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Direct chromatographic methods for the rapid determination of homogentisic acid in strawberry tree (*Arbutus unedo* L.) honey

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Abstract

Two rapid and direct chromatographic methods based on reverse phase-high performance liquid chromatography (RP-HPLC) and ion chromatography (IC) were developed for the determination of homogentisic acid (HA) in honey. This is the marker of the botanic origin of strawberry tree honey. The methods were validated and tested using 22 samples from Sardinia, Italy. The IC method is faster than the RP-HPLC one (6 min versus 13 min of total run), but it is slightly less sensitive (the limit of detection (LOD), is 26 mg kg^{-1} versus 15 mg kg^{-1}) and reproducible (relative standard deviation, RSD, of 10.4 and 4.4%, respectively). The whole dataset of validation parameters allows both the proposed methods to be considered as bias-free (by recovery tests, comparison of analytical results of the two independent methods and analysis of a synthetic sample) and precise (both the techniques show a repeatability better than 2% repeatability in the range between 70 and 600 mg kg^{-1}).

Keywords: Strawberry tree; Honey; Homogentisic acid; RP-HPLC; IC

1. Introduction

One of the most significant parameters for honey quality is its floral origin. The traditional method of investigation, the melissopalynological analysis [1], has shown in the past a number of limitations, especially when applied to honey samples with a low concentration of pollen. In particular, strawberry tree honey, one of the most typical Sardinian honeys famous for its unusual bitter taste, is characterized by a strongly under-represented sediment. This is due both to environmental (it is a honey produced in the late autumn) and botanical (the *Arbutus* flowers are in an upside-down position and this prevents direct nectar contamination) factors. Additionally, the consequences of the application of a recent EC directive [2] allowing the production of filtered honey (i.e. with a reduced amount of suspended particles) probably will diminish interest in a merely palynological approach.

These facts forced scientists to discover relationships between the botanical origin of the honey and the presence of one (or more) particular compounds in it. The efforts are promoted by the EU, which supports the development and adoption of harmonized analytical methods [3] useful to verify compliance with the quality specifications for each kind of honey and to avoid adulterations. In this connection, much experimental work has been done with the aim relating the botanical origin of the honey and qualitative and/or quantitative parameters of its constituents [4]. Among others, phenolic compounds and flavonoids are probably the most promising classes of compounds.

In the literature, there are a number of examples that suggest such compounds as honey botanical origin markers [5–16].

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On the other hand, relatively less work has been published on the composition of organic acids in honey of different botanical origin [17–25]. However, also these investigations appear to be helpful in providing additional information on honey samples from various sources.

In this connection the paper of Cabras et al. [17] that focused specific attention on 2,5-dihydroxyphenilacetic acid (homogentisic acid, HA) is of interest. The authors indicated HA as a specific marker of strawberry tree honey and proposed an analytical procedure for its determination: the analyte is first extracted with ethyl acetate from an aqueous solution and subsequently determined by means of a reverse phase-high performance liquid chromatography (RP-HPLC) method. In our laboratory, evidence was obtained of systematic errors in this procedure. A possible critical point of the proposed method was identified in the analyte extraction step. In particular, the acidity of the honey aqueous solution could not be high enough to allow a quantitative recovery of the analyte in the organic layer. Moreover, possible chromatographic interferences were recognized in most honey samples. On this basis, we think it useful to assess and optimize an alternative, direct chromatographic method for the accurate determination of HA in simply water-diluted honey.

The aims of the present work are: first, to check the possible presence of bias in the literature method and second, the optimization of the chromatographic conditions for the direct and accurate determination of HA in aqueous solution of strawberry tree honey.

2. Experimental

2.1. Materials

2.1.1. Samples

The study was carried out on 22 honey samples from Sardinia, Italy. Some of these samples were provided by the local beekeepers, the others were commercial samples.

2.1.2. Chemicals and solutions

Analytical standard-grade homogentisic acid (with assay >99%) was obtained from Fluka, Buchs, Switzerland. For the preparation of chromatographic mobile phases, water purified by a MilliQ system, Millipore, Bedford, MA, USA, methanol (HPLC grade, Riedel de Haen, Seelze, Germany), sulphuric acid (Merck, Milan), phosphoric acid (Merck, Milan) and $Na_2HPO_4{\cdot}12H_2O$ (Carlo Erba, Milan, 99%) were used. In the optimized RP-HPLC analytical procedure the gradient elution employed a 5×10^{-3} mol L^{-1} sulphuric acid solution in water (solvent A) and methanol (solvent B). The isocratic elution of the ion chromatography (IC) method used a mobile phase constituted by Na_2HPO_4 5×10^{-2} mol L^{-1} in water–methanol (90/10, v/v) pH 6.0 with phosphoric acid. All solvents used were previously passed through a 0.45 μm membrane filter from Millipore to remove any particulate impurities.

Table 1
Mobile phase composition in the RP-HPLC gradient elution of homogentisic acid

Time (min)	Solvent A (5×10^{-3} mol L^{-1} sulphuric acid in water) (%)	Solvent B (methanol) (%)
0–2.0	90	10
9.0	70	30
11.0	70	30
12.0	90	10

2.1.3. Chromatographic instrumentation

The HPLC equipment comprised of a Series 200 binary pump and UV–vis variable wavelength detector (Perkin-Elmer, Boston, MA, USA) equipped with a sampling valve and a 20 μL sample loop (Rheodyne, Rohnert Park, CA, USA).

The RP-HPLC methods were performed on a Spherisorb ODS2 column, 250 mm × 4.6 mm, 5 μm particle size (Alltech, Deerfield, IL, USA) used for verification of the method by Cabras et al. [17], and an Alltima C_{18} column 250 mm × 4.6 mm, 5 μm particle size (Alltech, Deerfield, IL, USA) fitted with a guard cartridge packed with the same stationary phase, for the proposed method. The optimized conditions employed the gradient elution program reported in Table 1, with 1.2 mL min^{-1} flow rate and UV detection at 291 nm operative wavelength.

The separation by ion chromatography method was obtained by means of a Dionex Ionpac AS4A-SC column 250 mm × 4 mm, 13 μm particle size (Dionex, Sunnyvale, CA, USA), using an isocratic elution (flow rate 2.0 mL min^{-1}) and UV detection as above.

Both methods were calibrated by aqueous standard solutions of HA, at least 6, in the calibration range 2.0–100 mg L^{-1}. Data acquisition was accomplished by the Turbochrom Workstation Software (Perkin-Elmer, Boston, MA, USA).

2.2. Sample preparation

Prior to each analytical determination honey was homogenized for 15 min with an Ultra-turrax mixer model T18 (IKA, Staufen, Germany). 1.0 g of homogenized sample is diluted to 10 mL by water and directly analyzed after filtration through a 0.45 μm PVDF filter (Alltech, Deerfield, IL, USA).

3. Results and discussion

3.1. Check of the bias of the literature method

To the best of our knowledge, the pk_{a1} of HA is still unknown. In any case, such a value presumably cannot be too far from that of a molecule with a close structure, 2-hydroxybenzeneacetic acid, which has $pk_{a1} = 4.1$ [26]. It is hence evident that, at a pH value of the aqueous solution