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An RP-HPLC determination of 5-hydroxymethylfurfural in honey The case of strawberry tree honey

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Abstract

The use of the RP-HPLC official method of the International Honey Commission (IHC) for the determination of 5-hydroxymethylfurfural (HMF) in strawberry tree honey (*Arbutus unedo*, a typical Sardinian honey) has brought to light a specific and heavy chromatographic interference that prevents accurate quantification. The interference has been identified as homogentisic acid (HA), i.e. the marker of the botanical origin of the honey. For this reason, an alternative RP-HPLC method is proposed. The bias-free method allows a complete separation of HMF from HA to the baseline level and is faster and more precise than the RP-HPLC official method: the detection and quantification limits are 1.9 and 4.0 mg kg^{-1}, respectively, whereas the repeatability is ca. 2% in the HMF concentration range of 5–140 mg kg^{-1}.

Keywords: Strawberry tree honey; 5-Hydroxymethylfurfural; Homogentisic acid; RP-HPLC

1. Introduction

5-Hydroxymethylfurfural (HMF) and congener compounds are spontaneously formed in carbohydrate-containing foods by the Maillard reaction (the nonenzymatic browning) or the acid-catalyzed dehydration of hexoses. HMF is practically absent in fresh and untreated foods [1], but its concentration tends to rise as a result of heating processes [2] or long-term storage. For this reason, HMF is a recognized parameter related to the freshness and quality of such foods.

Also in honey HMF is one of the most typical products of degradation: it is usually absent in fresh honey, but its concentration tends to increase as the honey ages, as a function of the low pH values, the botanical origin, the humidity and from thermal and/or photochemical stress, until it even reaches levels of some tenths of mg kg^{-1}. Codex Alimentarius (Alinorm 01/25 2000) and the European Union (EU Directive 110/2001) established the maximum HMF level consented in honey as 40 mg kg^{-1}, with the following exceptions: 80 mg kg^{-1} for honey from countries with tropical temperatures, and 15 mg kg^{-1} for honey with a low enzymatic level, respectively. The European quality standards adopt the official analytical methods [3] proposed by the International Honey Commission (IHC): spectrophotometric [4,5] and RP-HPLC [6] methods, that were recently compared [7]. Both spectrophotometer methods are fast but scarcely specific and sensitive; in particular systematic positive interference and the use of *p*-toluidine, a recognized carcinogenic compound, suggest the Winkler method be discarded [4]. On the other hand, the RP-HPLC method is more accurate and sensitive than spectrophotometric ones but quite slow. In short, as Anklam [8] states in his recent important review, ". . .the suitability of the analytical methods for HMF is unsatisfactory and requires further investigation. . .".

Moreover, in recent years the presence of HMF in foods has raised a toxicological concerns: the compound and its similar derivatives (5-chloromethyl and 5-sulphidemethylfurfural) have been shown to have cytotoxic [9], genotoxic [10] and tumoral [11,12] (colon-rectum, hep-

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atic and skin cancers) effects. However, further studies suggest that HMF does not pose a serious health risk [13], but the subject is still a matter of debate.

The growing attention of the scientific community with regard to the potentially toxic effects of HMF requires new efforts to be made to establish new rapid, reliable and sensitive methods to determine the analyte in real matrices. In this context, our research group, experienced in the analytical characterization and quality assessment of typical Sardinian honeys [14,15], decided to conduct research mainly devoted to (i) checking the official RP-HPLC method for HMF determination by analysis of honeys of uncommon botanical origin and (ii) improving the precision, accuracy and time of analysis of such a method.

The most typical of the Sardinian honeys, the strawberry tree honey, famous for its "bitter" taste, was chosen as the principal target matrix. From the chemical viewpoint, the strawberry tree honey appears to have been rarely studied: only a few papers are present in the literature, mainly concerning its physicochemical and melissopalynological characterization [16] and its organic acid profile [17]. In addition, high amounts of phenolic compounds have been found [18]: these are responsible for its high antioxidant property [19]. Recently Cabras et al. proposed a phenolic acid, the 2,5-dihydroxyphenylacetic, homogentisic, acid, HA, as a chemical marker of the botanical origin of strawberry tree honey [20]. In fact, HA is present in quite high amounts (hundreds of mg kg^{-1}) only in this botanical variety of honey.

2. Experimental

2.1. Materials

2.1.1. Samples

The study was carried out on 20 honey samples. Fifteen of them were strawberry tree honeys collected in different geographical areas of France and Italy: Corsica, France (samples 1 and 2), and four Italian regions: Umbria (samples 3 and 4), Tuscany (samples 5–7), Piedmont (samples 8 and 9) and Sardinia (samples 10–15). The last five samples (16–20) were Sardinian honeys of different botanical origin: orange (*Citrus Sinensis*), lavender (*Lavandula stoechas* L.), eucalyptus (*Eucalyptus camaldulensis*), thistle (*Carduus pycnocephalus*) and multifloral, respectively. Some of these samples were provided by the beekeepers, the others were commercial samples. They were produced in different years: sample 9 in 1998, samples 8 and 10–13 in 2000, samples 1, 14 and 3–7 in 2001, samples 2 and 15 in 2002 and the remaining in 2003.

2.1.2. Chemicals and reagents

Analytical standard-grade HMF and homogentisic acid (both with assay >99%) were obtained from Sigma–Aldrich, Milan, Italy and Fluka, Milan, Italy, respectively.

$K_4Fe(CN)_6{\cdot}3H_2O$ and $Zn(CH_3COO)_2{\cdot}2H_2O$ both from Merck, Milan, Italy (assay >99%), were used for the preparation of the Carrez solutions I (a 0.355 mol L^{-1} aqueous solution of $K_4Fe(CN)_6{\cdot}3H_2O$) and II (a 1 mol L^{-1} aqueous solution of $Zn(CH_3COO)_2{\cdot}2H_2O$), reagents utilized in the spectrophotometric determination of HMF with White's method. $NaHSO_3$ was purchased from Sigma–Aldrich, Milan, Italy (ACS reagent).

The chromatographic mobile phase consisted of ultra pure water (Merck, Milan, Italy), methanol (HPLC grade, Rieder de Haen, Milan, Italy) and a properly diluted H_2SO_4 solution (Merck, Milan, Italy). All solvents used were previously filtered through a 0.45 μm membrane, from Millipore, Bedford, MA, to remove any impurities.

2.1.3. Equipment

2.1.3.1. Sample preparation. Prior to each analytical determination the honey was homogenized for 15 min with an Ultra-turrax mixer mod. T18 (IKA, Staufen, Germany).

2.1.3.2. UV–vis. UV/vis measurements were made by means of a double beam spectrophotometer HITACHI Model U-2010 (Hitachi instruments, Milan, Italy), using 1 cm quartz cells.

2.1.3.3. RP-HPLC. The HPLC equipment comprised a Series 200 binary pump, a sampling valve (Rheodyne), a 20 μL sample loop and a Series 200 UV–vis variable wavelength detector, all from Perkin-Elmer, Milan, Italy. The separation was performed on an Alltima C_{18} column 250 mm × 4.6 mm, 5 μm particle size (Alltech, Sedriano, Italy) fitted with a guard cartridge packed with the same stationary phase. Data were elaborated using Turbochrom Workstation Software (Perkin-Elmer, Milan, Italy).

2.2. Literature methods

UV–vis [5] and RP-HPLC [6] determinations of HMF in honey samples were performed according to the procedure described in the literature.

3. Results and discussion

3.1. Comparative evaluation of HMF in honey samples by UV–vis (White) and RP-HPLC official methods

As a first step, the determination of HMF in all honey samples was performed using two of the most precise and reproducible IHC official methods: White's method and the RP-HPLC method. According to the suggestion of Bogdanov [3], we decided not to do use Winkler's method. Table 1 shows the analytical results.

Whereas the analytical data are in fair agreement for samples 16–20, also in consideration of the low amount of analyte present, heavy chromatographic interference prevented

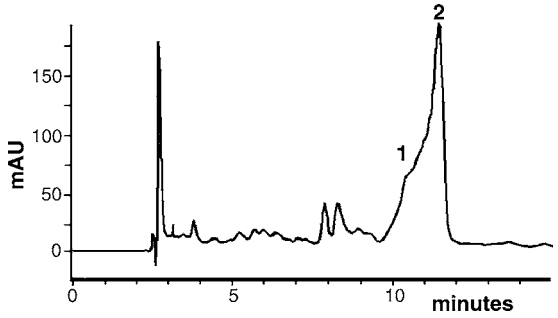

Fig. 1. Chromatogram of a typical strawberry tree honey (sample 1) recorded using the IHC official method. HMF peak (1) and interfering peak (2).

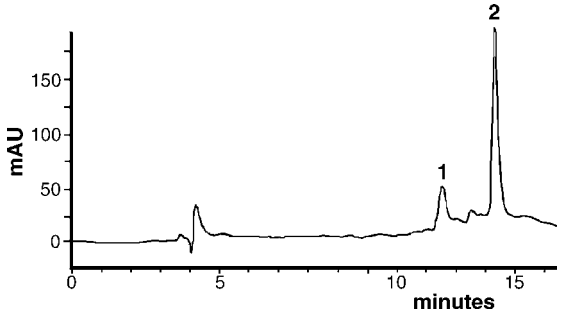

Fig. 2. . Chromatogram of a typical strawberry tree honey (sample 1) recorded using a gradient method (from water–methanol (90:10) (v/v) for 2 min to water–methanol (70:30) (v/v) in 5 min, 2 min of last isocratic step, initial conditions were re-established in 1 min and held for 5 min). Peak 1, HMF, peak 2, HA.

us from evaluating the HMF amount in all the strawberry tree honey samples. Fig. 1 shows a typical chromatogram obtained from a strawberry tree honey.

On the other hand, the interfering peak is completely absent in all five samples of honey of different botanical origin. Moreover, to the best of our knowledge, this interference has not yet been observed in any different varieties of honey. It is hence evident that in the strawberry tree honey a particular component is present, and its chromatographic interference prevents HMF determination by means of the RP-HPLC official method.

This fact shows severe limitation of the RP-HPLC official method. Several authors (Bogdanov [3], Fallico et al. [7]) suggested that this method "...seems to be the more appropriate for HMF determination in honey...", but the discovery of effective interference throws a long shadow on the general applicability of the method. In conclusion, an alternative, sensible, accurate and interference-free chromatographic method is needed.

3.2. Identification of the interfering compound

The extreme specificity of the HA in strawberry tree honey and the close retention times of HMF and HA observed, in the RP-HPLC official method at the typical phenolic acids zone, led us to think that HA could be an authoritative "candidate" as the interfering species in the chromatographic determination of HMF.

Our supposition was confirmed by the following findings:

- The chromatogram of pure HA dissolved in water recorded in the same conditions as the RP-HPLC determination of HMF shows a sharp peak at an RT of 11.46 min, i.e. very close to the HMF one (typical RT = 10.42 min) recorded in the same conditions.
- The spiking of a sample of multifloral honey (in which the HA was previously found absent) with a known amount of HA causes the loss of chromatographic resolution in the HMF zone of the chromatogram.
- The spiking of a sample of strawberry tree honey with a known amount of HA causes the right side of the overlapped peak system to rise.

In conclusion, the compound interfering in the RP-HPLC official method of HMF determination in strawberry tree honey was identified as the homogentisic acid, the marker of the botanical origin of this honey.

3.3. Optimization of a new RP-HPLC method

Several attempts to resolve completely the HMF/HA peaks system using different isocratic or gradient methods based on the water–methanol solvent couple have been unsatisfactory. In particular, a gradient method allowed us to achieve a sufficient resolution between HMF and HA peaks, but the presence of other signals between them, Fig. 2, does not allow a reproducible evaluation of the HMF peak.

Given that HMF and HA have different polarity, and that HA is an acid, its retention time might be strongly influenced by the pH of the mobile phase. The addition of moderate amounts of strong acid (i.e. sulphuric acid) to the eluent mixture could therefore be a decisive factor in optimizing the column selectivity.

Keeping this in mind, different isocratic and gradient methods were tested, varying the operative conditions, mainly flux and composition of the mobile phase (i.e. its concentration in sulphuric acid, water and methanol, ranging between 1×10^{-1} and 1×10^{-3} mol L^{-1} in water, 90 and 50% (v/v), 10 and 50% (v/v), respectively).

Finally, a gradient method described in Fig. 3 allowed us to obtain a complete separation of HMF from HA to the baseline level.

The total run time required was 12 min, i.e. 20% less than the official method [6].

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

Using the new RP-HPLC method, all samples of honey were re-analysed. In order to prevent the HMF breakdown observed by Känzig et al. [21], all analytical samples were previously treated with the Carrez solutions [5]. Just prior to each analytical determination, the sample was fil-

Table 1
Origin and HMF level in the honeys determined by the official methods [5,6] and the alternative HPLC method (with and without treatment with the Carrez solutions)

Sample	Origin	Year	*C* (mg/kg) literature RP-HPLC	*s*[a]	*n*[b]	*C* (mg/kg) White method	*s*[a]	*n*[b]	*C* (mg/kg) RP-HPLC Carrez-treated	*s*[a]	*n*[b]	*C* (mg/kg) RP-HPLC untreated	*s*[a]	*n*[b]
1	Strawberry tree	2001	Not valuable			12.88	1.2	8	12.21	0.33	4	13.04	0.51	10
2	Strawberry tree	2002	Not valuable			16.71	0.32	4	16.82	0.46	3	16.33	0.44	4
3	Strawberry tree	2001	Not valuable			75.7	0.06	2	77.94	0.09	2	85.25	3.01	8
4	Strawberry tree	2001	Not valuable			53.3	2.03	6	51.78	0.4	2	50.8	0.68	2
5	Strawberry tree	2001	Not valuable			71.24	0.62	4	70.02	2.45	4	72.37	0.84	4
6	Strawberry tree	2001	Not valuable			64.2	0.62	4	69.62	1.34	2	70.03	1.17	3
7	Strawberry tree	2001	Not valuable			69.76	4.34	8	72.4	2.83	4	70.01	1.89	6
8	Strawberry tree	2000	Not valuable			99.55	4.52	2	100.23	1.8	6	101.44	1.08	6
9	Strawberry tree	1998	Not valuable			136.92	0.97	4	137.54	3.13	4	138.2	3.29	4
10	Strawberry tree	2000	Not valuable			41.06	0.42	2	36.49	1.06	4	37.11	0.28	4
11	Strawberry tree	2000	Not valuable			45.71	2.28	4	47.8	1.02	4	49.87	1.35	6
12	Strawberry tree	2000	Not valuable			30.93	2.74	8	29.52	0.2	2	32.85	0.44	3
13	Strawberry tree	2000	Not valuable			70.69	3.01	4	75.76	0.05	2	72.13	0.85	2
14	Strawberry tree	2001	Not valuable			25.48	3.25	6	23.41	0.21	3	23.79	0.06	2
15	Strawberry tree	2002	Not valuable			3.42	0.82	4	<LOD[c]		3	<LOD[c]		2
16	Orange	2003	<2	–	2	3.56	0.16	4	<LOD[c]		3	<LOD[c]		4
17	Lavender	2003	<2	–	2	1.28	0.48	4	<LOD[c]		3	<LOD[c]		5
18	Eucalyptus	2003	2.36	0.02	2	3.53	0.2	6	<LOQ[d]		2	<LOQ[d]		3
19	Thistle	2003	3.98	0.14	2	5.43	0.76	5	4.8	0.45	2	5.14	0.2	2
20	Multifloral	2003	9.44	0.72	2	7.69	0.63	6	8.65	0.04	2	7.6	0.33	4

[a] Standard deviation.
[b] Number of replicates.
[c] LOD = 1.9 mg kg^{-1}.
[d] LOQ = 4.0 mg kg^{-1}.

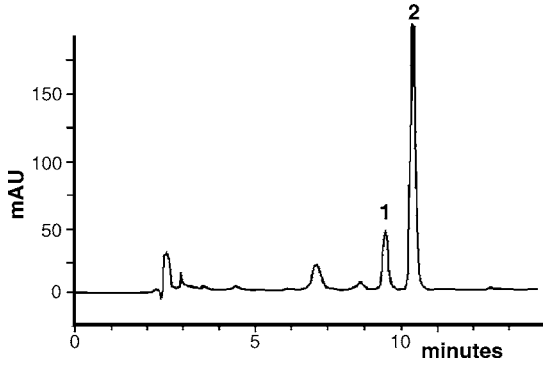

Fig. 3. Chromatogram of a typical strawberry tree honey (sample 1) recorded using the new chromatographic method (gradient from (H_2SO_4 1×10^{-2} mol L^{-1} in water)–methanol (90:10) (v/v) for 2 min to (H_2SO_4 1×10^{-2} mol L^{-1} in water)–methanol (70:30) (v/v) in 5 min, the last isocratic step was continued for 2 min and, finally, initial conditions were re-established in 1 min and held for 5 min, flow rate = 1.2 mL min^{-1}, operative wavelength = 291 nm). Peak 1, HMF, peak 2, HA.

tered through a 0.45 μm membrane. On the other hand, the HMF amount was measured in all samples also after simple dilution in water [6]. Table 1 also shows the analytical data.

3.5. Validation parameters

3.5.1. LOD and LOQ

These parameters were calculated, according to Long and Winefordner [22], by six repeated measurements of an aqueous standard solution at known concentration (0.1 mg L^{-1}). LOD was 0.2 mg L^{-1} for the aqueous solutions and LOQ was 0.4 mg L^{-1}, so the LOD and LOQ values in mg (of HMF) on kg^{-1} (of honey) were 1.9 and 4.0, respectively.

3.5.2. Precision

This was evaluated through repeatability [3,23] and reproducibility [3,23], tested on both Carrez-treated and untreated samples.

Repeatability: this was obtained from replicates of the complete analytical procedure, performed on each of 20 samples and expressed as the relative standard deviation (R.S.D.%). The average repeatability value of the proposed method is less than 2% in the concentration range between 5 and 140 mg kg^{-1}. The highest uncertainty (always less than 10%) was observed in the samples with the lowest HMF concentration (less than 5 mg kg^{-1}).

Reproducibility: this was obtained as the relative standard deviation of results of five analyses of a typical sample (sample 7) over 5 months and resulted equal to 3%.

Table 2 shows the comparison of these data with the validation parameters of the official RP-HPLC and spectrophotometric methods. It is evident that the proposed method is more sensitive and much more precise than all official methods.

Table 3
HMF recovery tests performed on three honey samples

Sample	*N* replicates	Range recovery%	Average recovery%	Average R.S.D.%
1	5	115.6–94.3	101.6	3.2
7	2	102.7–85.5	94.1	5.8
15	2	90.6–92.7	91.6	4.9

See Chapter 3.5.4.1 for additional experimental details.

3.5.3. Linearity

This was measured within the concentration interval between 2 and 800 mg kg^{-1}. A good linear relationship with concentration is observed over the whole range considered slope, intercept and correlation coefficient of the regression straight line resulting equal to 19.54 ± 0.05 AU kg mg^{-1}, 8.27 ± 17.5 AU and 0.999, respectively (AU = arbitrary units).

3.5.4. Bias

The absence of Certified Reference Materials meant we had to estimate the bias through recovery tests [24] and comparison with the results of analyses obtained by independent methods [24].

3.5.4.1. Recovery tests. Nine recovery tests were performed on three samples. After homogenisation, 1.5 mL of an aqueous solution of a freshly prepared, standard solution containing known amounts (200, 400 and 600 mg L^{-1}) of HMF were added to each of three weighted parts (15 g) of the sample. HPLC analysis was performed on samples obtained by diluting 1 g of spiked honey in ten parts of ultra pure water. The tests results are shown in Table 3.

Table 2
Comparison between validation parameters of different methods for HMF determination in honey

	UV–vis (Winkler)[a]	UV–vis (White)[a]	Official RP-HPLC[a]	This method[b]
R.S.D.%	7.9–15.2	3.7–22.0	6.1–10.9	0.1–9.4[c], 0.2–4.3[d]
Repeatability%	5.8–14.6	5.2–23.7	5.0–7.7	<1.8[c], <2.2[d]
Reproducibility%	22.1–42.7	10.5–60.5	17.3–30.8	<3
LOD (mg kg^{-1})				1.9
LOQ (mg kg^{-1})				4.0

[a] Ref. [3].
[b] Concentration range: 5–140 mg kg^{-1}.
[c] Carrez-treated samples.
[d] Untreated samples.

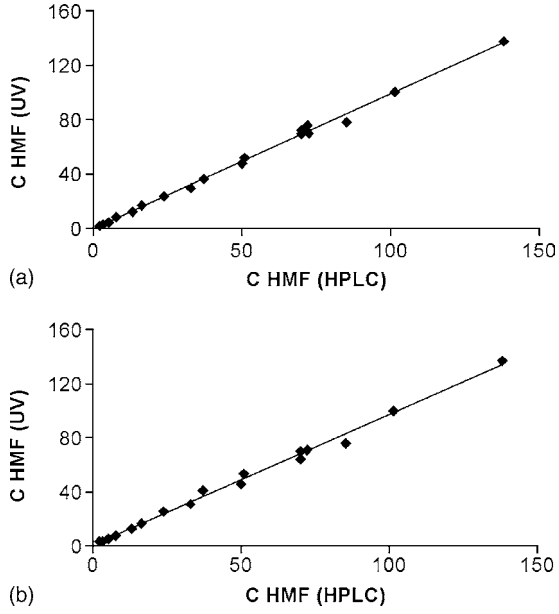

Fig. 4. Linear relationships between HMF concentrations determined by (a) RP-HPLC (Carrez-treated method) and UV method (White); (b) RP-HPLC (untreated method) and UV method (White).

The analysis of data reported in Table 3 shows a very good recoveries for the proposed analytical method.

3.5.4.2. Comparison between independent methods. Both the datasets obtained by analysing untreated and Carrez-treated samples (Table 1) were compared with those obtained by an independent analytical technique, i.e. White's official spectrophotometric method. A comparison between the results obtained on 16 honeys indicates that the 2 methodologies produce statistically compatible values of HMF concentrations: the average difference between experimental results (evaluated from the slope of the $C_{UV}/C_{RP\text{-}HPLC}$ plot in the concentration range 5–140 mg kg^{-1}, Fig. 4) was $0.96 \pm 0.02\%$ and $0.97 \pm 0.02\%$ for Carrez-treated and untreated samples, respectively.

By comparison with White's procedure, the proposed RP-HPLC method cannot therefore be proved to be affected by systematic errors ($p = 95\%$).

4. Conclusions

In this paper the comparative application to strawberry tree honey of two IHC official methods for the determination of HMF revealed specific and heavy chromatographic interference in the RP-HPLC method, completely preventing the quantification of the analyte. The interference, observed only for honey of this botanical origin, was identified as homogentisic acid (HA).

On the basis of these findings, a modified RP-HPLC gradient method is proposed. The method is more rapid, optimises the column selectivity and completely overcomes the interference of HA. Finally, the validation parameters show that the proposed method is sensitive, with a high linearity interval (from 2 to 800 mg kg^{-1}), bias-free (by recovery tests and comparison with data from the spectrophotometric (White's) method) and more precise (repeatability better than 2.5% and reproducibility better than 3% in the HMF concentration range between 5 and 140 mg kg^{-1}) than the RP-HPLC official method.

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