Enzymatic Determination of Citric Acid in Honey by Using Polyvinylpolypyrrolidone Clarification

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To characterize honey types, a citric acid determination may be useful. A citric acid determination on honey was carried out with previous polyvinylpolypyrrolidone (PVPP) clarification followed by the Boehringer-Mannheim GmbH enzymatic test. The sample solution was prepared from 2 g of honey in 100 mL of Milli-Q water. A volume of 10 mL of this sample was clarified with PVPP stirring for 1 min and filtered. The enzymatic determination was measured spectrophotometrically at 340 nm, using citrate lyase, L-malate dehydrogenase, and L-lactate dehydrogenase. With these conditions, there were no observed interference effects. The proposed method improves precision [coefficient of variation (CV) between 0.26% and 1.60%] and recovery (between 98.0% and 100.9%) on the direct enzymatic analysis (% CV between 1.02 and 2.66 and recovery between 84.0% and 115.6%). Furthermore, the cost was reduced 70% using a microtest. The method was applied to 20 honeys of Galicia (northwestern Spain), and the results ranged between 44.2 and 827.0 mg of citric acid/kg of honey (mean = 192.9 mg/kg).

Keywords: *Honey; citric acid; enzymatic analysis; polyvinylpolypyrrolidone (PVPP)*

INTRODUCTION

Organic acids comprise a small proportion of honey (<0.5%). These acids are one group of constituents that contribute to the flavor of honey (White, 1979a). The level of acidity of honey also contributes to its stability toward microorganisms (White, 1979b).

In honey, 19 organic acids have been identified (Crane, 1990). Citric acid was identified in honey by Nelson and Mottern (1931); however, the origin of this acid, as well as that of other organic acids present in honey, is not very well-known. It could originate from glucose, fructose, or sucrose of the nectar by the action of enzymes that the bee adds at ripening (Echigo and Takenaka, 1974). It is also possible that citric acid may be present already in the nectar (White, 1979b).

After D-gluconic and L-malic acids, citric acid is found in greatest concentration (Cherchi et al., 1994). Even when it is not the major organic acid, citric acid analysis in honey is interesting because its quantitation may be useful for characterizing different honey types. Talpay (1988) used the content of citric acid as a reliable analytical property for the differentiation of two main types of honey: floral honey and honeydew honey.

The number of references to citric acid in honey is small. It has been isolated, with other acids, by paper chromatography, ion-exchange chromatography and silicic acid partition chromatography (Stinson et al., 1960). Citric acid and other acids were determined by Cherchi et al. (1994), using a high-performance liquid chromatographic (HPLC) method with two columns connected in series after sample purification by solid-phase extraction. In that work, the levels of citric acid found in different types of floral honey (multifloral, strawberry-tree, asphodel, and red gum honeys) ranged from 42.5 to 207.6 mg/kg.

An enzymatic method for determining citric acid has been developed by several authors for different foods (Boehringer-Mannheim GmbH, 1995). The citric acid content of honey using a direct enzymatic method, without clarification, was determined by Tourn et al. (1980) and Talpay (1988). There are no data regarding precision and recovery in the Tourn et al. (1980) work. The citric acid values found by Talpay (1988) in floral honeys ranged from 36.5 to 1454.2 mg/kg, and the values found in honeydew honey ranged from 447.6 to 3019.8 mg/kg; precision ranged from 1.61% to 1.72%, and the recovery value was 92.0 ± 6.25%.

The purpose of this paper is to develop a simple, precise, accurate, specific, and low-cost method for determining citric acid in honey because of its possible usefulness in characterizing this foodstuff.

MATERIALS AND METHODS

Samples. The work was carried out on 20 floral samples from Galicia (northwestern Spain). The samples were harvested in the autumn of 1994 and stored in darkness at room temperature until the analysis 4 months later. The botanical origin of the samples was determined according to the procedure of Louveaux et al. (1978), after the sediment in the honeys was treated and dyed using the method of Terradillos et al. (1994). One sample was *Castanea sativa* honey, 7 samples

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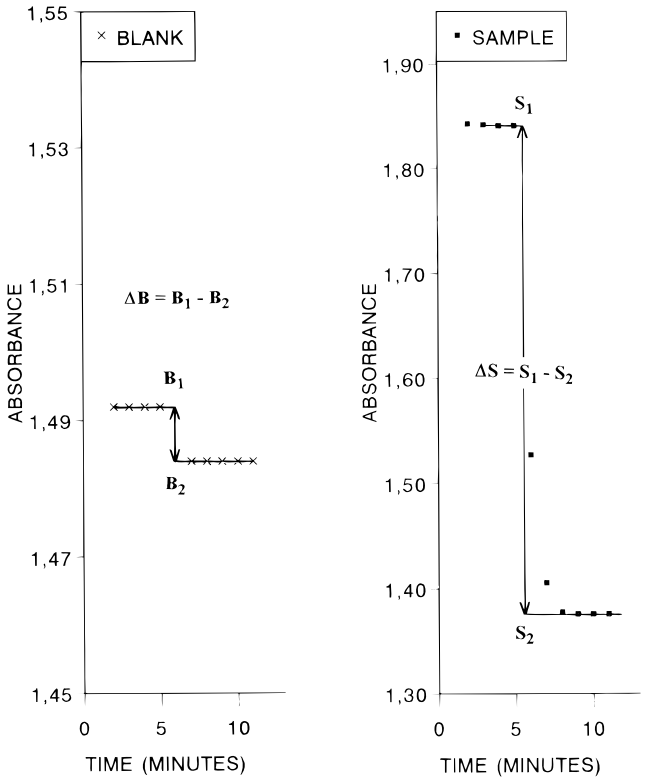

Figure 1. Absorbances at 340 nm measured to determine citric acid in honey using the enzymatic method with previous clarification with PVPP.

were *Eucalyptus* sp. honeys, 1 sample was *Rubus* sp. honey, and 11 samples were multifloral honeys.

Reagents and Apparatus. (a) A Boehringer-Mannheim GmbH (1995) enzymatic test was used for 3 × 10 determinations (catalog no. 139 076). The test combination contains the following: (a1) powder mixture, consisting of glycylglycine buffer (pH 7.8), 136 units of L-malate dehydrogenase (L-MDH), 280 units of L-lactate dehydrogenase (L-LDH), 5 mg of reduced nicotinamide-adenine dinucleotide (NADH), and stabilizers; (a2) 12 units of citrate lyase lyophilisate (CL); (a3) citric acid standard solution. Also used were (b) polyvinylpolypyrrolidone (PVPP) Sigma P-6755 and (c) a Kontron Uvikon 810 P UV−vis double-beam spectrophotometer.

Procedure. *Sample Solution*. Approximately 2 g of honey was dissolved in 20 mL of Milli-Q water. It was transferred with Milli-Q water to a 100 mL volumetric flask, filled up to the mark, and stirred. Ten milliliters of this solution was pipetted; after stirring with 0.100 g of PVPP for 1 min, the mixture was filtered. This filtered solution was used for the assay.

Spectrophotometry Measurements at 340 nm. Into a 1.5 mL cuvette we pipetted 0.30 mL of solution a1, and 0.60 mL of sample solution. The contents were mixed, and the absorbance was read, at room temperature, at 340 nm (S_1) when stable (after ≈5 min). The reaction was started by the addition of 0.006 mL of solution a2 and mixing; after completion of the reaction (≈5 min), the absorbance of the solution (S_2) was read.

The blank was measured following the same procedure with 0.60 mL of Milli-Q water instead of 0.60 mL of sample solution (B_1 and B_2). The absorbance differences for both, sample and blank, were determined, and the absorbance difference of the blank was subtracted from the absorbance difference of the sample. Occasionally a negative value with $(B_1 - B_2)$ was obtained. This value was then to be added to $(S_1 - S_2)$ according to the calculation formula (Figure 1)

$$\Delta A = (S_1 - S_2) - (B_1 - B_2)$$

Calculations. The calculations were carried out as specified by the supplier, Boehringer-Mannheim GmbH (1995), for other foodstuffs. For honey, following our procedure, the citric acid was calculated, as the anhydrous acid, as follows:

$$\text{mg of citric acid/kg of honey} = \frac{4604}{\text{sample wt in g}} \times \Delta A$$

$$4604 = \frac{0.906 \times 192.1}{6.30 \times 1 \times 0.600 \times 1000} \times \frac{100}{1000} \times 1000 \times 1000$$

In these equations $\Delta A = (S_1 - S_2) - (B_1 - B_2)$, $(S_1 - S_2)$ is the absorption of the sample, $(B_1 - B_2)$ is the absorption of the blank, 0.906 = final volume (mL), 192.1 = mol weight of anhydrous citric acid, 6.30 = absorption coefficient of NADH at Hg 340 nm (L mmol^{-1} cm^{-1}), 1 is the light path (cm), 0.600 = sample volume (mL), 1000 = mL in 1 L, 100/1000 = g of citric acid in 100 mL of final solution, 1000 = mg in 1 g, and 1000 = g in 1 kg.

RESULTS

Tables 1 and 2 show the repeatability and recovery results of the enzymatic method applied for determining citric acid in honey both without clarification and with the PVPP clarification studied in this paper.

Repeatability. The precision (Table 1) was established by measuring the citric acid content of 10 solutions from each of 4 samples (10, 17, 12, and 1). These samples contains low, medium, high, and very high citric acid levels, respectively. The precision (% CV) of the direct enzymatic method without clarification ranged between 1.02% and 2.66%. In contrast, the precision of the developed method with PVPP clarification ranged between 0.26% and 1.60%.

Recovery of Added Citric Acid. The accuracy (Table 2) of the method was established by adding increasing amounts of citric acid standard to 1 g of the three lowest citric acid content honeys (10, 2, and 4) and dissolving in 100 mL, covering the concentration range present in the samples analyzed (≈45−800 mg/kg). The citric acid reference solution (Boehringer-Mannheim GmbH no. 139 076) included in the enzymatic test kit was used. The recovery of the samples without clarification ranged from 84.0% to 115.6%. In contrast, the recovery of the samples with PVPP clarification ranged from 98.0% to 100.9%.

Specificity. The method is specific to determine citric acid. Intermediates of the tricarboxylic acid cycle and related metabolites do not react (Boehringer-Mannheim GmbH, 1995).

Citric Acid Contents of the Galician Honeys Analyzed. Table 3 shows the citric acid contents of the 20 Galician honeys analyzed by using the method with PVPP clarification. The mean citric acid concentration was 192.9 mg/kg with a range of values from 44.2 to 827.0 mg/kg.

DISCUSSION

Although the Talpay (1988) work had adequate precision, the recovery without PVPP clarification was poor and variable (92.0 ± 6.25%) and could be improved.

Several problems were found when Talpay's (1988) method was applied. First, the method used different amounts of honey for floral and honeydew honeys [2 g of honey in 20 mL of Milli-Q water (10 g/100 mL) for floral honeys and 0.5 g in 20 mL of Milli-Q water (2.5 g/100 mL) for honeydew honeys], so it was necessary to know beforehand the origin of the honey. Second, according to the recommendation of the supplier for the enzymatic analysis (1−80 μg of citric acid/cuvette), only

Table 1. Study of Precision of the Determination of Citric Acid Content (in Milligrams per Kilogram) of Honeys Using the Direct Enzymatic Method and the Enzymatic Method with a Previous Clarification with PVPP

	honey sample							
	10		17		12		1	
	direct	clarified	direct	clarified	direct	clarified	direct	clarified
n	10	10	10	10	10	10	10	10
mean	44.9	44.2	132.7	135.6	424.0	428.4	817.2	827.0
SD[a]	1.19	0.707	2.82	0.978	6.497	2.198	8.323	2.186
% CV[b]	2.66	1.60	2.12	0.72	1.53	0.51	1.02	0.26

[a] Standard deviation. [b] Coefficient of variation.

Table 2. Study of the Recovery of the Determination of Citric Acid Content (in Milligrams per Kilogram) of Honeys Using the Direct Enzymatic Method and the Enzymatic Method with a Previous Clarification with PVPP

	recovery (%)	
added (mg/kg)	direct	clarified
	Sample 10	
25	84.0	100.4
175	95.3	98.0
375	96.4	99.9
775	98.0	98.5
mean	93.4	99.2
SD[a]	6.38	1.134
% CV[b]	6.83	1.14
	Sample 2	
25	115.6	99.6
175	110.2	99.0
375	102.5	100.1
775	96.7	99.2
mean	106.3	99.5
SD[a]	8.33	0.486
% CV[b]	7.84	0.49
	Sample 4	
25	104.0	99.6
175	100.7	100.4
375	99.8	100.9
775	97.3	100.9
mean	100.5	100.5
SD[a]	2.769	0.614
% CV[b]	2.76	0.61

[a] Standard deviation. [b] Coefficient of variation.

Table 3. Citric Acid Contents of the Honeys Analyzed with the Enzymatic Method Using a Previous Clarification with PVPP

sample	botanical origin	citric acid (mg/kg)
1	*Castanea sativa*	827.0
2	*Eucalyptus* sp.	48.3
3	*Eucalyptus* sp.	61.5
4	*Eucalyptus* sp.	49.9
5	*Eucalyptus* sp.	76.8
6	*Eucalyptus* sp.	56.4
7	*Eucalyptus* sp.	74.9
8	*Eucalyptus* sp.	69.2
9	*Rubus* sp.	98.0
10	multifloral	44.2
11	multifloral	259.3
12	multifloral	428.4
13	multifloral	293.9
14	multifloral	99.9
15	multifloral	403.4
16	multifloral	317.0
17	multifloral	135.6
18	multifloral	313.1
19	multifloral	149.8
20	multifloral	51.9
mean		192.9
SD[a]		196.2
V_{min}		44.2
V_{max}		827.0

[a] Standard deviation.

floral honeys with citric acid content ≤400 mg/kg and honeydew honeys with citric acid content ≤1600 mg/kg could be analyzed with this method when 2 mL of the honey solution was used for carrying out the test. Unfortunately, it was found that many honeys did not lie in the range of the enzymatic test. Finally, Talpay (1988) did not mention the wavelength for measuring the citric acid in honey and absorption coefficients of NADH at 334, 340, and 365 nm are different. This is especially important because the absorbance measured depends on the wavelength employed, among other factors.

To resolve the difficulties above, 2 g of honey in 100 mL of Milli-Q water and 2 mL of this solution previously clarified with PVPP were used for carrying out the test for both floral and honeydew honeys. Under these conditions, all honeys with citric acid content between 25 and 2000 mg/kg could be analyzed. A major reduction of the sample would not allow this determination in honeys with low content of citric acid. The method would be made uniform to floral honeys and the majority of honeydew honeys. The absorption coefficients of NADH at 334, 340, and 365 nm are 6.18, 6.30, and 3.40 L $mmol^{-1}$ cm^{-1}, respectively. The absorbances of a honey solution measured at these wavelengths provided the values of 1.00, 0.92, and 0.62, respectively. The chosen wavelength was 340 nm due to the best relation of absorption coefficient of NADH/absorbance of the honey solution. With this wavelength the absorbance B_1 (solution a1 and Milli-Q water) is near 1.50 units of absorbance. If 10 g/100 mL was used, as in the Talpay (1988) method, the absorbance S_1 (solution a1 and sample solution) of some honeys exceeded 3.00 units of absorbance. This absorbance is too high for a correct spectrophotometric determination, so the reduction of the sample is more suitable because the high absorbance S_1 of some honeys would be reduced.

With regard to the precision (Table 1), our results without PVPP clarification were similar to those found by Talpay (1988). However, the recovery percents were not constant in the samples. In sample 10, the recovery increased when the amount of citric acid added was increased. In sample 2, on the contrary, the recovery decreased when the amount of citric acid added was increased. Meanwhile, in sample 4, the recovery (although the mean was close to 100) was not constant and it also decreased when the amount of citric acid added was increased (Table 2). The mean recovery improves the mean recovery of 92.0 ± 6.25% obtained by Talpay (1988), but the standard deviation is worse, so the interferences continued.

An attempt was made to eliminate the possible interferences by clarifying the diluted sample solutions. The Carrez clarification, which is used in other methods of enzymatic honey analysis (Huidobro et al., 1993), could not be used for the citric acid determination due to a too low recovery rate because of adsorption of citric acid. Employing activated charcoal (which is used for organic acid determination in several foodstuffs) improved neither the precision nor the recovery.

Even when honey solutions (2 g/100 mL) were not turbid and strongly colored, an attempt was made to eliminate the interferences with PVPP clarification (unusual in honey), which is recommended by the supplier (Boehringer-Mannheim GmbH, 1995) for determining citric acid in turbid and strongly colored foodstuffs (such as wine or fruit juices). With this clarification, the light color of the diluted samples was not visibly modified. Surprisingly, previous interferences were eliminated and significant improvements were achieved, with very good precision and recovery (Tables 1 and 2).

Moreover, the cost of the enzymatic analysis was reduced 70%, because only 30% of the sample volume and reagent volumes specified by the supplier (Boehringer-Mannheim GmbH, 1995) were used. Applying this reduction, the same concentration of citric acid was obtained in 0.600 mL of sample for a total volume of 0.906 mL as in 2 mL of sample for a total volume of 3.02 mL.

In conclusion, the enzymatic method to determine citric acid in honey was modified and improved. This proposed method modified the previous direct method with a sample reduction and a previous clarification with PVPP of the solution of honey and measurement at 340 nm. Moreover, the cost was reduced 70% using this microtest. The enzymatic determination was rapid, simple, precise, accurate, interference-free, and low cost for practical application even in other foods with a high sugar content.

With regard to the honey's citric acid contents, 7 of 20 honey samples analyzed had a higher content of citric acid than the samples analyzed by HPLC by Cherchi et al. (1994) because of the different types of floral honey. Sample 1 could be floral or honeydew honey on the basis of the citric acid content. The botanical origin has showed that this sample was a *C. sativa* honey. The observed value of 827.0 mg/kg was close to the mean value of 751.1 mg/kg obtained by Talpay (1988) with a recovery of 92.0 ± 6.25% in *C. sativa* honeys. Attempts to characterize honeys with different botanical origins on the basis of their citric acid contents could be interesting because of the simplicity of this measurement.

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