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Rapid and Sensitive Determination of Phenol in Honey by High-Performance Liquid Chromatography with Fluorescence Detection

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The objective of this research was to develop a novel high-performance liquid chromatographic (HPLC) method involving a simple sample preparation procedure for the rapid, low-cost, and sensitive quantitation of phenol in honey at levels of regulatory and practical importance. After proper dilution of honey with water, the samples were analyzed by a gradient HPLC system, using a reversed-phase column with fluorescence detection at excitation and emission wavelengths of 270 and 300 nm, respectively. The eluents applied were water−acetonitrile−85% orthophosphoric acid (10:10:0.01, v/v/v) and water−85% orthophosphoric acid (20:0.01, v/v). The retention time of phenol was found to be 14.1 min, and the limit of quantitation for phenol in honey was set at 5 μg/kg. Overall recovery was 98%. The proposed method has been successfully applied to real sample analysis.

KEYWORDS: Phenol; honey; sample preparation; HPLC; fluorescence detection

INTRODUCTION

Phenol, which is also referred to as hydroxybenzene, has been widely used as a bee repellent over the past 70 years. Commonly, the phenol crystals are dissolved in water to make a concentrated solution of up to 90%, and this solution is sprinkled on a cloth mounted on a frame called a phenol board. The phenol board is placed on top of the beehive and a black cover is added. The heat from the sun vaporizes the phenol, which then has a repelling effect on the bees, thus enabling the beekeeper to collect honey with a minimum of disturbance of the bees. Careless use of phenol in collecting honey may result in contamination with significantly high residues of phenol, which imparts an objectionable medicinal taste to the honey (*1*, *2*). Therefore, the use of phenol as a bee repellent has been discouraged in the United States for many years, although its use in other countries is still common (*3*).

However, phenol may also occur naturally in honey at low levels (*4*). Many different phenolic substances are known as characteristic components of honey responsible for the honey-specific flavor and taste (*5*). Several countries have adopted maximum residue limits of 50−100 μg/kg for phenol in honey because the natural background falls well below this level (*4*).

The relationship between nutrition and health is gaining public acceptance and consumers are increasingly health conscious worldwide. For this reason, many countries are considering greater regulation of chemicals, such as phenol, used in beekeeping. Increased regulation will require reliable analytical techniques for the rapid detection of phenol residues in honey.

Sporns (*3*) first introduced an analytical procedure for phenol residues in honey using high-performance liquid chromatography with ultraviolet detection (HPLC-UV) following steam distillation of the samples. Later Daharu and Sporns (*1*) evaluated reverse-phase HPLC-UV, fluorometric, and colorimetric methods for the analysis of phenol in honey and beeswax. All these methods required an initial steam distillation prior to analysis. Takeba et al. (*6*) determined the concentrations of phenol in honey by HPLC with amperometric detection preceded by steam distillation and solid-phase extraction. For the detection of bee repellents, including phenol, in honey, Kwan and Sporns (*7*) developed an analytical method involving ether extraction of the water-diluted samples followed by gas chromatography with flame ionization detection. These methods are all capable of determining phenol in honey at parts per million levels, however, they involve a laborious sample preparation step. Therefore, the purpose of this study was to simplify sample preparation for HPLC analysis while maintaining the detection limit that renders possible the measurement of phenol in honey at sufficiently low concentrations.

MATERIALS AND METHODS

Origin of Honey Samples. Honeys were randomly selected from a large number of samples obtained from diverse parts of the world or purchased in retail outlets in Hungary and other countries. All samples were kept in sealed jars and analyzed immediately or stored at −18 °C until required. A wide range of honey samples labeled as eucalyptus, avocado, wildflower, clover blend, Alaskan fireweed, Middle Eastern citrus and date honeys, Central European honeydew honeys, acacia, monofloral, and multifloral honeys were examined.

Silanization of Glassware. Glassware was silanized before testing to prevent phenol from adsorbing to glass surfaces. Volumetric flasks were rinsed with absolute methanol and were dried under vacuum. After

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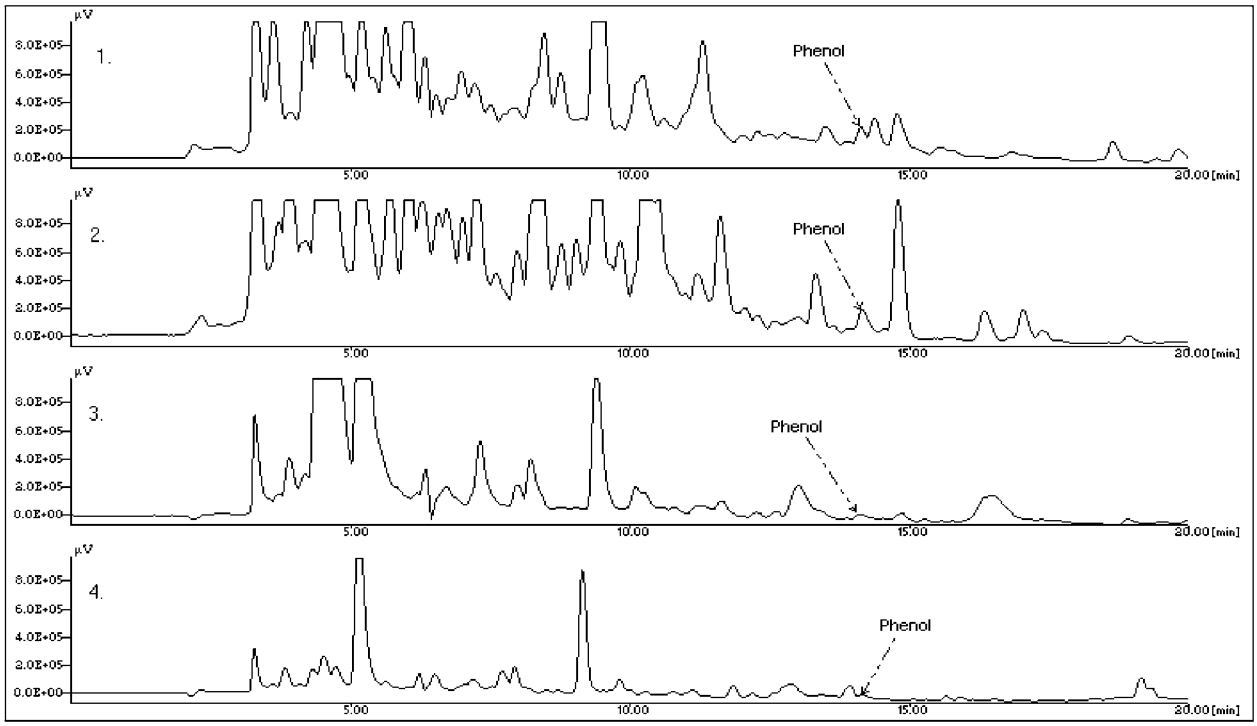

Figure 1. Typical chromatograms of various types of honey: (1) date honey containing 28.3 μg/kg of phenol; (2) honeydew honey containing 53.0 μg/kg of phenol; (3) fireweed honey containing 16.5 μg/kg of phenol; and (4) eucalyptus honey containing 6.2 μg/kg of phenol.

this procedure, flasks were filled with 7.5% dimethyldichlorosilane (Supelco, Bellefonte, PA) in toluene (Merck, Darmstadt, Germany) and were capped and let stand overnight. Flasks were then rinsed with anhydrous toluene and acetone (Merck). Before first use, silanized glassware was washed with methanol and water, and was useable for up to 6 months unless scraping or strong alkali effect occurred.

Chemicals. HPLC grade water purified by a Milli-Q ultrapure water system (Millipore, Bedford, MA) was used for the analyses. HPLC Chromasolv gradient grade acetonitrile was obtained from Riedel-de Haën (Seelze, Germany) and analytical grade phenol, 85% orthophosphoric acid (H_3PO_4), and methanol were purchased from Merck.

Sample Preparation. Only glassware was used for storage of chemicals and samples. Sampling and homogenization were performed according to AOAC Method 920.180 (*8*). A 1-g (to the nearest 0.1 mg) honey sample was measured into a 10-mL volumetric flask and 5 mL of HPLC grade water was added to it. This mixture was homogenized for 1 min with an RX3 vortex mixer (Velp Scientifica, Usmate, Italy), diluted to 10-mL volume with water, and then mixed again. The dissolved sample was filtered through a Millex-GV_{13} hydrophilic membrane filter with a pore diameter of 0.22 μm (Millipore). One hundred-microliter aliquots of this filtered sample were injected into the HPLC column.

HPLC Conditions. Two Jasco PU-980 pumps (Jasco, Tokyo, Japan) with a high-pressure dynamic mixer (Knauer, Berlin, Germany) and a Jasco AS-950-10 autosampler were employed. The eluate was monitored with a Jasco FP-920 fluorescence detector ($\lambda_{ex} = 270$ nm, $\lambda_{em} = 300$ nm), with the emission slit width set at 10 nm, and a 1000-fold gain was thus obtained. HPLC separations were performed on a LiChrospher 100 RP C18 reversed-phase column (250 × 4 mm, 5 μm particle size; Merck) protected by a LiChrospher 100 RP C18 guard column (4 × 4 mm, 5 μm particle size; Merck), the latter being replaced every 250 injections. Both columns were operated at 30 °C by means of a Jones 7955 column thermostat (Jones Chromatography, Hengoed, UK).

A gradient elution was employed, using solvent system A (water−acetonitrile−85% H_3PO_4, 10:10:0.01, v/v/v) and solvent system B (water−85% H_3PO_4, 20:0.01, v/v), with the gradient program being as follows: A:B = 10:90 (0 min), 90:10 (20 min). The flow-rate was maintained at 1 mL/min. Data acquisition and evaluation were done with BORWIN 1.21.60 chromatographic software (JMBS Developpements, Grenoble, France).

Calibration Procedure. A stock solution of phenol was prepared by dissolving pure phenol in methanol at a concentration of 1 mg/mL. This stock solution was diluted with HPLC grade water to obtain two working solutions containing 10 and 1 μg/mL of phenol. A base standard was prepared then by weighing 1 mL of 1 μg/mL working solution into a volumetric flask and diluting it to 10-mL volume with HPLC grade water. Aliquots from this base standard were appropriately diluted to obtain three calibration solutions at concentrations of 10, 5, and 1 ng/mL. A calibration curve was generated by linear regression, using the peak area of phenol plotted against the corresponding concentrations. The phenol contents of test samples were determined by this calibration curve.

Linearity and Limits of Detection and Quantitation. To determine linearity and limits of detection and quantitation, the calibration curve was extended to both the lower and higher concentration ranges, i.e., from 0.1 to 50 ng/mL. Three replicates were measured for 11 known concentrations, equally divided over the calibration curve.

Recovery, Repeatability, and Reproducibility. Honeys containing minute quantities of phenol were selected and recovery was determined by adding phenol to the honey samples at five different concentrations (25, 50, 75, 100, and 250 μg/kg). The measurements were performed with three replicates.

Repeatability (within-day precision) studies were carried out on three types of honey, each containing different levels of phenol. Measurements were repeated nine times on the same day.

Five samples of honey, each containing various amounts of phenol, were selected for reproducibility (between-day precision) tests. Phenol contents were determined in triplicate on each of three different days.

RESULTS AND DISCUSSION

The calibration curve was linear over the concentration range of 0.1−50 ng/mL ($Y = AX$, where $A = 189\,382$). On the basis of 33 measurements (11 concentrations, 3 replicates), the V_y and R^2 values were equal to 15 164 and 0.9998, respectively. The limit of detection ($3V_y/A$) of the HPLC instrument was thus 0.24 ng/mL of phenol, being equivalent to 0.024 ng of injected phenol because 0.1-mL aliquots of the prepared sample were injected into the HPLC column. The limit of quantitation, defined as the lowest concentration of phenol in honey with a signal-to-noise ratio of 3, was set at 5 μg/kg.

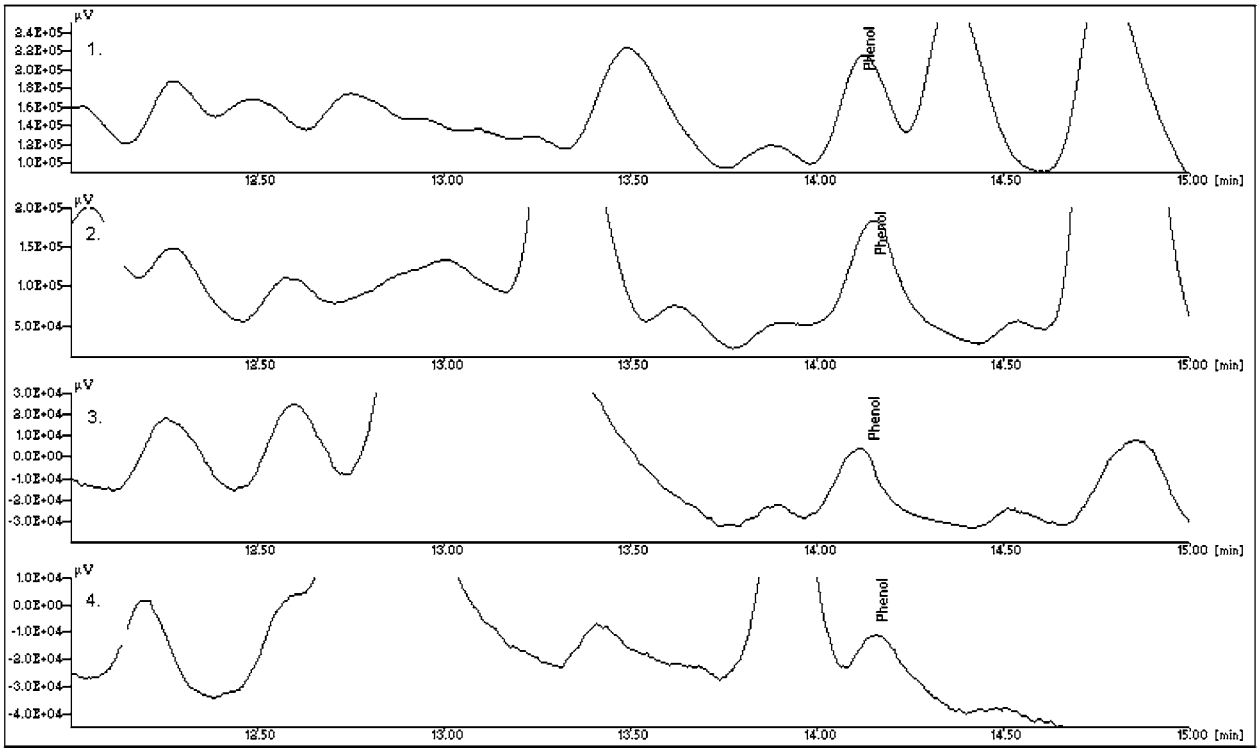

Figure 2. Magnified details of typical chromatograms of various types of honey: (1) date honey containing 28.3 μg/kg of phenol; (2) honeydew honey containing 53.0 μg/kg of phenol; (3) fireweed honey containing 16.5 μg/kg of phenol; and (4) eucalyptus honey containing 6.2 μg/kg of phenol.

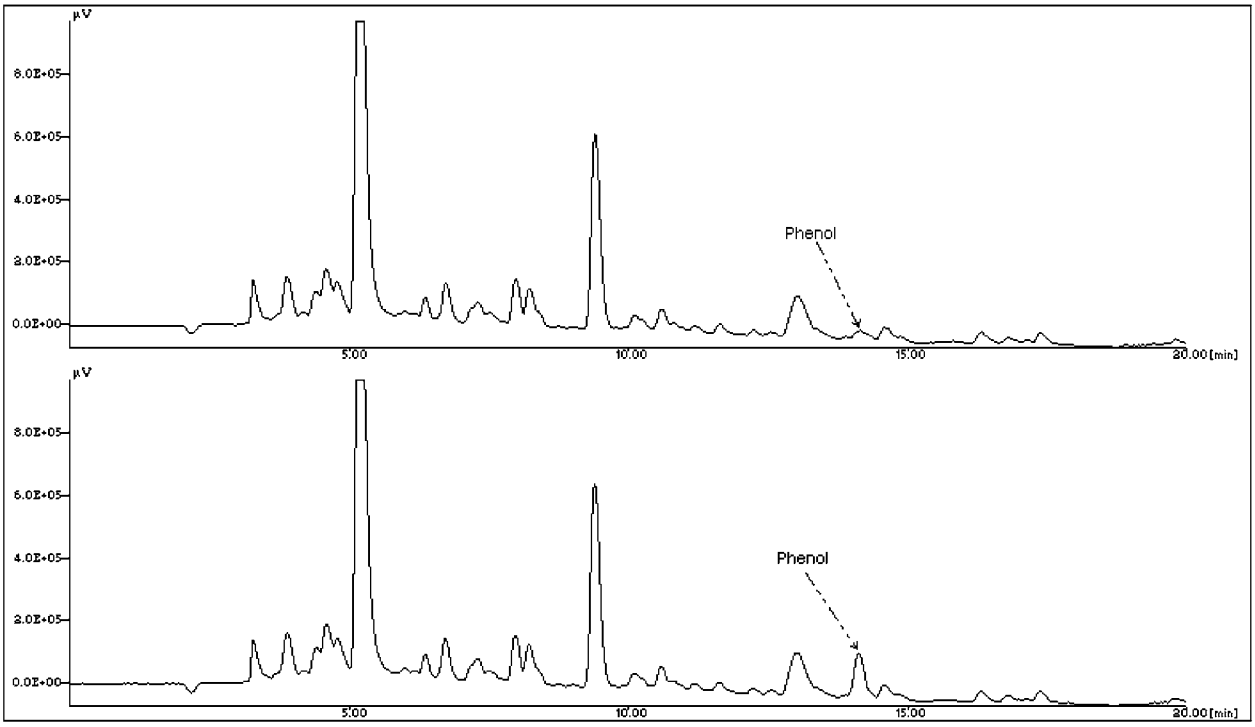

Figure 3. Acacia honey containing 12.9 μg/kg of phenol (top) and the same sample spiked with 50 μg/kg of phenol (bottom).

It should be emphasized that the use of a high-sensitivity fluorescence detector is of prime importance to ensure the low detection limit of the method, and the emission slit width must be set at 10 nm, or even narrower, because the wavelengths of excitation and emission lights differ only slightly. If a wider slit setting is applied, in the case of a 1000-fold gain, the noise caused by the scattered light may render impossible the measurements.

Although the chromatograms of the various types of honey differed considerably from one another, no interference with the phenol peak was observed (**Figures 1** and **2**). It is worth noting that use of plastic containers for storage of solutions should be avoided, otherwise ghost peaks interfering with the phenol peak may appear in the chromatograms. The retention time of phenol was found to be 14.1 min. The eluents applied were acidified with H_3PO_4, which did not influence the retention time of phenol. This was needed because there are certain unidentified components in honey with variable retention times that may have caused interference without acidification of the eluents.

Table 1. Accuracy Data Based on Recovery of the Phenol Added to Honey at Five Concentrations

phenol added (μg/kg)	mean concn ± SD (μg/kg)[a]	RSD (%)	recovery (%)
0	13.5 ± 0.6	4.5	
25	39.4 ± 1.1	2.8	104
50	60.9 ± 3.0	5.0	95
75	88.7 ± 1.5	1.7	100
100	108.1 ± 1.7	1.5	95
250	252.2 ± 5.5	2.2	96
overall mean			98

[a] $N = 3$.

Table 2. Repeatability (Within-Day Precision) of Phenol Quantitation in Honey

honey no.	mean concn ± SD (μg/kg)[a]	RSD (%)
1	6.6 ± 1.2	18.2
2	64.7 ± 0.4	0.7
3	158.5 ± 2.1	1.4

[a] $N = 9$.

Table 3. Reproducibility (Between-Day Precision) of Phenol Quantitation in Honey

honey no.	mean concn ± SD (μg/kg)[a]	RSD (%)
1	6.7 ± 0.9	13.4
2	9.3 ± 0.7	7.5
3	55.5 ± 0.7	1.3
4	65.3 ± 2.9	4.5
5	165.4 ± 7.8	4.7

[a] $N = 9$.

Figure 3 shows the chromatogram of a honey sample, declared to be natural acacia honey, containing 12.9 μg/kg of phenol and that of the same sample spiked with 50 μg/kg of phenol.

To study the accuracy of the method, a standard addition procedure was evaluated. Fifteen out of eighteen samples of honey were spiked with phenol at five concentrations, and all samples were subjected to analysis by this method. As indicated in **Table 1**, recovery in spiked samples averaged between 95% and 104%, with an overall mean of 98%.

The precision of the method was assessed by the analysis of repeatability and reproducibility. Within-day precision (repeatability) was good, with relative standard deviation (RSD) values of 0.7−18.2% (**Table 2**). The RSD percentage of between-day precision (reproducibility) ranged from 1.3 to 13.4 (**Table 3**).

The suitability of several HPLC columns for use in phenol quantitation in honey had been tested and finally a LiChrospher 100 RP C18 reversed-phase column (250 × 4 mm, 5 μm particle size) was selected because it did not produce deformed chromatographic peaks even when a large size sample (100 μL) was injected. In addition, no considerable change was observed in the theoretical plate number even after long-term usage of the column, as is demonstrated by the peak parameters of phenol in **Table 4**.

The applicability of the method can be best illustrated by results obtained from the analysis of commercial honey samples. In the years 2000 and 2001, phenol content was determined in 272 samples of honey by the proposed method in our laboratory.

Table 4. Changes in HPLC Column Efficiency after Long-Term Usage

no. of injections	theoretical plate no.	peak width (min) at half-height	asymmetry
576	48 081	0.15	1.26
1857	47 246	0.15	1.17

Phenol concentration was found to be below the generally accepted maximum residue limit of 50 μg/kg in 221 samples (81.25%), 159 of which contained phenol below the 5-μg/kg quantitation limit of the method. Values close to this limit of quantitation are likely to have resulted from the natural background (*4*). In the case of 62 samples, however, beekeepers probably used phenol but proper use of the bee repellent led to phenol contents of less than 50 μg/kg in honey. In contrast, 51 samples (18.75%) contained phenol levels of above 50 μg/kg, and in 15 cases the results even exceeded 500 μg/kg, which was a clear sign of the careless use of phenol in collecting honey. These results suggest considerably lower levels of phenol in honey than those obtained by Canadian authors (*2*, *7*), who determined that as high as 60−65% of all the honey samples tested by them had phenol concentrations exceeding 1 ppm (average 5 ppm), whereas our findings indicate that only 5.5% of honey samples contained more than 0.5 ppm phenol.

In conclusion, a simple method for the quantitation of phenol in honey has been developed, and its accuracy and precision have been tested. The method needs small sample size and offers considerable savings over cost of the conventional methods in terms of sample manipulation and time for analyses. The sample preparation step is accomplished within approximately 10 min without steam distillation or ether extraction procedures, and automatic sampling can be exploited in extending the capacity for analysis with unattended chromatographic operation. Because of its advantages, the newly developed method might be particularly suitable to laboratories where large throughput of compliance samples is obligatory.

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