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Validation of a Spectrometric Method Based on Prussian Blue Reaction Used for the Determination of Ascorbic Acid from Honey and Propolis

Simona DOBRINAS*, Alina SOCEANU, Nicoleta MATEI and Ionela DANCIU

Department of Chemistry, Ovidius University of Constanta, 124 Mamaia Blvd, 900527 Constanta, Romania

Abstract The objective of the present work was to validate and to apply a spectrometric method based on Prussian Blue reaction for the determination of ascorbic acid from different types of honey and propolis. The formation of Prussian Blue is the base of the qualitative determination of Fe(II) using hexacianoferrate as reagent. A molecular absorption spectrometer, with double beam optical system was used. In order to validate the spectrometric method the following performance parameters were tested: working range, linearity, detection and quantitation limits, method precision (repeatability, intermediate precision) and accuracy. The proposed spectrometric method was successfully applied for the determination of ascorbic acid from honey and propolis, from different rural and urban areas of Dobrogea.

Keywords: acid ascorbic, honey, propolis, Prussian Blue reaction, spectrometry.

1. Introduction

Honey has been used as a food and medical product since the earliest times. It is naturaly produced by honeybees, *Apis mellifera*, from the nectar of blossoms or from exudates of trees and plants giving nectar honeys or honeysdews, respectively. Being the only available natural sweetener, honey was a important food for Homo sapiens from his beginings [1]. In most ancient cultures honey was used for both nutritional and medical purposes [2-5].

Honey contains essential elements (calcium, phosphorus, iron, sodium, potassium), as well as compounds like water, proteins, carbohydrate, vitamins. All these substances give honey its nutritional and healing properties.

On the account of the nutritional value (303 kcal/100 g honey) and fast absorption of its carbohydrate, honey is a suitable food for humans of any age [7]. Honey is particularly recommended for children and sportsmen, because it helps improving the organism efficiency (children, elderly and invalids) [8-11].

Apart from honey, other products such as bee wax, propolis and royal jelly can be obtained from bee keeping, which are all income generating products [6].

Propolis (also known as bee glue) has been known since ancient times for its medicinal properties: antiseptic, antimycotic, bacteriostatic, astringent, choleric, spasmolytic, anti-inflammatory anaesthetic and antioxidant. Propolis contains a high concentration of flavonoids, used in cosmetics and food preparations for their antimicrobial properties [12-14].

Vitamins such as thiamin, riboflavin, pyridoxin, vitamin A, niacin and ascorbic acid were reported as contained in honey, but the amount of vitamins and minerals is low. The contribution of honey to the Recommended Dietary Allowances (RDA) of 75 mg/day and 90 mg/day have bees estabilished for adult women and men, respectively, of 45 mg/day for children [15, 16].

Ascorbic acid is an essential water-soluble vitamin, and the nutritional importance of it is well known. Therefore, adequate intake of vitamin C

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from foods and/or food supplements is vital for normal functioning of the human body [16].

Various methods have been employed for the analysis of ascorbic acid in food, including electrochemical methods [17], spectrometry [18-20], fluorescence spectrometry [21] and chromatography [22-25].

The aim of this work was to validate and to apply a spectrometric method based on Prussian Blue reaction for the determination of ascorbic acid from different types of honey and propolis.

For the method validation, the Eurachem – Romania guide no. 12/2008 [26] was followed.

The proposed spectrometric method was successfully applied for the determination of ascorbic acid of honey and propolis from different rural areas of Dobrogea region, Romania.

2. Experimental

Reagents and solutions

All reagents were of analytical-reagent grade, purchased from Merck (Fe^{3+}, KCl, HCl) or Fluka (hexacyanoferrate) and all solutions were prepared using distilled-deionized water.

For the spectrometric method the following reagents were prepared: 2.0 mM Fe(III) solution, 2.0 mM hexacyanoferrate solution, 0.1 M KCl and 10 mM HCl solutions.

Sample preparation

Honey was produced in private apiary from different areas from Romania: Pecineaga, Bragadiru, Viisoara and Babadag. Honey samples were of from different flowers: linden, polyfloral, acacia, sunflower.

Propolis was purchased from local producers as follows: propolis 1 (Favisan Proposalv, Timis region), propolis 2 (SRL Bietan, Tarnave region), propolis 3 (SC Natura, Sibiu region) and propolis 4 (Plafar, Dobrogea region).

Solutions of 100 mL were obtained from aliquots of 1g of honey and propolis samples, respectively, disolved in deionized water. Solutions where then transferred into Teflon vessels before analysis.

Sample analysis

The reagents: 2.0 mM Fe (III) chloride salt, 2.0. mM potassium hexacyanoferrate solution, 0.1 M KCl, 10 mM HCl, than mixed in 50 mL calibrated flasks with distilled-deionized water. After 10 minutes, the absorbance of the coloured complex was determined at the 700 nm by a molecular absorbtion spectrometer, Jasco 550 UV-VIZ, with double beam optical system.

3. Results and Discussions

3.1. Validation of the spectrometric method

In order to validate the spectrometric method based on Prussian Blue reaction, the following performance parameters were tested: linearity and range, detection and quantitation limits, precision (repeatability, intermediate precision) and accuracy.

Liniarity and range

To demonstrate an acceptable linear range, standard solutions covering the range between 50% and 150% of the nominal standard concentration (0.00025M) were prepared by diluting specific volume of the stock standard to get several concentrations (0.00044, 0.00088, 0.00132, 0.00176, 0.00220 şi 0.00265 mg/L). [27, 28].

Results have shown that the method is linear over the specified range (0.00044-0.00265 mg/L) with R^2 of 0.9994.

The working concentration range was established by analyzing the lowest and the highest concentration values of the proposed concentration range, ten times each of them.

The test of homogeneity variance was applied for these values and identical measurements on 10 samples of ascorbic acid to the lowest and highest concentration (x_1 and x_{10}), achieving 10 values information $y_{i,1-10}$ were performed (**Table 1**).

Table 1. Experimental results obtained for homogeneity test

i/j	$x_{i(mg/L)}$	$y_{i,1}$	$y_{i,2}$	$y_{i,3}$	$y_{i,4}$	$y_{i,5}$	$y_{i,6}$	$y_{i,7}$	$y_{i,8}$	$y_{i,9}$	$y_{i,10}$
1	0.0004	0.0311	0.0312	0.0315	0.0310	0.0314	0.0316	0.0308	0.0309	0.0313	0.0307
10	0.00265	0.1785	0.1787	0.1788	0.1781	0.1784	0.1782	0.1783	0.1780	0.1786	0.1779

The Fisher test was applied as a test of variance in homogeneity. F-tests were undertaken to evaluate the regression and lack of fit significances [29]. The calibration is considered suitable if F is less than the one-tailed tabulated value (Ftab) at a P selected confidence level [30].

In order to evaluate significance differences the P values were determined ($P=s_1^2/s_2^2$ for $s_1^2>s_2^2$ and $PG=s_2^2/s_1^2$ for $s_2^2>s_1^2$) and compared with the Ftab value for n-1=9 degrees of freedom. The results showed that the calculated P value (equal with 1) was below the F_{tab} value [31] (equal with 5.35), meaning that for our working range, the dispersions deviation wasn't significant, so that the working range was correctly chosen.

To assess the linearity of the analytical methods, a graphical representation of the relative responses (absorbance/concentration) was necessary, according to the logarithmic value of the concentration. It was necessary to obtain a horizontal line across the linearity domain with a positive deviation at low concentrations and negative deviation at high concentrations. Deviations should not exceed 5% of the average relative responses.

The values obtained for the dispersion of b coefficient (slope deviation, s_b), for the dispersion of a coefficient (intercept - intersection with ordinate deviation, s_a), for the dispersion of the entire population of y values (standard deviation for the entire population of y values s_0), and for the correlation coefficient of determination (R^2) are given in **table 2**.

Table 2. Characteristics of the linear calibration function

b	a	s_a	s_b	s_0	R^2
67.390	0.0023	0.0017	1.0673	0.0019	0.9994

Lower values of parameters like slope and intercept (table 2) indicated high precision of the proposed method. Also, the mean slope and intercept values are within the 95% confidence interval. Goodness of fit of the regression equations was supported by high regression coefficient values and lower calculated *F*-values.

Detection limit (LOD) and quantification limit (LOQ)

LOD and LOQ were calculated according to equations Eq. (1) and (2) [32].

$$LOD = \frac{3 \cdot s_a - a}{b} \quad (1)$$

$$LOQ = \frac{10 \cdot s_a - a}{b} \quad (2)$$

The LOD and LOQ values obtained were 0.005 mg/L and 0.017 mg/L, respectively. This low LOD and LOQ permit the detection of ascorbic acid at low concentrations.

Precision

Precision was primarily expressed as a relative standard deviation (RSD%). It can be discussed at three different levels: repeatability, intermediate precision and reproducibility, but in this study will only reffer to repeatability and intermediate precision.

Repeatability was demonstrated by measuring a standard solution of ascorbic acid of 2.5 mM concentration, one honey sample, as well as one propolis sample, all of six replicates.

The results obtained (RSD%) for the six replicates of the standard solution, honey and propolis samples were 19.46%, 0.128% and 22.95%, respectively, much lower than those given by Horwitz equation ($RSD < 2^{(1-0,5 \lg c)}$) for the working range 0.00044-0.00265 mg/L, for which RSD% should be between 32 and 45.3%.

These results show that the current method for ascorbic acid analysis is repeatable.

Intermediate precision was demonstrated measuring a standard solution (n=6), honey and propolis samples, in the same laboratory, by different operators, in different days.

The relative standard deviation was calculated. The results obtained (RSD%) for each working day and each analyst, and also those obtained by combining results from different days indicated that proposed method has excellent repeatability and intermediate precision. This fact is also illustrated by the RSD% values ranging between 0.092%-23.71%, values lower than those of Horwitz equation (32-45.3%).

Accuracy

Accuracy was demonstrated by the recovery test. The honey and propolis samples were fortified with known amounts (0.1 mL, 0.2 mL, 0.3 mL) of ascorbic acid standard solution of 0.1 mg/L. After each addition of the ascorbic acid the final concentrations were determinated. Accuracy was calculated according to equation (3):

$$R\% = \frac{CF - CU}{CA} \times 100 \quad (3)$$

where: CF - the concentration of analyte measured in fortified sample, CU - the concentration of analyte measured in unfortified sample, CA - added analyte concentration in the sample (measured value, not one determined by the method of testing).

The recovery was between 97-102%, being in the limits of 85-110%, given by the Horwitz equation for this level of concentration. These results reveal that ascorbic acid could be accurately determined by the proposed analytical method.

3.2. Analysis of honey and propolis samples

Ascorbic acid concentrations (three replicates) in honey and propolis samples are presented in **Table 3.**

Table 3. Ascorbic acid concentration in studied samples

No.	Sample	Concentration (mg/kg)
1	linden honey	0.0067
2	polyfloral honey	0.0059
3	acacia honey	0.0063
4	sunflower honey	0.0025
5	propolis 1	0.4115
6	propolis 2	0.4372
7	propolis 3	0.3875
8	propolis 4	0.3613

As presented in **table 3**, the AA concentrations determined in the honey samples varied from 0.025 to 0.0067 mg/kg, depending on the flowers type visited by bees to collect plant materials from which honey is produced.

Propolis contains higher amounts of flavonoids than honey. The reduction of ascorbic acid by oxidation can be inhibited by the reducing action of the flavonoids[33]. Therefore the concentrations of AA were higher in propolis than in the honey samples.

4. Conclusions

In order to validate the spectrometric method based on Prussian Blue reaction, the following performance parameters were tested: linearity and range, , detection and quantification limits, method precision (repeatability, intermediate precision) and accuracy.

The method is sensitive, precise and accurate, demonstrating that it can be used to determine the ascorbic acid in bee products honey and propolis samples.

Bee products contain a variety of compounds with significant biological action in the human body. However, the chemical composition of these products is not consistent, nor reproducible, it varies according to the geographical area and the flowers sources.

5. References

* E-mail address: sdobrinas@univ-ovidius.ro

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