concordance between the retention data of phenol. (F) Variation of the stationary phase. A good way to prove the traceability of the procedure is to use a different stationary phase to compare retention data. Thus, when replacing the monolithic column by the RP-AmideC_{16} column, the mean ± S.D. values for the retention times of phenol in different samples ($n = 5$) were 4.14 ± 0.04 min, which again indicates a very good agreement between retention data.

Finally, three samples of each honey from different botanical origin were analyzed and phenol was found at concentrations of between 15 and 318 ng g^{-1} (Table 4). The higher phenol levels appeared in biercol honey, while lower levels corresponded to citrus honey. These results are in agreement with those found by other authors [11,15]. An ANOVA on ranks (Kruskal–Wallis) test was then used to evaluate whether statistically significant differences existed between the phenol content of the different honey groups. The Dunn test was selected to carry out all the pairwise multiple comparison procedures. The difference in the median values shows that there was a statistically significant difference between the phenol contents in the citrus and biercol honey samples ($H = 11.16$, $p = 0.025$). Thymol was only found in three samples of heather and biercol honeys at levels of between 142 and 346 ng g^{-1}. Carvacrol was not found in any of the samples above the detection limit. Detection limits in the honey samples using the proposed procedure are between 1 and 4 ng g^{-1} depending on the phenol.

4. Conclusion

Monolithic columns offer a good alternative for the rapid analysis of residual phenols in honey. The procedure used reversed phase liquid chromatography with fluorescence detection and gradient elution. The recovery study carried out in several honey samples provided good results, detection limits in the honey samples ranging between 1 and 4 ng g^{-1}, depending on the phenol. Phenol levels of between 15 and 318 ng g^{-1} were found, while thymol was only found in three samples at levels between 142 and 346 ng g^{-1} and carvacrol was not found in any sample above its detection limit. The chromatographic peaks were identified by comparing the retention data obtained for the standards and the sample spiked with the standards under identical conditions and by comparison of the fluorescence spectra while the solute passed through the flow-cell. The sample preparation step was minimal.

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