Enzymatic Determination of Total D-Gluconic Acid in Honey

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The total D-gluconic acid (free D-gluconic acid and D-glucono-δ-lactone) content of 20 Galician (northwestern Spain) honeys has been determined using an enzymatic method. To the author's knowledge, this is the first time this method has been applied to honey. The solution of honey was adjusted to pH 10.5 with 0.1 N KOH; 10 min at room temperature was allowed for hydrolysis of D-glucono-δ-lactone to D-gluconic acid. After this, it was necessary to adjust to test pH (7.8) with 0.1 N HCl. With these conditions, there were no observed interference effects. The enzymatic determination was measured spectrophotometrically at 340 nm, using gluconate kinase and 6-phosphogluconate dehydrogenase. The method combines precision (% CV was 0.30, at worst), good recovery (99.8%), simplicity, and low cost because this cost was reduced by 60% using a microtest. The total D-gluconic acid content of the honeys analyzed ranges between 3.91 and 11.71 g/kg (mean = 7.37 g/kg), which is in keeping with value ranges obtained by other authors using HPLC methods.

Keywords: *Honey; total D-gluconic acid; D-glucono-δ-lactone; enzymatic analysis*

INTRODUCTION

Organic acids comprise a small proportion of honey (<0.5%); however, they are one group of constituents that contribute to the flavor of any honey (White, 1979, 1992). The level of acidity probably also contributes to the stability of honey against microbiological attack (White, 1978). In honey, 19 organic acids certainly or probably present have been identified (Crane, 1990).

Years ago it was thought that citric acid predominated, with small amounts of formic, acetic, butyric, malic, and succinic acids (Nelson and Mottern, 1931). Nevertheless, it has been found (Stinson *et al.*, 1960) that the predominant acid in honey is gluconic acid. This acid, in equilibrium with the gluconolactone, originates largely from the activity of the glucose oxidase, which the bee adds at ripening (White *et al.*, 1963), with some contribution from bacterial action during the ripening (Ruiz-Argüeso and Rodríguez-Navarro, 1973). The glucose oxidase−glucose reaction also produces hydrogen peroxide, which is the chief source of the antibacterial action of honey (White and Subers, 1963).

D-Gluconic acid has been isolated and identified, with other acids, by paper chromatography, ion-exchange chromatography, and silicic acid partition chromatography (Stinson *et al.*, 1960).

White (1978), as other authors, considered that a need exists for an analytical procedure to determine the content of total D-gluconic acid (D-gluconic acid and D-glucono-δ-lactone) in honey. Total D-gluconic acid was 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 this study other acids were determined too. Total D-gluconic acid is found in concentrations of grams per kilogram, and the others are in minor concentration (milligrams per kilogram) in honey.

Boehringer-Mannheim GmbH (1989) has determined total D-gluconic acid using an enzymatic method in various foods such as wine or meat. In the literature, we have not found data about total D-gluconic acid contents of honeys determined by this method. It struck us as surprising that nobody had applied this simple method to honey.

A HPLC method does not separate the D-gluconic acid and the D-glucono-δ-lactone, so both are determined as total D-gluconic acid in honey. Also, this method is expensive and laborious because of the use of two columns connected in series and solid-phase extraction. The purpose of this work has been to simplify the analysis of total D-gluconic acid in honey and make it cheaper by applying, for the first time, the enzymatic method (Boehringer-Mannheim GmbH, 1989).

MATERIALS AND METHODS

Samples. The work was carried out on 20 floral samples from Galicia (northwestern Spain). The samples were harvested in autumn 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 were *Eucalyptus* sp. honeys, 1 sample was *Rubus* sp. honey, and 11 samples were multifloral samples.

Reagents and Apparatus. (a) A Boehringer-Mannheim GmbH (1989) enzymatic test was used for approximately 25 determinations (Catalog No. 428 191). The test combination contains the following: (a1) powder mixture, consisting of triethanolamine buffer (pH 7.8), 60 mg of nicotinamide adenine dinucleotide phosphate (NADP), 150 mg of adenosine 5′-

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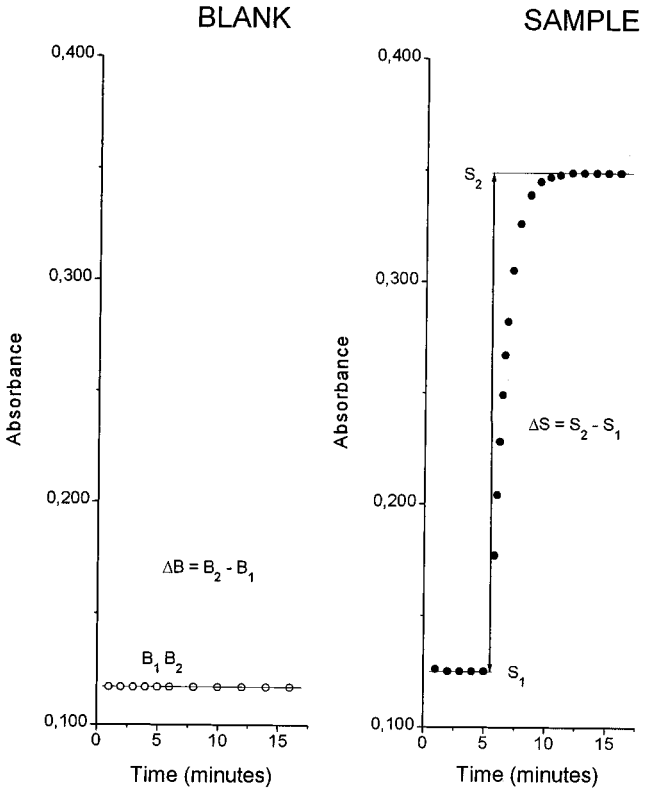

Figure 1. Absorbances at 340 nm measured to determine total D-gluconic acid in honey using the enzymatic method.

triphosphate (ATP), magnesium sulfate, and stabilizers; (a2) 110 units of 6-phosphogluconate dehydrogenase suspension (6-PGDH); (a3) 13 units of gluconate kinase suspension. Also used were (b) a Kontron Uvikon 810 P UV−vis double-beam spectrophotometer, (c) a Sentron 1001 pH meter, and (d) a Metrohm 649 magnetic stirrer.

Procedure. *Sample Solution*. Approximately 2.5 g of honey was dissolved in 50 mL of Milli-Q water, and the pH was adjusted to approximately 10.5, using the necessary quantity of 0.1 N KOH; the mixture was stirred for 10 min with the magnetic stirrer. After this time, the pH was adjusted to approximately 7.8 (test pH) using the necessary quantity of 0.1 N HCl. The mixture 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 and transferred to a 100 mL volumetric flask and made up to 100 mL with Milli-Q water.

Spectrophotometry Measurements at 340 nm. Into a 1.5 mL cuvette, we pipetted 0.40 mL of solution a1, 0.80 mL of sample solution, and 0.008 mL of 6-PGDH suspension a2. The contents were mixed, and the absorbance was read, at room temperature, at 340 nm (S_1) when stable (after approximately 5 min). The reaction was started by addition of 0.008 mL of gluconate kinase suspension (a3) and mixed; after waiting for completion of the reaction (approximately 5−10 min), the absorbance of the solution (S_2) was read.

The blank was measured following the same procedure with 0.80 mL of Milli-Q water instead of 0.80 mL of sample solution (B_1 and B_2). In our case, with the enzymatic test employed by us, after solution gluconate kinase suspension (a3) was added, there was no variation of the absorbance, so $B_2 - B_1 = 0.000$.

The absorbance differences for both blank and sample were determined, and the absorbance difference of the blank was subtracted from the absorbance difference of the sample (Figure 1):

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

Calculations. The calculations were carried out as specified by the supplier, Boehringer-Mannheim GmbH (1989), for other foodstuffs. For honey, following our procedure, the total D-gluconic acid is calculated as follows:

$$\text{g of total D-gluconic acid/kg of honey} = \frac{47.31}{\text{sample wt in g}} \times \Delta A$$

$$\text{factor of } 47.31 = \frac{1.216 \times 196.1}{6.3 \times 1 \times 0.800 \times 1000} \times \frac{100}{1000} \times \frac{100}{10} \times 1000$$

In these equations $A = (S_2 - S_1) - (B_2 - B_1)$, $(S_2 - S_1)$ is the absorption of the sample and $(B_2 - B_1)$ is the absorption of the blank, 1.216 = final volume (mL), 196.1 = mol weight of D-gluconic acid, 6.3 = absorption coefficient of NADH at Hg 340 nm (L $mmol^{-1}$ cm^{-1}), 1 is the light path (cm), 0.800 = sample volume (mL), 1000 = mL in 1 L, 100/1000 = g of D-gluconic acid in 100 mL of final solution, 100/10 is a dilution factor, and 1000 = g in 1 kg.

Table 1. Precision of the Enzymatic Method for Measuring Total D-Gluconic Acid Content (Grams per Kilogram) of Honeys

	honey sample		
	14	8	11
	3.90	7.28	11.72
	3.92	7.28	11.71
	3.92	7.25	11.70
	3.92	7.25	11.71
	3.90	7.28	11.72
	3.92	7.26	11.72
	3.92	7.29	11.71
	3.90	7.28	11.70
	3.90	7.28	11.72
	3.93	7.25	11.70
mean	3.91	7.27	11.71
SD[a]	0.0116	0.0156	0.00876
% CV[b]	0.30	0.22	0.07

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

RESULTS

Repeatability. Precision was very good. It was established by measuring the total D-gluconic acid content of 10 solutions from each of 3 honey samples (14, 8, and 11) with low (3.91 g/kg), medium (7.27 g/kg), and high (11.71 g/kg) total D-gluconic acid levels, respectively. The greatest coefficient of variation (corresponding to the low total D-gluconic acid level) was 0.30% (Table 1).

Recovery. The free acidity and lactonic acidity are a mean of 75% and 25% in milliequivalents of total acidity, respectively (White, 1962). The total D-gluconic acid is found in high percent (≈90%) of grams in relation to all acids (Cherchi *et al.*, 1994). So the amounts of total D-gluconic acid, which are added in the recovery, are formed by 75% of free acid, originated from sodium salt of D-gluconic acid (Sigma G-9005) and 25% of free acid originated from D-glucono-δ-lactone (Merck Art. 288). The recovery was established by adding increasing amounts of total D-gluconic acid, covering the concentration range present in the samples analyzed (approximately 4−12 g/kg), to a honey sample containing 1.96 g/kg of total D-gluconic acid and using the method to determine the total D-gluconic acid content (Table 2). The mean recovery was 99.8%, and the coefficient of variation (% CV) was 0.40%.

Specificity. The method is specific to determine D-glucono-δ-lactone together with free D-gluconic acid as total D-gluconic acid (Boehringer-Mannheim GmbH, 1989).

Table 2. Study of the Recovery of the Enzymatic Method To Determine Total D-Gluconic Acid (Grams per Kilogram) in Honey

present	added (g/kg)	found (g/kg)	recovery (%)
	2.00	3.96	100.0
	2.00	3.97	100.5
	2.00	3.96	100.0
	4.00	6.00	100.3
	4.00	5.99	100.0
	4.00	5.94	99.8
1.96			
	8.00	9.94	99.8
	8.00	9.91	99.4
	8.00	9.90	99.3
	10.00	11.90	99.4
	10.00	11.88	99.2
	10.00	11.94	99.8
mean			99.8
SD[a]			0.403
% CV[b]			0.40

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

Table 3. Total D-Gluconic Acid Contents of the Honeys Analyzed

sample	botanical origin	total D-gluconic acid (g/kg)
1	*Castanea sativa*	9.53
2	*Eucalyptus* sp.	4.52
3	*Eucalyptus* sp.	7.95
4	*Eucalyptus* sp.	8.30
5	*Eucalyptus* sp.	8.64
6	*Eucalyptus* sp.	6.31
7	*Eucalyptus* sp.	5.43
8	*Eucalyptus* sp.	7.27
9	*Rubus* sp.	4.44
10	multifloral	5.54
11	multifloral	11.71
12	multifloral	7.34
13	multifloral	7.49
14	multifloral	3.91
15	multifloral	7.30
16	multifloral	7.79
17	multifloral	9.67
18	multifloral	8.58
19	multifloral	9.40
20	multifloral	6.33
mean		7.37
SD[a]		1.99
V_{min}		3.91
V_{max}		11.71

[a] Standard deviation.

Total D-Gluconic Acid Contents of the Galician Honeys Analyzed. The total D-gluconic acid contents of the 20 honey samples analyzed are shown in Table 3. The mean total D-gluconic acid concentration was 7.37 g/kg, with a spread of values from 3.91 to 11.71 g/kg. These results lie within the range of total D-gluconic acid contents found by Cherchi *et al.* (1994) in other honeys using high-resolution liquid chromatography.

DISCUSSION

There are, at least, two reasons to determine D-glucono-δ-lactone (as free D-gluconic acid) together with original free D-gluconic acid:

(a) In aqueous solution D-glucono-δ-lactone suffers hydrolysis to D-gluconic acid at the pH of an enzymatic test ($\approx$7.8).

(b) During the determination, D-gluconic acid reacts and upsets the equilibrium D-gluconic acid/D-glucono-δ-lactone, so there is a displacement of the equilibrium and D-glucono-δ-lactone is converted into D-gluconic acid.

Therefore, total hydrolysis of D-glucono-δ-lactone to D-gluconic acid is guaranteed by the preliminary use of 0.1 N KOH (to adjust the pH to 10.5) for 10 min at room temperature (Boehringer-Mannheim GmbH, 1989).

For the enzymatic analysis at 340 nm, the recommendation of Boehringer-Mannheim GmbH (1989) is for an optimum quantity of 5−60 μg of D-gluconic acid and hydrolyzed D-glucono-δ-lactone/cuvette (in 0.10−2.00 mL of sample for a total volume of 3.04 mL).

On the basis of the study by Cherchi *et al.* (1994), the total D-gluconic acid concentration in different honeys is between 1.3 and 13.8 g/kg. It follows that 0.80 mL of solution prepared from 2.5 g of honey in 100 mL of water and diluted 1:10 (as per the method given in the proposed procedure) contains 3.9−41.9 μg of total D-gluconic acid in a total volume of 1.216 mL, meeting the conditions of the enzymatic test because this is equal to the 6.5−69.0 μg using 2 mL of sample.

A concentration of about 0.25 g of honey/100 mL was necessary for the determination. In these conditions, interferences would not be important, so we thought that a previous clarification would not be required. This was confirmed in the work.

The cost of the enzymatic analysis was reduced by 60%, because only 40% of the sample volume and reagent volumes specified by the supplier [Boehringer-Mannheim GmbH (1989)] were used.

In conclusion, the proposed method meets the conditions of precision, recovery, sensitivity, simplicity, and low cost required for an analytical method to be useable.

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