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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.