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RP-HPLC determination of water-soluble vitamins in honey

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ABSTRACT

The assessment and validation of reliable analytical methods for the determination of vitamins in sugar-based matrices (e.g. honey) are still scarcely explored fields of research. This study proposes and fully validates a simple and fast RP-HPLC method for the simultaneous determination of five water-soluble vitamins (vitamin B_2, riboflavin; vitamin B_3, nicotinic acid; vitamin B_5, pantothenic acid; vitamin B_9, folic acid; and vitamin C, ascorbic acid) in honey. The method provides low detection and quantification limits, very good linearity in a large concentration interval, very good precision, and the absence of any bias. It has been successfully applied to 28 honey samples (mainly from Sardinia, Italy) of 12 different botanical origins. While the overall amount of the analytes in the samples is quite low (always below 40 mg kg^{-1}), we have observed a marked dependence of some of their concentrations (i.e. vitamin B_3 and vitamin B_5) and the botanical origin of the honey. This insight might lead to important characterization features for this food item.

1. Introduction

Honey is the chief hive product, currently produced in almost every country of the world. Historically it was one of mankind's earliest food sources, and has been valued throughout later civilizations. It essentially consists of a supersaturated solution of sugars, mostly fructose and glucose, but with a significant quantity of minor compounds including polyphenols and flavones, enzymes, organic acids, Maillard reaction products, furanic aldehydes and acids, amino acids and proteins, minerals and water-soluble vitamins.

The qualitative and quantitative characterization of the minor constituents of honey represents a powerful tool to determine its botanical and geographical origin, as well as their effects on physical, chemical and biological properties. For this reason, the development of new analytical methods for the determination of these analytes has received increasing attention in recent years.

The importance of vitamins in nutrition was initially understood in the 1920s and 1930s, and the occurrence of vitamins in honey was studied in the past, mainly by biological assay methods. Chemical methods of analysis were introduced only in the 1940s, particularly for determination of ascorbic acid, i.e. the only one—at that time—considered to be of interest due to its biochemical significance [1]. Moreover, determination of vitamins in foods is often a challenging task due to their instability. Aging, storage conditions, and processing of foods cause vitamin loss, which varies widely depending on the type of food and a number of specific parameters (e.g., temperature, oxygen, light, moisture, pH) [2]. In the case of honey, has been shown that commercial filtration reduces its vitamin content due to the almost complete removal of pollen. Another factor that causes the loss of vitamins in honey is the oxidation of ascorbic acid by hydrogen peroxide produced by glucose oxidase [1]. Until now, only a few methods have been proposed for the determination of vitamins in honey, unlike for most of the other minority organic compounds. Besides the early contribution by Kitzes et al. [3] that reported an array of microbiological methods for the determination of B-group vitamins, almost all methods are devoted to the determination of one specific vitamin. For example, the concentration of ascorbic acid has been evaluated by chromatographic and titrimetric procedures [4–6], whereas riboflavin and its vitamers have been recently analyzed by liquid chromatography [7]. However, to the best of our knowledge, no study devoted to the contemporary determination of the most important water-soluble vitamins in honey has ever been published.

Driven by our interest in assessing and validating analytical methods for the determination of minor compounds and elements in honey [8–12] and honey-based [13] products, we conducted experimental work to fully validate a RP-HPLC method capable of determining five water-soluble vitamins (WSV) in honey: vitamin B_2 (riboflavin), vitamin B_3 (nicotinic acid), vitamin B_5 (pantothenic acid), vitamin B_9 (folic acid) and vitamin C (ascorbic acid).

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2. Experimental

2.1. Samples

The 28 honey samples used in this study were of the following botanical types: 5 from eucalyptus (*Eucalyptus calmadulensis* Denhn), 3 from citrus (*Citrus* spp.), 3 from asphodel (*Asphodelus microcarpus* Salzm.), 3 from sulla (*Hedysarium coronarium* L.), 3 from thistle (*Galactites tomentosa* Moench), 3 from strawberry-tree (*Arbutus unedo* L.), 2 from lavender (*Lavandula* spp.), 2 from acacia (*Robinia pseudacacia* L.), and the last four from linden (*Tilia* spp.), heather (*Erica arborea* L.), rosemary (*Rosmarinus officinalis* L.) and multifloral, respectively. Except for linden and acacia honeys (all from northern Italy), the remaining samples were obtained from local beekeepers (Sardinia, Italy). The attribution of the botanical origin for unifloral honey was confirmed by melissopalinological analysis [14]. All samples were stored at 4 °C from the time immediately following their extraction from the honeycomb to the time of their analysis. Prior to each analytical determination, each sample was homogenized for 15 min with an Ultra-turrax mixer mod. T18 (IKA, Staufen, Germany).

2.2. Chemicals and reagents

All reagents were analytical grade. Riboflavin, D-pantothenic acid hemicalcium salt, folic acid, nicotinic acid and trifluoroacetic acid were purchased from Sigma Aldrich (Milan, Italy). L-Ascorbic acid was obtained from Lancaster (Eastgate White Lund, Morecambe, England), sodium dihydrogen phosphate and sodium hydroxide were obtained from Carlo Erba (Milan, Italy). Acetonitrile (HPLC grade) was purchased from Riedel de Haen (Milan, Italy). Only ultra pure water, purchased from Merck (Milan, Italy), was used throughout each phase of the method.

2.3. RP-HPLC equipment

The HPLC equipment consisted of a Series 200 binary pump, a sampling valve, a 20 μL sample loop and a Series 200 UV–vis variable wavelength detector, all from PerkinElmer, Milan Italy. Separation was performed on an Alltima C18 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 (PerkinElmer, Milan, Italy). Each sample was prepared and injected in triplicate.

2.4. Standard preparation

The stock standard solution was prepared by weighing in a 100 mL volumetric flask, 10.0 mg of vitamin B_2; 25.0 mg of vitamin B_5; 10.0 mg of vitamin B_9 and by adding 40 mL of water. Then, 4 mL of NaOH were introduced. After complete dissolution, 50 mL of phosphate buffer 1 M (pH = 5.5), 10.0 mg of vitamin B_3 and 10.0 mg of vitamin C were added, and the solution was topped up to the mark with water. The standard solution was kept in the dark at 4 °C and was prepared fresh daily.

2.5. Sample preparation

10 g of homogenized honey were weighed and dissolved in 10 mL of ultra pure water. Then, 1 mL of NaOH 2 M (in order to favor the complete solubilization of the honey) and 12.5 mL of phosphate buffer 1 M (pH = 5.5) were added, and the solution was topped up to the mark with ultra pure water in a 25 mL volumetric flask. Sample solutions were injected through a PVDF (13 mm and 0.45 μm) model 6722 filter from Alltech, Sedriano, Italy. Also, the honey solution was stored in the dark at 4 °C until injection.

2.6. RP-HPLC analysis

Chromatographic separation was accomplished by optimizing (in terms of both program of elution gradient and sensitivity) the method described by Heudi et al. [15] previously proposed for measures in polyvitamin premixes. Further details on the assessment and experimental conditions of the chromatographic method are reported in Section 3.1 (optimization of chromatographic method).

3. Results and discussion

3.1. Botanical origin of honey samples

The qualitative melissopalinological analysis was applied to determine the botanical origin of honey samples. In detail, the 5 samples of eucalyptus honey were characterized by overrepresented pollen, reaching more than 90% for *Eucalyptus* type, accompanied by other rare pollens such as *Echium*, *Rubus*, *Trifolium pretense*, *Trifolium repens*, *Cistus*, *Hedysarum* and Cruciferae. The 3 samples of citrus honey showed a relatively low and variable percentage of *Citrus* pollen, with the most significant contaminants being *Carduus*, *Echium* and *Trifolium*. Asphodel honey samples were also characterized by underrepresented pollen, having large, often isolated, grains and a spectrum mainly composed of spring flowering species: *Brassica*, *Cistus*, *Erica*, *Lavandula*, *Pisticia*, *Prunus*, *Acacia*, *Galactites* and *Trifolium*. The spectra of sulla samples showed more than 50% *Hedysarum* pollen, with other contaminant pollens such as *Eucalyptus*, *Cistus*, *Trifolium*, *Echium*, Cruciferae. *G. tomentosa* is the first source of thistle honeys with a pollen content varying from 5 to 25%; pollen spectra included various other species such as *Acacia*, *Borago*, *Brassica*, *Cistus*, *Citrus*, *Echium*, *Eucalyptus*, *Hedysarum*, *Lotus*, *Trifolium*. Strawberry-tree honeys were characterized by underrepresented pollen of *Arbutus* (from about 8 to 20%); moreover, in addition to fall or winter flowering plant species (e.g. *Smilax*, *Hedera*, *Asparagus*, *Inula*, *Rosmarinus* and *Rhamnus*), pollen from spring and summer flowers was found—particularly *Citrus* and *Eucalyptus*. The 2 lavender samples showed underrepresented pollen of *Lavandula* in their sediment with percentages from 6 to 10%, as reported in literature [16]. Even for rosemary, heather, acacia and linden honey samples the pollen spectra correspond to those reported in the schedules of Italian monofloral honeys [16], while *Erica* pollen is dominant in heather honeys (more than 45%). Acacia and linden samples also correspond to the standards reported in the schedules of Italian monofloral honeys [16], while the multifloral one is a typical spring Sardinian product composed of various melliferous species as *Lavandula*, *Asphodelus*, *Echium*, *Trifolium*, *Carduus, Cistus* and *Rosmarinus* [17].

3.2. Optimization of chromatographic method

The excellent contribution of Heudi et al. [15] deals on the RP-HPLC determination of the most important water-soluble vitamins in premixes used for fortification of food products. However, compared to premixes, honey presents a higher matrix complexity. This issue suggested that a careful optimization of the procedure was needed prior to applying it to honey. The optimization work mainly addressed the program of the chromatographic elution and a proper choice of the UV wavelength in order to maximize both resolution and sensitivity. An aqueous solution of trifluoroacetic acid (0.025%, v/v), solution A, and acetonitrile, solution B, were still adopted as components of the mobile phase, whereas the optimized elution program for honey matrix is reported in Table 1.

Table 1
Elution program in the RP-HPLC gradient elution of water-soluble vitamins in honey.

Time (min)	Solvent A (TFA aqueous solution, 0.025%, v/v) (%)	Solvent B (acetonitrile) (%)
0[a]	100	0
5[a]	100	0
11[a]	75	25
11[b]	75	25
19[b]	55	45
20[b]	60	40
22[b]	100	0

Flow rate: 1.0 mL min^{-1}.
[a] Operative wavelength: 254 nm.
[b] Operative wavelength: 210 nm.

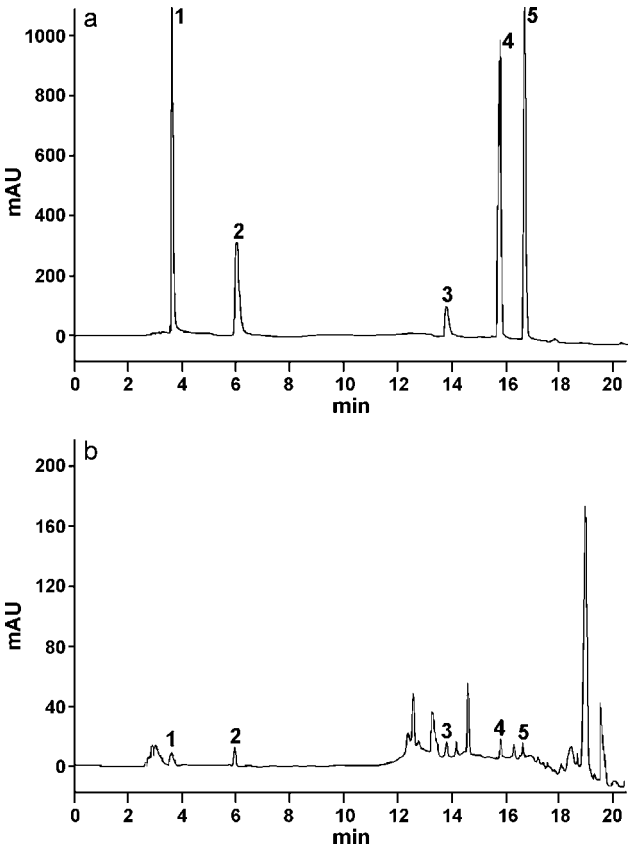

Fig. 1. (a) Chromatogram of a standard mixture of vitamin C (1), vitamin B_3 (2), vitamin B_5 (3), vitamin B_9 (4) and vitamin B_2 (5), each at a concentration of 50 mg L^{-1}; (b) chromatogram of an eucalyptus honey, (1) vitamin C; (2) vitamin B_3; (3) vitamin B_5; (4) vitamin B_9; (5) vitamin B_2.

In addition, a preliminary screening performed on selected samples of honey of all botanical origins considered revealed the substantial absence of four WSV (i.e. vitamin B_1, thiamine; vitamin B_6, pyridoxine; vitamin B_8, biotine; vitamin B_{12}, cyanocobalamine): for this reason our efforts were directed to the optimization of analytical responses of the remaining WSV (i.e. vitamin B_2, riboflavin; vitamin B_3, nicotinic acid, vitamin B_5, pantothenic acid, vitamin B_9, folic acid and vitamin C, ascorbic acid).

The best sensitivity compromise for all analytes was reached using two different operative wavelengths (254 nm for B_3 and C vitamins, and 210 nm for B_2, B_5 and B_9 vitamins). The chromatographic separation was performed at a flow rate of 1.0 mL min^{-1}. The method proposed is rapid: all analytes were completely eluted within 17 min and the whole chromatographic run was completed in 22 min. Fig. 1 shows the chromatograms obtained by analyzing a mixture of standards (Fig. 1a), and an authentic honey sample (Fig. 1b).

3.3. Water-soluble vitamins in honey

The sample analytes were identified by comparison to the retention times of WSV solutions and the attribution was confirmed by spiking the peak in the real sample with a standard solution containing known amounts of pure vitamin. To determine the retention times the reference standards were injected both individually and as a mixture. Quantification was performed by the external standard method based on peak areas of the eluted WSV. For each vitamin, four concentration levels inside the relevant linearity interval were chosen.

Table 2 reports the amount of the WSV in all honey samples analyzed, and Table 3 reports the average WSV concentration for honeys of different botanical origin.

Further, our data confirmed that honey is not a vitamin-rich food: the highest average value of the sum of the concentrations of the five analytes is less than 36 mg kg^{-1} (observed for Citrus honeys), and the total amount of WSV found in the only sample of Rosemary honey analyzed is even less than 4.8 mg kg^{-1}.

Concerning the abundance of each analyte, vitamin C was quantified in almost all honeys; only in two samples (of linden and multifloral origin) its concentration was lower than the LoD. Despite its pervasiveness, vitamin C seems not to be abundant in honey: the highest concentration observed in our study was 5.8 ± 0.5 mg kg^{-1} in a eucalyptus honey, and the average concentration for unifloral honeys ranges from 1.3 ± 0.8 mg kg^{-1} for sulla honey to 3.2 ± 0.7 mg kg^{-1} for eucalyptus honey.

The presence of the vitamin B_3 was detected in all the samples analyzed, even if in five of them (two strawberry tree honeys, and one each of eucalyptus, lavender and rosemary) the concentration was under its LoQ. Generally, the concentration of vitamin B_3 in honey is higher than those measured for vitamin C. In particular, the range of the concentrations measured for vitamin B_3 spans from 27.80 ± 0.04 mg kg^{-1} to 2.1 ± 0.3 mg kg^{-1} for samples of citrus and eucalyptus origins, respectively. This observation suggests that the botanical origin of the honey influences its content of vitamin B_3. Although the low number of available samples for the unifloral honeys kept us from reaching conclusions of general significance regarding this issue, the evident differences in the average concentration of vitamin B_3 for each botanical origin seems to support this hypothesis.

Vitamin B_5 seems to be much less common than vitamin C and B_3. It was quantified only in 12 honey samples and was qualitatively identified in a further nine samples. On the other hand, when vitamin B_5 is present in honey, it often reaches very high concentrations (sometimes the highest among all WSVs); in particular this happened for the three samples of asphodel honey (average vitamin B_5 concentration of 16 ± 6 mg kg^{-1}).

Furthermore, vitamin B_9 and vitamin B_2 were quantified in about half of the samples, and their presence was observed in at least 75% of the samples for the former and 60% for the latter. For both vitamins the highest concentrations observed are under 10 mg kg^{-1}. Despite this, vitamin B_9 is the most abundant WSV in eucalyptus honey (five samples, average concentration of 5.6 ± 0.4 mg kg^{-1}) while meaningful concentrations of riboflavin are always detectable in asphodel and citrus honeys (among three samples, average concentration of 3.7 ± 0.3 mg kg^{-1} and 2.2 ± 0.2 mg kg^{-1}, respectively).

Table 2
Water-soluble vitamins in Sardinian honey samples.

Sample	Water-soluble vitamin concentration in honey (mg kg^{-1} ± SD[a])					
	Origin	B_2	B_3	B_5	B_9	C
1	*Eucalyptus*	<0.25[b]	<0.75[c]	<1.75[c]	6.6 ± 0.3	5.8 ± 0.5
2	*Eucalyptus*	1.49 ± 0.03	2.2 ± 0.7	<1.75[c]	5.4 ± 0.2	2.0 ± 0.3
3	*Eucalyptus*	<0.75[c]	2.86 ± 0.06	5.8 ± 0.7	4.32 ± 0.08	3.0 ± 0.4
4	*Eucalyptus*	2.90 ± 0.08	2.1 ± 0.3	4.4 ± 0.4	4.54 ± 0.02	2.85 ± 0.04
5	*Eucalyptus*	1.90 ± 0.02	3.4 ± 0.3	4.73 ± 0.02	6.92 ± 0.01	2.3 ± 0.1
6	*Sulla*	<0.25[b]	4.9 ± 0.6	4.73 ± 0.09	<0.50[c]	1.5 ± 0.4
7	*Sulla*	<0.25[b]	5.8 ± 0.8	6.5 ± 0.4	<0.15[b]	0.84 ± 0.09
8	*Sulla*	<0.75[c]	3.8 ± 0.2	4.4 ± 0.6	<0.50[c]	1.5 ± 0.7
9	*Citrus*	2.0 ± 0.1	27 ± 2	7 ± 1	<0.50[c]	2.0 ± 0.4
10	*Citrus*	2.6 ± 0.2	27.80 ± 0.04	8.09 ± 0.07	<0.50[c]	1.6 ± 0.4
11	*Citrus*	1.86 ± 0.05	22 ± 1	<1.75[c]	<0.15[b]	3 ± 2
12	*Acacia*	<0.25[b]	6 ± 1	<1.75[c]	<0.50[c]	1.015 ± 0.005
13	*Acacia*	<0.25[b]	4.29 ± 0.02	<1.75[c]	<0.15[b]	1.3 ± 0.2
14	*Asphodel*	4.8 ± 0.2	6.91 ± 0.02	18 ± 1	3 ± 1	4 ± 2
15	*Asphodel*	4.6 ± 0.2	6.71 ± 0.05	12 ± 1	<0.15[b]	2 ± 1
16	*Asphodel*	1.7 ± 0.2	3.7 ± 0.1	19 ± 6	<0.15[b]	1.0 ± 0.1
17	*Thistle*	3.03 ± 0.01	6.6 ± 0.7	<1.75[c]	1.54 ± 0.08	1.4 ± 0.2
18	*Thistle*	9.2 ± 0.2	6.8 ± 0.3	<1.75[c]	2.3 ± 0.2	1.9 ± 0.1
19	*Thistle*	<0.25[b]	12.3 ± 0.2	<1.75[c]	<0.50[c]	3.5 ± 0.2
20	*Strawberry-tree*	2.1 ± 0.6	12.4 ± 0.6	<1.75[c]	0.52 ± 0.03	3.08 ± 0.02
21	*Strawberry-tree*	<0.25[b]	<0.75[c]	28 ± 6	<0.50[c]	4.4 ± 0.8
22	*Strawberry-tree*	<0.25[b]	<0.75[c]	<0.58[b]	<0.15[b]	4 ± 1
23	*Lavender*	1.92 ± 0.01	<0.75[c]	<0.58[b]	<0.15[b]	2.2 ± 0.3
24	*Lavender*	6 ± 1	5.5 ± 0.7	<0.58[b]	3 ± 2	2.3 ± 0.2
25	*Heather*	<0.25[b]	5.92 ± 0.01	<0.58[b]	<0.50[c]	2.7 ± 0.9
26	*Rosemary*	<0.25[b]	<0.75[c]	<0.58[b]	1.7 ± 0.2	1.5 ± 0.2
27	*Linden*	<0.25[b]	7.0 ± 0.3	<0.58[b]	1.28 ± 0.05	<0.10[b]
28	*Multifloral*	1.1 ± 0.5	8 ± 1	<0.58[b]	1.8 ± 0.3	<0.10[b]

$n = 3$.
[a] Standard deviation.
[b] Below its limit of detection.
[c] Below its limit of quantification.

4. Validation

4.1. LoD and LoQ

The limit of detection, LoD, was calculated according to the upper limit approach method, ULA1 [18], approved by the International Union of Pure and Applied Chemistry. For all analytes, each regression line was obtained by analyzing four different aqueous solutions at concentrations very close to the expected LoD. Each measure was performed in triplicate. According to the ULA1 method the limit of quantification, LoQ, is equal to 3 × LoD. As shown in Table 4, LoD values varied from 0.10 mg kg^{-1} (vitamin C) to 0.58 mg kg^{-1} (vitamin B_5), while LoQ values ranged from 0.30 mg kg^{-1} (vitamin C) to 1.75 mg kg^{-1} (vitamin B_5).

The high LoD value obtained for vitamin B_5 is due to its low UV absorbtivity with respect to the remaining WSVs.

4.2. Linearity

Linearity was explored within a range of three to four decades of concentration, spanning for each WSV from values close to the LoQ to hundreds of mg L^{-1}. As shown in Table 4, very good correlation coefficients (R^2) were observed for all vitamins, ranging from 0.9929 (vitamin B_3) to 1 (vitamin B_2). These observations also exclude any deviation from linearity for analyte amounts that largely exceed the concentrations usually found in honey.

Table 3
Average concentration of water-soluble vitamins in Sardinian honey of different botanical origin.

Origin	Average water-soluble vitamin concentration in Sardinian honey (mg kg^{-1} ± SD[a])					
	B_2	B_3	B_5	B_9	C	Σ[b]
Eucalyptus ($n = 5$)	<1.458	<2.262	<3.686	5.6 ± 0.4	3.2 ± 0.7	<16.2
Sulla ($n = 3$)	<0.417	5 ± 1	5.2 ± 0.7	<0.383	1.3 ± 0.8	<12
Citrus ($n = 3$)	2.2 ± 0.2	26 ± 2	<5.613	<0.383	2 ± 2	<36
Asphodel ($n = 3$)	3.7 ± 0.3	5.8 ± 0.1	16 ± 6	<1.1	2 ± 2	<28
Acacia ($n = 2$)	<0.25	5 ± 1	<1.75	<0.325	1.2 ± 0.2	<8.5
Lavender ($n = 2$)	4 ± 1	<3.125	<0.58	<1.575	2.2 ± 0.4	<11.5
Thistle ($n = 3$)	<4.16	8.6 ± 0.8	<1.75	<1.447	2.3 ± 0.3	<18.3
Strawberry-tree ($n = 3$)	<0.87	<4.633	<10.11	<0.39	4 ± 1	<20
Heather ($n = 1$)	<0.25	5.92 ± 0.01	<0.58	<0.50	2.7 ± 0.9	<10.0
Rosemary ($n = 1$)	<0.25	<0.75	<0.58	1.7 ± 0.2	1.5 ± 0.2	<4.8
Linden ($n = 1$)	<0.25	7.0 ± 0.3	<0.58	1.28 ± 0.05	<0.10	<9.2
Multifloral ($n = 1$)	1.1 ± 0.5	8 ± 1	<0.58	1.8 ± 0.3	<0.10	<11.6

[a] Standard deviation.
[b] Sum of average concentrations of water-soluble vitamins for honey of the same botanical origin.

Table 4
Validation data.

Vitamin	Sensitivity		Linearity		Repeatability		Reproducibility		Bias
	LoD (mg kg^{-1})	LoQ (mg kg^{-1})	Concentration range (mg L^{-1})	R^2	$CV\%_{exp,r}$[a]	$HorRat_r$[b]	$CV\%_{exp,R}$[c]	$HorRat_{exp,R}$[d]	Recovery (%±SD[e])
B_2	0.25	0.75	0.01–100	1	2.6	0.32	19.8	0.98	100 ± 10
B_3	0.25	0.75	0.05-500	0.9929	6.9	0.76	18	1.06	99 ± 7
B_5	0.58	1.75	0.5-1000	0.9993	7.6	0.91	7.6	0.55	103 ± 7
B_9	0.15	0.50	0.05-100	0.9999	2.9	0.37	12.8	1.00	98 ± 8
C	0.10	0.30	0.05-500	0.9989	7.3	0.76	3.3	0.20	104 ± 3

[a] Experimental coefficient of variation of repeatability.
[b] The ratio between $CV\%_{exp,r}$ and the theoretical repeatability data ($CV\%_{H,r}$) according to Horwitz's theory.
[c] Experimental coefficient of variation of reproducibility.
[d] The ratio between $CV\%_{exp,R}$ and the theoretical reproducibility data ($CV\%_{H,R}$) according to Horwitz's theory.
[e] Standard deviation.

4.3. Precision

Precision was determined by means of repeatability and reproducibility measures.

Repeatability was evaluated through five consecutive repetitions of the analysis of the same honey sample, and expressed in terms of experimental variation ($CV\%_{exp,r}$). Average repeatability ranged from 2.6% (vitamin B_2) to 7.6% (vitamin B_5).

Reproducibility was verified through five repetitions of the analysis of the same honey sample, performed over different analytical sessions, within 1 month. It was expressed by the experimental variation, $CV\%_{exp,R}$. Reproducibility values ranged from 3.3% (vitamin C) to 18% (vitamin B_3). Precision data are reported in Table 4.

In order to eliminate any uncertainty regarding the acceptability of the level of precision of the data, a fitness-for-purpose evaluation based on Horwitz's theory [19] was used. According to this theory, it is possible to calculate a theoretical CV% (CV% H) value on the basis of the concentration level expected and to compare it with the experimental CV% ($CV\%_{exp}$). If the ratio between experimental and theoretical CV% (HorRat ratio) is less than 1.5 the precision level of the experimental data can be considered acceptable. Table 4 shows that HorRat ratio values for repeatability and reproducibility are always lower than 1.5 for all WSVs. Therefore, the reproducibility parameters can be considered acceptable.

4.4. Bias

Due to the lack of certified reference materials and independent analytical methods, recovery tests were performed to check the absence of bias in the proposed method. After homogenization, 40 g of a honey sample were divided into four 10 g aliquots. Aliquots 2, 3, and 4 were treated with a suitable and increasing volume of a freshly prepared bulk solution containing 100 mg L^{-1} of water-soluble vitamins, while no addition was made to aliquot 1. Each aliquot underwent all the analytical procedures previously described and was analyzed in triplicate. The procedure always produced quantitative average recoveries (criteria: two-tail *t*-test, $p = 0.95$), as reported in Table 4.

On the basis of validation results discussed, the proposed method can be considered accurate.

5. Conclusions

For the first time, a quick and simple RP-HPLC method to simultaneously determine five water-soluble vitamins (B_2, B_3, B_5, B_9 and C) in honey has been developed and validated in terms of sensitivity, linearity, precision, bias and accuracy. Low detection and quantification limits, very good linearity in large concentration intervals, acceptable precision, and absence of any bias are the key criteria of the validation protocol. The proposed method was successfully applied to 25 Sardinian honey samples of 10 different botanical origins, in addition to three monofloral honeys (acacia and linden) from northern Italy. The amount of WSV found in honey is quite low: the overall concentration of the five analytes never exceeded 40 mg kg^{-1} and often appeared to be significantly dependent on the botanical origin of the matrix. Vitamin C and vitamin B_3 were present in almost all the honey samples. Interestingly, the concentration of vitamin C was generally low (the highest concentration detected was slightly over 5 mg kg^{-1}) and invariant with respect the origin of the honey (probably due to the aforementioned interactions between ascorbic acid, glucose oxidase and catalase in honey). In contrast, the concentration of vitamin B_3 was observed to be as high as ca. 28 mg kg^{-1} and it seemed to be strongly dependent on the botanical origin of the samples. The quantification of vitamins B_5, B_2 and B_9 was successful only in ca. 50% of the samples analyzed: while vitamin B_5 can reach concentrations of some tens of mg kg^{-1} in honeys of selected botanical origin (i.e. asphodel honey), the concentrations of vitamins B_2 and B_9 are always below 10 mg kg^{-1}. We can presume an influence of the origin of the samples on the concentration of these analytes as well, but the low number of samples analyzed keeps us from drawing such a conclusion at this time. In conclusion, although the concentration of WSV in honey may be too low to generate interest in the field of nutrition, its potential correlation to the botanical origin of the samples may prove useful to determine the origin of honeys.

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