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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

of honey, the condition of the acid–base equilibria does not allow the quantitative extraction of HA in ethyl acetate. This theoretical evaluation has been supported by the relevant experimental data.

First, an exactly weighted amount (between 25 and 125 mg) of HA was dissolved in 500 mL of water in a marked flask (solution 1). 2.0 mL of solution 1 was extracted with 10 mL of ethyl acetate and analyzed by the procedure described by Cabras et al. [17]. Recoveries were between 48 and 57%, depending on the HA concentration in the initial aqueous solution. In addition, similar results were obtained for an eucalyptus honey spiked with 1000 mg kg^{-1} HA.

The presence of a heavy bias in the extraction phase of the literature method has been ascertained, attention was paid to the assessment of direct chromatographic methods, where the always critical extraction step was unnecessary.

3.2. Assessment of a new RP-HPLC method

We first tried to directly analyze a diluted aqueous solution of strawberry tree honey samples: 1g of homogenized honey was diluted up to 10 mL with ultra pure water, filtered through a 0.45 μm filter and immediately injected according to the operative conditions described by Cabras et al. [17]. Unfortunately, all samples analyzed showed severe chromatographic interference, which always prevented us from evaluating the concentration of HA.

Several attempts to completely resolve the chromatographic interference were made using different isocratic or gradient elution methods, based both on the composition of the eluent mixture (methanol–aqueous H_2SO_4 solution) and on different RP-C_{18} analytical columns (see Section 2). Finally, the optimization of the operative conditions, i.e. the adoption of the gradient elution program (Table 1) and the use of an Alltima C_{18} column having a higher density C18 coverage, allowed us to obtain a complete separation of HA from the interference peak to the baseline level (Fig. 1).

Using the operating condition of Table 1, the total run time was 13 min, i.e. 35% less than the literature method.

3.3. Analysis of the honey samples with the new RP-HPLC method

The new RP-HPLC method was tested for all samples of honey. The quantification of HA in honey samples was obtained from the calibration function by analysis of standard aqueous solutions at different concentrations. The standard solution must be prepared daily. The samples were analyzed in duplicate.

Table 2 shows the analytical data.

It can be noted that HA is always present, in quite high amounts (between 70 and 600 mg kg^{-1}), in all samples of strawberry tree honey, while the analyte is always under the detection limit in honey samples of different botanical origin (i.e. multifloral; orange, *Citrus sinensis*; thistle, *Carduus pycnocephalus*; lavender, *Lavandula stoechas* L.; and eucalyptus, *Eucalyptus camaldulensis*).

3.4. Assessment of an independent IC method

The need to validate the RP-HPLC procedure made us decide to develop an alternative analytical method in order to determine HA in honey samples. In particular, it seems to be possible to apply the IC to separate HA from other components present in honey samples. The use of a Dionex Ionpac AS4A-SC column, one of the most widely used in inorganic and organic ion analytical separations and of an isocratic elution with a mobile phase constituted by a Na_2HPO_4 5×10^{-2} mol L^{-1} in water–methanol (90/10, v/v), allowed us to obtain promising results. They were further improved by lowering to 6 the pH value of the mobile phase by the addition of phosphoric acid. Using a flow rate of 2.0 mL min^{-1} the HA retention time was in this connection reduced to 6 min (Fig. 2), and this fact makes the method particularly suitable for screening purposes.

All the honey samples were re-analyzed by the IC method after homogenization, dilution in water and filtration through a 0.45 μm membrane filter. Table 2 also shows these analytical data. HA was present in all the strawberry tree honey samples, in concentrations ranging between 73.16 and

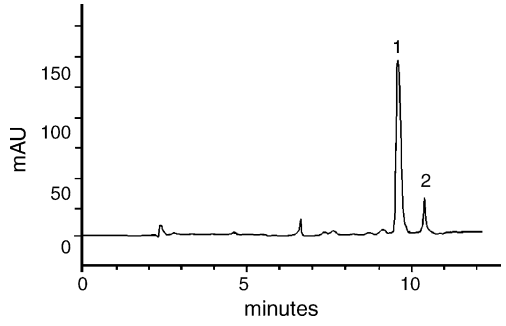

Fig. 1. Chromatogram of an aqueous solution of a typical strawberry tree honey (sample 1) recorded using the optimized RP-HPLC gradient method. Experimental details are in Section 3.2. Peak 1: interference; peak 2: HA.

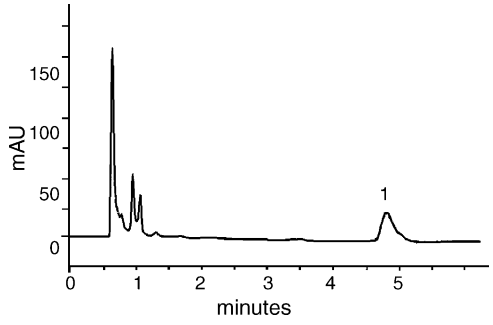

Fig. 2. Chromatogram of an aqueous solution of a typical strawberry tree honey (sample 1) recorded using the IC method. Experimental details are in Section 3.2. Peak 1: HA.

Table 2
Nature and HA level in the strawberry tree honey samples determined by the proposed RP-HPLC and IC methods

Strawberry tree honey sample	C_{HA} (mg kg^{-1}) RP-HPLC	SD (mg kg^{-1})	C_{HA} (mg kg^{-1}) IC	SD (mg kg^{-1})
1	367.0	1.2	367.1	1.0
2	353.1	0.8	351.9	0.2
3	73.13	0.17	80.10	0.02
4	439.2	8.1	441.6	0.2
5	181.9	0.1	181.3	0.7
6	418.4	0.1	416.2	1.3
7	371.6	0.1	369.2	0.6
8	357.1	0.4	354.0	0.0
9	445.3	0.2	444.9	0.5
10	358.6	0.2	360.2	0.8
11	435.0	0.1	438.4	0.4
12	465.7	0.1	466.3	0.3
13	385.5	3.8	390.9	0.2
14	422.1	0.3	427.5	0.1
15	396.8	0.1	396.9	0.8
16	227.4	0.1	230.2	0.7
17	384.2	0.1	373.1	0.5
18	455.7	0.4	436.2	1.0
19	297.0	0.0	292.6	0.3
20	440.6	0.7	445.7	0.2
21	604.4	0.2	577.5	0.8
22	315.1	0.0	305.7	0.8

SD is the standard deviation.

604.4 mg kg^{-1} (RP-HPLC method) and between 80.10 and 577.5 mg kg^{-1} (IC method). The mean values for both methods were 372.5 and 370.3 mg kg^{-1} for RP-HPLC and IC methods, respectively. Curiously, these values are very close to the ones reported by Cabras and coworkers (378 mg kg^{-1}) [17], and this despite the significant bias affecting their analytical procedure. Finally, the analytical values provided by both methods are in excellent agreement: the relative bias is typically less than 1.5%.

3.5. Validation

The limit of detection (LOD) was calculated according to IUPAC guidelines [27] using the confidence interval method through the calibration curves obtained, for both procedures, before every analytical session. The instrumental LOD was 1.5 mg L^{-1} for the RP-HPLC method and 2.6 mg L^{-1} for IC procedure, i.e. 15 and 26 mg kg^{-1}, respectively.

The precision was evaluated through repeatability and reproducibility.

3.5.1. Repeatability

This was obtained from replicate analyses, performed on each sample within the same analytical session, and expressed as the relative standard deviation (RSD, %). The proposed methods are characterized by a repeatability normally lower than 2% in the concentration range 70–600 mg kg^{-1}.

3.5.2. Reproducibility

This was obtained as the relative standard deviation of results for seven analyses of a synthetic sample over a period of time of 2 months and resulted equal to 4.4% for RP-HPLC and 10.4% for IC.

3.5.3. Bias

The bias of both chromatographic methods was evaluated by means of three different approaches: through recovery tests on fortified analytical samples, by comparison of analytical results obtained using the RP-HPLC and IC methods and by the analysis of a control sample.

Recovery tests were performed on four strawberry tree honeys and one eucalyptus honey. After homogenization, each sample was divided in four aliquots (ca. 15 g, exactly weighted). The first aliquot was kept unchanged, whereas 1.5 mL of aqueous solution of HA containing 2000, 4000 and 6000 mg L^{-1}, respectively, were added to the remaining aliquots. After homogenization for 15 min and 1:10 dilution in ultra pure water, the samples were analyzed. The test results, shown in Table 3, indicate excellent recovery factors for both analytical methods.

The dataset obtained by analyzing all honey samples by RP-HPLC technique was compared with that obtained by the IC method (Table 2). The comparison indicates that the two methodologies produce statistically identical results ($p = 95\%$; $t_{exp} = 0.95$, $t_{0.05/2,20} = 2.09$): the average difference between experimental values (evaluated from the slope of the $C_{IC}/C_{RP\text{-}HPLC}$ plot in the concentration range 70–600 mg kg^{-1}) was $0.98 \pm 0.02\%$.

This result suggests the absence of systematic errors (bias) in procedures.

Finally, a control sample was prepared by spiking an aliquot of eucalyptus honey with 210.8 mg kg^{-1} HA after testing the absence of analyte in the matrix. For 7 days, the

Table 3
HA measured concentrations and average recovery values relative to RP-HPLC and IC determination of HA in different honey samples

Honey sample	RP-HPLC				IC		
	C_{HA} in unspiked sample (mg kg^{-1})	C_{HA} in spiked sample[a] (mg kg^{-1})	Recovery (%)	RSD (%)	C_{HA} in spiked sample[a] (mg kg^{-1})	Recovery (%)	RSD (%)
Strawberry tree 1	367.0	864	98.3	0.8	795	90.4	1.0
Strawberry tree 2	353.1	949	109.5	8.9	937	108.1	2.6
Strawberry tree 3	73.13	633	103.4	2.7	575	94.0	9.8
Strawberry tree 4	439.2	905	95.8	3.8	966	102.3	4.9
Eucalyptus	<LOD	521	95.5	1.9	563	103.2	3.9

[a] After spiking with 1.5 mL of aqueous HA solution, $C = 6000$ mg kg^{-1}.

synthetic sample was randomly analyzed using both the chromatographic methods. From the dataset it is again evident that both RP-HPLC and IC methods afford analytical results (202.5 ± 9.0 mg kg^{-1} and 204.3 ± 16.9 mg kg^{-1}, respectively) that are statistically indistinguishable from the true value ($p = 95\%$; $t_{RP\text{-}HPLC} = 0.93$, $t_{IC} = 0.38$, $t_{0.05/2,6} = 2.45$). The lack of any distinguishable trend relative to the analytical value of the sample in the control charts supports the absence of bias in the proposed methods.

4. Conclusions

In this paper, the shortcomings of a procedure for the determination of homogentisic acid, the marker of botanical origin of strawberry tree honey, have been pointed out and discussed. This fact led us to develop two rapid and direct chromatographic methods (RP-HPLC and IC) to overcome the analytical problem. The procedures were tested by analyzing 22 strawberry tree honey samples from Sardinia, Italy. They revealed quite high concentrations of analyte (between 73.16 and 604.4 mg kg^{-1}), whereas HA was always under the LOD in samples of different botanical origin. This confirms the reliability of HA as a specific marker of botanical origin of strawberry tree honey. The validation parameters allow us to consider both the proposed methods as bias-free and precise. In particular, the IC method appears to be faster but slightly less sensitive and repeatable than the RP-HPLC one, and for these reasons can fruitfully be proposed as a screening method.

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