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Analysis of Water-Soluble Vitamins in Honey by Isocratic RP-HPLC

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Abstract An isocratic RP-HPLC method has been developed for the identification and quantification of water-soluble vitamins in honey. The mobile phase tested was an aqueous solution of sulphuric acid and the detection was carried out simultaneously by UV and fluorescence. The separation of vitamins C (L-ascorbic acid), B_1 (thiamine), B_3N (nicotinamide), B_3H (nicotinic acid), B_5 (D-pantothenic acid) and B_6 (pyridoxine) is achieved in these conditions in 15 min. The addition to the mobile phase of methanol 2 %v/v reduced significantly the analysis time in the separation of these vitamins up to 10 min. Moreover, in presence of a cationic surfactant hexadecyltrimethylammonium bromide (CTAB) in the mobile phase, the separation of vitamin C, B_1, B_3N, B_3H, B_2 (riboflavin) and B_6 is possible in 6 min. The combination of both mobile phases, H_2SO_4/methanol and H_2SO_4/methanol/CTAB, has been applied to the analysis, in isocratic mode, of several monofloral honeys (rosemary, thyme, lavender, chestnut, echium) and a honeydew honey in a short time analysis.

Keywords Water-soluble vitamins · Honey · HPLC · Cationic surfactant · CTAB

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Introduction

Honey is the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants, the living parts of plants secretions or the excretions of plant-sucking insects on the living parts of plants. The bees collect and transform these secretions by combining with specific substances of their own. Next, they deposit, dehydrate, store and leave them in honeycombs to ripen and mature (European Union 2001). Honey contains at least 181 substances; it is a supersaturated solution of sugars and their content of water is about 18 %. Other minor components are minerals, organic acids, proteins, free amino acids, enzymes, flavonoids, phenolic acids and vitamins (White 1979; Sato and Miyata 2000; Bogdanov et al. 2004; Álvarez-Suarez et al. 2010; Lachman et al. 2010).

At present, there is an increasing commercial interest to produce unifloral honeys due to the therapeutic uses of certain honey varieties. It would be important for the customers to have nutritional information of honey on the label, which is regulated for all foodstuffs by European Community (European Union 1990, 2001, 2006, 2008).

The vitamin's content in honey is mainly water soluble due to its aqueous nature containing a high quantity of sugars and a low percentage of lipids. Vitamin C has been specially determined in honey because of its antioxidant effect (Castro et al. 2001; Gheldof et al. 2002; Hayakawa et al. 2008; León-Ruiz et al. 2011) and it is often found in almost all honeys. Only Casella and Gatta (2001) in one orange honey sample, Álvarez-Suárez et al. (2010a, b) in six honey samples and Castro et al. (2001), in spite of a high number of honeys studied, did not detect the presence of ascorbic acid in any sample. Vitamin C analysis is one of the indicators more often used to give the nutritional quality of a food since it is very vulnerable to chemical and enzymatic oxidation, accelerated further by factors such as light, oxygen or heat.

In the last 10 years, very few references appear on the determination of vitamins in honeys, and the analytical

methods have been developed for the determination of only one specific vitamin or a few of them. Anyway, it is not possible to find a determination of all water-soluble vitamins in honey with the same analytical method. In fact, Viñas et al. (2004a, b) have determined the different structures of vitamin B_6 and vitamin B_2. Vitamin B_6, as pyridoxine, is detected in one eucalyptus honey and in another multifloral honey at levels lower than 0.021 mg/100 g. Vitamin B_6 was not detected in rosemary or orange blossom honey. On the other hand, vitamin B_2, as riboflavin, is detected in chestnut tree honey, eucalyptus, cane and multifloral at levels up to 0.074 mg/100 g.

Ciulu et al. (2011) have determined vitamins C, B_2, B_3, B_5 and B_9. However, in this work, neither vitamin B_1 nor vitamin B_6, which often appears in some honeys, was not analysed. They have developed a RP-HPLC gradient mode to determine five water-soluble vitamins in honeys, with an analysis time of 18 min. The presence of vitamin B_3H was detected in all samples analysed, but in some of them the concentration was under its limit of detection. Vitamin B_9 and vitamin B_2 were quantified in about half of the samples, and vitamin B_5 seems to be much less common than the other vitamins.

The aim of this work is the study of the vitamin's composition of the Protected Denomination of Origin *La Alcarria Honey* (multifloral, rosemary and lavender) located in central Spain, using a fast and simple chromatographic method working in isocratic mode. The quantification of the vitamins content in different honeys allows their characterisation in function of their types and the discrimination between them which is very important in the quality controls of honey. Other interesting honeys which are not into the Denomination *La Alcarria Honey*, as chestnut, echium and thyme, have also been studied.

Materials and Methods

Reagents and Solutions

All reagents were of analytical grade. The reagents were D-pantothenic acid, L-ascorbic acid and nicotinamide (Fluka, Madrid, Spain); folic acid, nicotinic acid, pyridoxine hydrochloride, thiamine hydrochloride and riboflavin (Sigma, Barcelona, Spain); *n*-butanol, ethanol, methanol and *n*-propanol (Scharlab, Barcelona, Spain); hexadecyltrimethylammonium bromide (CTAB) (Merck, Darmstadt, Germany); polyoxyethylene(23)laurylether (Brij-35) (Aldrich Madrid, Spain), sulphuric acid (Merck, Darmstadt, Germany) and ortho-phosphoric acid (Panreac, Barcelona, Spain).

Standard solutions of thiamine (B_1), nicotinamide (B_3N), nicotinic acid (B_3H), D-pantothenic acid (B_5), pyridoxine (B_6) and L-ascorbic acid (C) were dissolved in ultra-pure water. Riboflavin (B_2) was dissolved in acid media (0.5 mL of H_3PO_4 conc. in 25 mL ultra-pure water) and the folic acid (B_9) in alkali media (0.2 mL of NaOH 0.1 M in 25 mL ultra-pure water). All solutions were stored in darkness at 4 °C.

Equipment

The chromatographic system used is equipped with a pump model 250 (Perkin-Elmer), a thermostatic oven Jet-Stream Plus (Knauer), 20-μL injection valve (Rheodyne) and two detectors: a programmable UV/VIS detector model 785A (Applied Biosystems) and a fluorescence detector 200 (Perkin-Elmer). An interface 950 (Perkin-Elmer) was used to transfer data to the computer. Two chromatographic columns μBondapack C_{18} 10 μm, 150×3.9 mm from Waters were used. One of them was previously saturated with CTAB. Mobile phase flow was 0.9 mL/min.

The pH measures were realised in a pH-meter model 781 pH/Ion Meter from Metrohm.

Mobile Phase Selection

Firstly, a simple mobile phase was prepared with H_2SO_4 0.01 % (v/v) at pH 2.5. The next mobile phases were obtained from this one but modifying different parameters: pH (ranged between 2.5 and 3.7), temperature (ranged between 25 °C and 40 °C) and finally by addition of a low percentage of a short chain alcohol as methanol (MeOH), ethanol (EtOH), *n*-propanol (*n*-PrOH) and *n*-butanol (*n*-BuOH). The alcohol percentages were varied from 1 % to 10 % (v/v).

In a second study, a surfactant was added to the mobile phase to improve the separation. The surfactants tested were one non-ionic (polyoxyethylene-23-laurylether, Brij 35) at a concentration of 5 mM and in presence of methanol 2 % (v/v) and another cationic (hexadecyltrimethylammonium bromide, CTAB) at concentrations ranging between 10^{-4} and 0.02 M, lower and higher than its critical micellar concentration. The separation was studied also in presence of MeOH, *n*-PrOH and *n*-BuOH ranged between 1 % and 10 % (v/v).

Analytical Features

The analytical features of the chromatographic method were determined for the mobile phase in absence of CTAB [H_2SO_4 0.01 %/MeOH 2 % (v/v) at pH 3.5 and 25 °C] and in presence of the surfactant [H_2SO_4 0.01 %/CTAB 0.01 M/MeOH 2 % (v/v) at pH 2.75 and 25 °C]. In both cases, the analytical characteristics of the chromatographic method were obtained ranging the water-soluble vitamins concentrations from 0.05 to 10.0 mg/L (thiamine and pantothenic acid), 0.025 to 10.0 mg/L (pyridoxine), 2×10^{-3} to 62 mg/L (riboflavin), 0.2 to 100 mg/L (nicotinamide and nicotinic acid) and 0.1 to 800 mg/L (vitamin C). The analytical wavelength in UV detection was 254 nm changing to 200 nm at 7.5 min for the

detection of vitamin B_5. Fluorescence detection wavelengths for vitamin B_6 and vitamin B_2 were 290/396 nm and 450/520 nm excitation/emission, respectively.

The validation of the chromatographic method has been realised and the validation analytical parameters estimated for all vitamins.

The calibration method, fitting peak area with the quantity of vitamin injected, was used to determine the sensitivity, limit of detection and limit of quantification. The sensitivity of the method was defined as the slope of calibration graph. The limit of detection, LOD, was calculated as the amount of vitamin that yields a signal equal to three times the standard deviation of intercept, s_a. The same definition was used for the limit of quantification, LOQ, adding ten times the s_a value (Miller and Miller 2005). The robustness was calculated as the relative standard deviation (RSD) corresponding to the slope of the calibration curve measured in four different days.

Finally, precision of retention time and peak area of each vitamin was determined by means of repeatability and reproducibility measures. In all cases, precision was expressed in terms of relative standard deviation, RSD.

The accuracy of the method was evaluated by the addition of a known quantity of B group vitamins standards to the honey samples and calculating the difference between its content in absence and in presence of the standard. The recovery is given in percentage. In the case of vitamin C, the accuracy was determined by comparison with an official method.

Vitamin Determination in Honey Samples

The honey samples analysed in this study were one honeydew and six monofloral honeys. The honey type of nectar samples were as follows: two thyme honeys (*Thymus vulgaris* L.), one rosemary (*Rosmarinus officinalis* L.), one chestnut (*Castanea sativa* Mill.), one lavender (*Lavandula latifolia* L.) and one echium (*Echium vulgare* L.). All honeys were directly provided by the beekeepers and had not been industrially processed. They were stored at −20 °C until analysis.

Samples were prepared by dissolving an amount between 5 g and 15 g of honey in 25 mL of ultra-pure water, and filtered through a nylon syringe filter of 0.45 μm prior to their injection in the chromatographic system. The vitamins B_1, B_3H, B_3N, B_5 and B_6 were analysed using the mobile phase H_2SO_4 0.01 %/MeOH 2 % (v/v) at pH 3.5 and 25 °C, and vitamins C and B_2 were analysed with the mobile phase H_2SO_4 0.01 %/CTAB 0.01 M/MeOH 2 % (v/v) at pH 2.75 and 25 °C.

Results and Discussion

This work has been developed to separate water-soluble vitamins in honey samples with a chromatographic method in isocratic mode as a fast way to be used in quality control of honeys. The chromatographic method should separate the water-soluble vitamins with a good resolution in the shortest time possible.

The first mobile phase used was H_2SO_4 0.01 % (v/v)/pH=2.5 at 25 °C, which had been utilised by our research group in the determination of vitamin C in honeys (León-Ruiz et al. 2011), and its possibilities were investigated in the separation of B complex vitamins. This mobile phase in the experimental conditions mentioned above gives a poor separation of vitamins C, B_1, B_3N, B_3H, B_5 and B_6, with bad resolution between the different peaks. In addition, vitamins B_2 and B_9 are not being eluted in a reasonable analysis time. In the case of vitamin B_2, this fact has high importance due to its usual presence in honey types. From these results, the mobile phase was modified to improve the vitamin separation.

Influence of pH, Temperature and the Addition of a Short-Chain Alcohol to a Mobile Phase H_2SO_4 0.01 % (v/v)

In order to achieve the separation of the vitamins reducing the retention time of vitamins B_2 and B_9, the influence of pH and temperature was studied on the chromatographic separation.

The pH has been varied between 2.5 and 3.7 values. The vitamins have very different chemical structures, and for this reason the influence of pH is very different over each one. In some case, such as the pairs B_1–B_3H and B_3N–B_6, the elution order is reversed around 3.2 and 3.0, respectively. From the differences observed in the retention factors for each vitamin as a function of pH, the best separation conditions are obtained at pH=3.5. Once the value of pH was setting, the temperature was varied between 25 and 45 °C. As it is expected, an increase in temperature produces a decrease in the retention times of vitamins. When the temperature reaches high values, the chromatographic peaks corresponding to nicotinic acid (B_3H) and thiamine (B_1) lose resolution, and they are overlapping completely at 40 °C. For this reason, and taking into account that an increase in temperature would produce the degradation of vitamins, 25 °C was selected in the rest of the work.

Therefore, the mobile phase H_2SO_4 0.01 M/pH 3.5 at 25 °C provides the separation of six water-soluble vitamins in 16 min. The chromatogram of this separation is shown in Fig. 1a. The analysis time is lower than those reported before in isocratic mode with hydro-organic mobile phases (Ekinci and Kadakal 2005) or by Micellar Liquid Chromatography in presence of SDS/*n*-pentanol (Monferrer-Pons et al. 2003; Capella-Peiró et al. 2004). Nevertheless, using this mobile phase, it is not possible to elute the vitamins B_2 and B_9 in a short time lower than 30 min since these vitamins are the most hydrophobic of the water-soluble vitamins and they have a long retention at any pH and temperature if a low content of

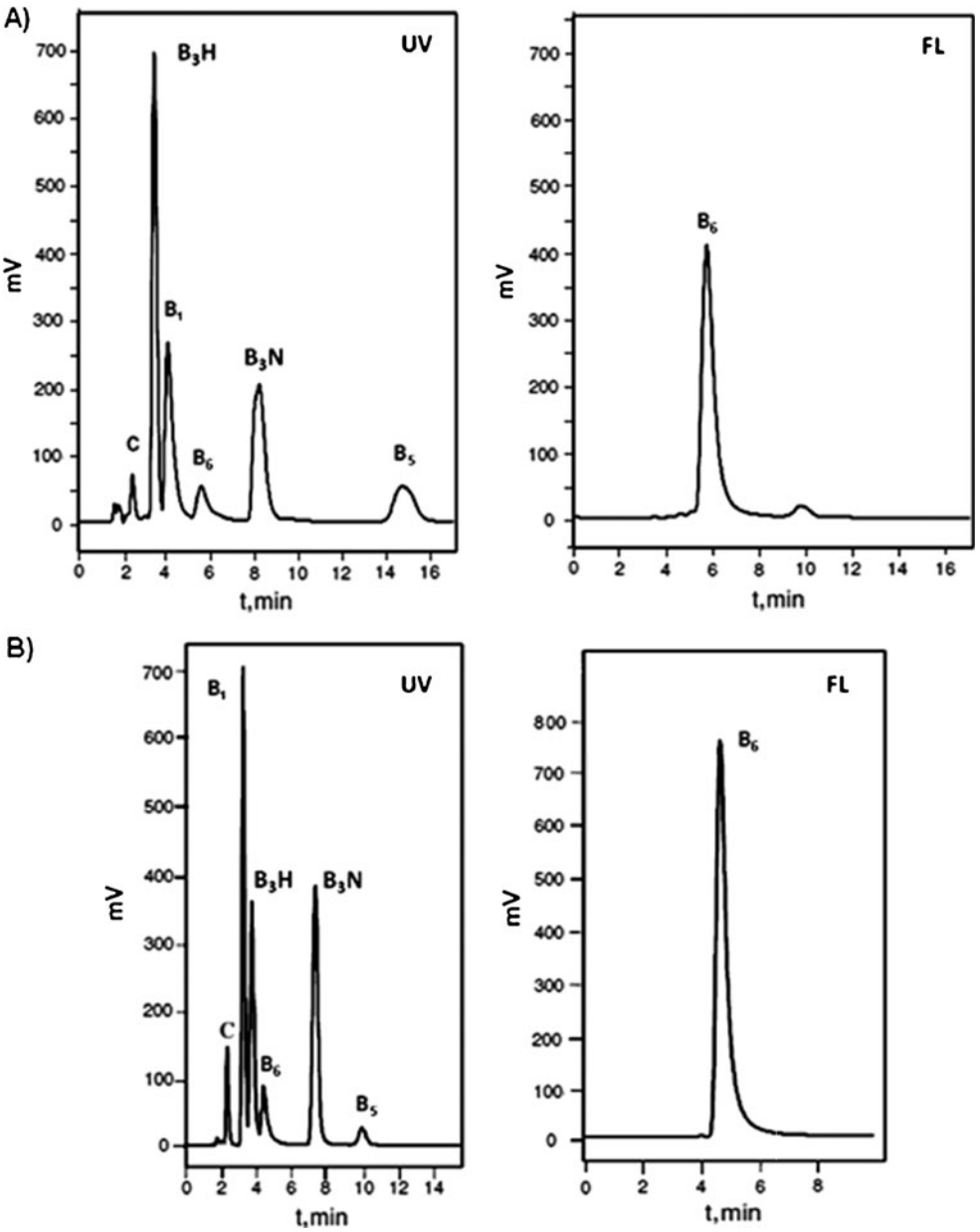

Fig. 1 Chromatographic separation of vitamins **a** with a mobile phase H_2SO_4 0.01 %, pH=3.5 at 25 °C and **b** with a mobile phase H_2SO_4 0.01 %/ MeOH 2 %, pH=3.5 at 25 °C with UV and fluorescence detection in each case

organic modifier is used in the absence of a surfactant. For this reason, in Fig. 1 these vitamins are not separated.

In the next step in this study, when a short-chain alcohol (MeOH, EtOH, *n*-PrOH and *n*-BuOH) is added to the mobile phase, *n*-butanol is the only one that allows the elution of vitamin B_2 in a short time with 8.3 %v/v, but the peaks of the other six vitamins are overlapped at a column dead time. The best improvement in the separation with the other alcohols between 2 % and 10 %v/v is obtained in the presence of 2 %v/v MeOH. The chromatogram is obtained with the alcohol in a shorter analysis time (10 min versus 15 min) with good resolution as it is shown in Fig. 1b. It is also important to indicate the change in the elution order of thiamine (B_1) and nicotinic acid (B_3H), produced by the presence of methanol in the mobile phase with respect to its absence. On the other hand, the pantothenic acid molecule (vitamin B_5) does not contain a characteristic chromophore group and hence it exhibits only very weak absorbance at 200 nm owing to the presence of carbonyl groups (Blake 2007). This vitamin was detected in UV spectrum at 200 nm changing the wavelength detection at 7.5 min.

As a summary of this study, it can be said that a mobile phase containing H_2SO_4 at pH=3.5, with or without methanol, allows the separation in isocratic mode of six vitamins: ascorbic acid (C), thiamine (B_1), nicotinamide (B_3N), nicotinic acid (B_3H), pantothenic acid (B_5) and pyridoxine (B_6). However, vitamin B_2, which is very important in honey analysis, cannot be eluted in an appropriate time with this mobile phase. Therefore, in the next step in this study, it was proceeded to introduce a surfactant in the mobile phase, with the aim to attempt the separation of the originally proposed eight vitamins including vitamin B_2.

Study of Vitamin's Separation using a Surfactant in the Mobile Phase

As it is well known, in presence of a surfactant, in the mobile phase the molecules are located between the stationary phase, the formed micelles and the bulk mobile phase. This fact modifies their retention in the chromatographic system and yields unique selectivity for many compounds (Armstrong and Nome 1981; Ruíz-Ángel et al. 2009a, b). The presence of a surfactant in the mobile phase was used to separate vitamin B_2 from the other vitamins in an appropriate analysis time.

In a previous study realised by our research group, the separation of six water-soluble vitamins (B_1, B_2, B_3H, B_3N, B_6 and B_9) using an elution gradient was achieved in the presence of an anionic surfactant, sodium dodecylsulfate, SDS, and *n*-propanol in 11 min (Almagro et al. 2002). This surfactant offers a good separation in gradient elution, but it is not possible in isocratic mode. For this reason, and looking for a different surfactant that allows the separation in isocratic mode, it was tried on with a non-ionic [polyoxyethylene(23)lauryl eter, Brij 35] and another cationic (hexadecyltrimmethylammonium bromide, CTAB) surfactants in concentrations above their critical micellar concentration which are 9×10^{-5} M for Brij 35 and 9×10^{-4} M for CTAB (Mittal and Lindman 1984).

The non-ionic surfactant was added to the mobile phase in a concentration 5 mM, and it does not offer good separation possibilities since all peaks of the vitamins appear at retention times close to the column dead time. The use of this surfactant was also tested in presence of MeOH 2 %, and the separation of the vitamins was not possible.

The cationic surfactant CTAB was added to the mobile phase in concentrations that ranged between 10^{-4} and 0.02 M, but for concentrations lower than 5 10^{-3} M the chromatogram baseline is not stabilised until at least 2 h, and the repeatability between chromatographic injections is bad. From the separations obtained with the different surfactant concentrations, it is found that 0.01 M is the minimum surfactant concentration that achieves the stabilisation of the column in a reasonable time, and it also could separate an acceptable number of water-soluble vitamins. Therefore, 0.01 M was the chosen surfactant concentration to separate the vitamins. However, the obtained peaks are wide and they have low efficiencies. To solve it, an organic solvent is also added to micellar mobile phases to improve peak efficiencies and to reduce retention times, giving rise to the so-called hybrid micellar mobile phases. In this study, the same alcohol in low quantity (methanol 2 %v/v) and the same than in absence of surfactant was chosen to improve peak efficiency and the separation. Thus, the first mobile phase tested was H_2SO_4 0.01 %/CTAB 0.01 M/MeOH 2 %, pH=3.5. Using this mobile phase and in these conditions, riboflavin (vitamin B_2) can be eluted in isocratic mode in 6 min, shorter time than the ones found in the literature both in isocratic (Viñas et al. 2004a; Ekinci and Kadakal 2005) and gradient mode (Moreno and Salvadó 2000; Vidović et al. 2008; Ciulu et al. 2011). However, vitamin B_3H elutes in a long time and the peaks of the vitamins B_1 and B_6 overlap at dead time. Therefore, a study of vitamin retention as a function of pH was performed again, this time in presence of CTAB.

The pH does not affect the retention of vitamins B_2, B_3N, B_1 and B_6, but for pH values higher than 3 the retention of vitamins C and B_3H is dramatically altered. According to these results, it is essential to modify the pH of the mobile phase in presence of CTAB respecting to the same in its absence so that the mobile phase chosen in presence of the surfactant was H_2SO_4 0.01 %/CTAB 0.01 M/MeOH 2 % at 25 °C and pH 2.75. Using this mobile phase, the separation of six vitamins (C, B_1, B_2, B_3N, B_3H and B_6) was achieved in isocratic mode, by UV at 254 nm and fluorescence detection at excitation/emission wavelengths 290/396 nm for B_6 and 450/520 nm for B_2, as it is shown in Fig. 2 with UV (a) and fluorescence detection (b). Folic acid (vitamin B_9), very unstable in acid medium, was not detected.

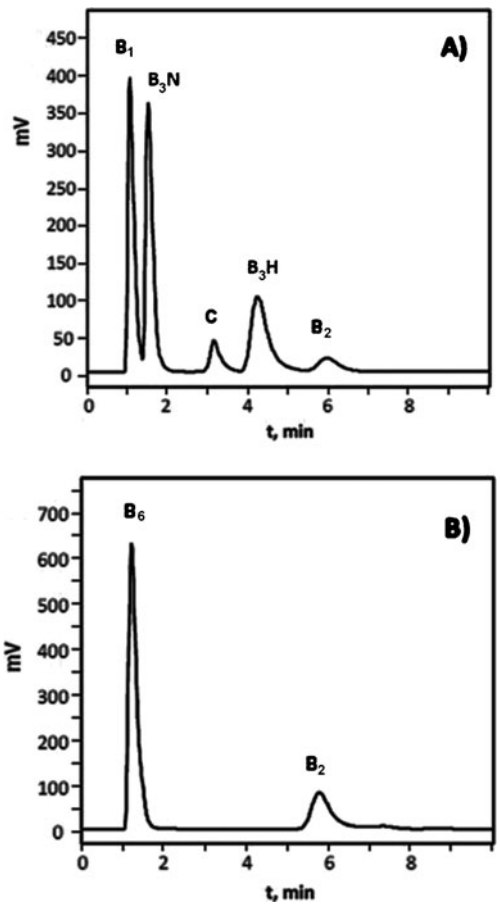

Fig. 2 Chromatographic separation of vitamins in a mobile phase H_2SO_4 0.01 %/CTAB 0.01 M/MeOH 2 %, pH=2.75, 25 °C. **a** UV detection and **b** fluorescence detection

Table 1 Analytical characteristics for chromatographic method

Vitamin	t_R min	r	Sensitivity (L/mg)	Robustness (n=4), %RSD	LOD (mg/L)	LOQ (mg/L)	Repeatability n=5, % RSD (t_R/peak area)	Reproducibility n=4, % RSD (t_R/peak area)
H_2SO_4 0.01 %/MeOH 2 % at pH=3.50 and 25 °C								
B_1	2.77	0.9999	28,836	4.6	0.055	0.182	0.20/1.2	0.8/8.4
B_3H	3.58	0.9999	53,367	2.0	0.048	0.160	0.4/4.3	2.3/5.7
B_3N	7.32	0.9999	29,263	6.1	0.067	0.222	0.2/1.7	2.4/2.6
B_5	9.79	0.9999	41,344	7.3	0.037	0.124	0.1/5.2	0.8/9.1
B_6[a]	4.39	0.9998	854,273	7.1	0.063	0.209	0.2/1.7	1.7/8.4
H_2SO_4 0.01 %/CTAB 0.01 M/MeOH 2 % at pH=2.75 and 25 °C								
B_2	5.67	0.9986	51,169	3.5	0.43	1.42	0.10/0.88	1.51/8.48
B_3H	4.59	0.9985	33,136	3.9	0.38	1.27	1.97/1.53	3.72/2.99
B_3N	1.43	0.9997	34,560	1.3	0.63	2.11	1.21/1.34	1.05/4.70
C	2.97	0.9981	37,832	2.6	0.64	2.13	0.19/0.35	4.16/3.76
B_2[a]	5.86	0.9983	411,030	8.0	0.15	0.49	0.45/1.49	1.49/8.84

[a] Fluorescence detection

Analytical Features with H_2SO_4 0.01 %/MeOH 2 % as Mobile Phase

The analytical features of the chromatographic method were determined using the mobile phase in absence of CTAB for the determination of vitamins B_1, B_3H, B_3N, B_5 and B_6. As discussed above, vitamins B_2 and B_9 cannot be eluted in a time shorter than 30 min, and for this reason they are not included in this section. Vitamin B_6 was determined using fluorescence detection which provides high sensitivity. In Table 1, the values are gathered for retention time, sensitivity, robustness, limit of detection, limit of quantification, and repeatability and reproducibility of retention time and peak area. The values found have a good sensitivity for all vitamins, and as can be expected, it is significantly higher for vitamin B_6 with fluorescence detection. The limits of detection are below 0.1 mg/L and the limits of quantification are lower than 0.3 mg/L; thus, they are very adequate for this analysis in these samples. These detection limits are similar to those found in the literature in isocratic separations and in some cases lower than them, although the experimental conditions are different (Ekinci and Kadakal 2005; Burini 2007). Only in two determinations of one vitamin in its different vitamers was found lower detection limits for vitamins B_2 (Viñas et al. 2004b) and B_6 (Viñas et al. 2004a). The precision parameters can be considered acceptable. Consequently, the use of this mobile phase allows the quantification of vitamins B_1, B_3H, B_3N, B_5 and B_6 in an analysis time of 10 min. However, the analysis of vitamin B_2 is not possible.

Analytical Features with H_2SO_4 0.01 %/CTAB 0.01 M/MeOH 2 % as Mobile Phase

The analysis of vitamin B_2 is only possible in the presence of a cationic surfactant. Thus, the mobile phase H_2SO_4 0.01 %/CTAB 0.01 M/MeOH 2 % at 25 °C and pH 2.75

Table 2 Amount of water-soluble vitamins found in different types of honeys (n=4)

Mobile phase	H_2SO_4 0.01 %/MeOH 2 %/pH=3.50					H_2SO_4 0.01 %/CTAB 0.01 M/MeOH 2 %/pH=2.75	
Honey	B_1 (mg/100 g)	B_3N (mg/100 g)	B_3H (mg/100 g)	B_5 (mg/100 g)	B_6 (mg/100 g)	C (mg/100 g)	B_2 (μg/100 g)
Rosemary	<0.005	<0.22	<0.05	<0.04	<0.06	1.90±0.01	<0.43
Thyme (1)	3.2±0.2	<0.06	0.192±0.001	0.27±0.06	0.022±0.002	149±10	<0.43
Thyme (2)	2.3±0.2	<0.06	<0.05	0.23±0.05	0.022±0.001	109±4	<0.43
Echium	0.26±0.02	0.02±0.01	0.05±0.01	<0.04	0.044±0.004	2.2±0.1	<0.43
Lavender	0.03±0.01	<0.06	0.31±0.02	<0.04	<0.06	0.98±0.04	<0.43
Honeydew	0.4±0.1	0.013±0.003	0.11±0.04	<0.04	<0.21	1.93±0.06	16.52±0.06
Chestnut	0.97±0.02	0.07±0.01	0.04±0.03	<0.04	<0.06	3.4±0.2	<1.42

was used to determine the analytical characteristics of vitamins C, B_3H, B_3N and B_2. Vitamins B_1 and B_6 were not determined with this mobile phase because their retention time is close to the column dead time; they are determined with the mobile phase in absence of CTAB. The retention time, sensitivity, robustness, limit of detection, limit of quantification, and repeatability and reproducibility of retention time and peak area are gathered in Table 1. In the case of vitamin B_2, these were also determined using fluorescence detection.

The sensitivity of this method is good for all vitamins, although in the case of vitamin B_3H this is lower (and limit of quantification higher) than in the absence of surfactant. Thus, the quantification of vitamin B_3H in honey samples has been carried out in the absence of CTAB.

As it was expected for vitamin B_2, the sensitivity obtained with FL detection is significantly higher than with UV detection. Limits of detection are lower than 0.7 mg/L with UV detection and lower than 0.2 mg/L for vitamin B_2 with fluorescence detection. The robustness

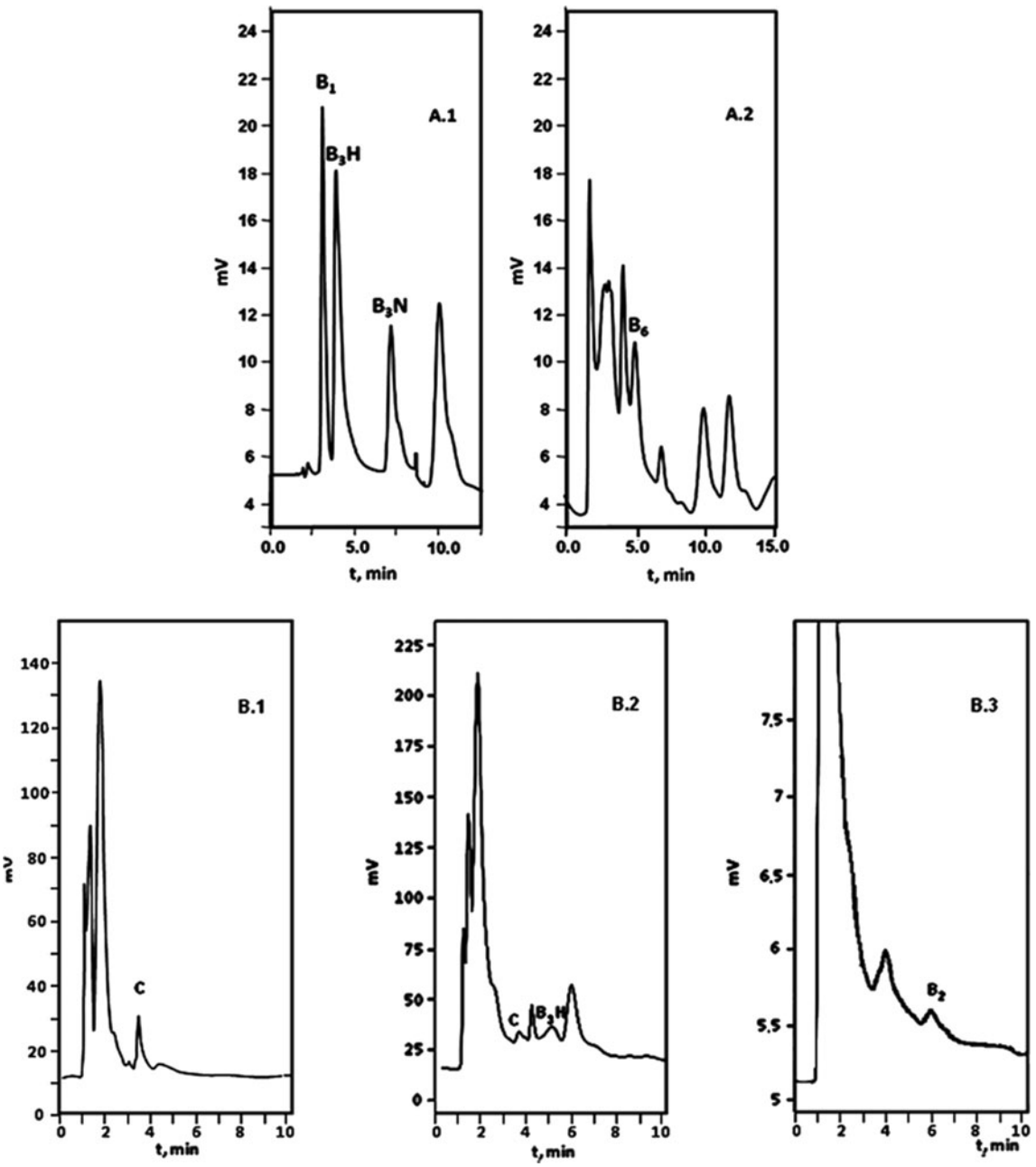

Fig. 3 Chromatograms of honey samples with **a** mobile phase H_2SO_4 0.01 %/MeOH 2 %, pH=3.5, 25 °C (*1* Echium UV, *2* Echium FL) and **b** mobile phase H_2SO_4 0.01 %/CTAB 0.01 M/MeOH 2 %, pH=2.75, 25 °C (*1* Echium UV, *2* Honeydew UV, *3* Honeydew FL)

gives RSD values between the slopes of calibration curves interdays lower than 5 % in all cases, by UV detection. The robustness obtained for vitamin B_2 by fluorescence is worse than by UV detection with a RSD value of 8 %.

The accuracy of the method was evaluated by adding different concentrations of the vitamins to honey samples, and the recoveries were calculated over the content determined in each sample when the sample contains the vitamin and directly if the sample does not contain it. The results are discussed in the next section.

Analysis of Water-Soluble Vitamins in Different Honey Samples

Both mobile phases chosen in the absence and in the presence of CTAB have been used to analyse real honey samples with different botanical origin. Table 2 summarises the results of the water-soluble vitamins concentrations found in all samples. As can be seen in this table, vitamin C is present in all samples, highlighting the thyme honeys whose amount is ten times higher than in the other honeys, something already found in an earlier work carried out by our group (León-Ruiz et al. 2011). Vitamin B_1 also appears in all honey samples with the exception of rosemary. In honey samples that belong to the Protected Denomination of Origin *La Alcarria Honey*, only vitamin C is detected in rosemary and B_1, B_3H and C in lavender.

Figure 3 shows the chromatograms corresponding to some of the samples analysed with both mobile phases as examples and using UV and fluorescence detection.

After the content of the different vitamins in honey samples was determined, 2.0, 4.0 and 6.2 mg/L of each vitamin were added to the samples. The results as the average of three determinations with their standard deviation are B_1 102.6±18.4 %, B_2 101.1±3.35 %, B_3H 109.4±5.7 %, B_3N 86.3±4.0 %, B_5 90.1±3.1 % and B_6 95.0±2.8 %. A *t* test was used to determine if there is a significant difference between the experimental average and the expected recovery (100 %), at 95 % confidence level. In all cases, $t_{experimental}$ was less than the critical value; therefore, we conclude that there are not statistically significant differences between found recoveries and 100 %. According to AOAC acceptable recovery, as a function of the concentration (AOAC 2002), experimental recoveries were acceptable for the added concentrations.

In the case of vitamin C, the HPLC method was already tested in a previous research (León-Ruiz et al. 2011), compared with the titration with the 2,6-dichloroindophenol method (AOAC 2005), and no statistically significant differences were found.

Conclusions

A simple RP-HPLC method in isocratic mode has been developed to detect seven water-soluble vitamins (B_1, B_2, B_3H, B_3N, B_5, B_6 and C) in honeys. The combination of two mobile phases, H_2SO_4 0.01 %/MeOH 2 %/25 °C/pH 3.50 and H_2SO_4 0.01 %/CTAB 0.01 M/MeOH 2 %/25 °C/pH 2.75, provides a suitable method for the chromatographic separation, identification and quantification of these vitamins.

The analytical features using both mobile phases are good, with high sensitivity and low limits of detection. The presence of CTAB is especially useful for the determination of vitamins C and B_2.

The method has been applied successfully in the vitamin's determination in honeys with different botanical origins.

Additionally, for beekeepers it is very important to add the nutrition labelling as the amount of vitamins present in honey. According to the European Union (Commission Directive 2008; Council Directive 1990) on nutrition labelling for foodstuffs, 15 % of the recommended daily allowance (RDA) supplied by 100 g should be taken into consideration in deciding what constitutes a significant amount. In the case of studied honeys, all of them meet the requirement with respect to thiamin except lavender. In reference to vitamin C, only the thyme honeys follow the European Directive.

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