Enzymatic Determination of Glycerol in Honey

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The glycerol content of 33 honeys of Galicia (northwestern Spain) has been determined using the Boehringer-Mannheim enzymatic method modified for this purpose. As far as we know, it is the first time this method has been applied to honey. Volumes of 0.5 mL of potassium hexacyanoferrate(II)–trihydrate solution (Carrez I) and zinc acetate–dihydrate solution (Carrez II) were used and, following clarification, about 4 mL of 0.1 N NaOH. The enzymatic determination was performed "spectrophotometrically" at 365 nm using pyruvate kinase, lactate dehydrogenase, and glycerokinase in double the quantities recommended by the supplier. The method combines precision (CV% less than 1.1%), good recovery (102.2%), sensitivity (30 mg/kg), simplicity, and low cost. The glycerol content of the honeys analyzed ranges between 50.0 and 366.2 mg/kg (mean 137.6 mg/kg), which is in keeping with values obtained by other authors using other methods (HPLC, GC).

INTRODUCTION

The polyol glycerol occurs as a minor constituent in honey and is thought to be produced by microorganisms present in the nectar and honeydew which bees collect. Laub and Marx (1987), having found a qualitative relationship between the number of these microorganisms and the quantity of glycerol, analyzed more than 100 honeys; over 79% of those containing more than 200 mg/kg glycerol showed the presence of microorganisms and spores, whereas only 14% of the honeys with less than 200 mg/kg glycerol contained spores. The mean glycerol content was 172 mg/kg, and the maximum value was 601 mg/kg; 45% of the honeys had less than 100 mg/kg, 27% had between 100 and 200 mg/kg, 18% had between 200 and 299 mg/kg, and 8% had between 300 and 399 mg/kg. Only two (2%) contained more than 400 mg/kg.

Spencer and Sallans (1956), Spencer and Shu (1957), Peterson et al. (1958), and Hajny et al. (1960) observed that, under suitable conditions, some osmophilic yeasts isolated from honey convert 60% of a 10–20% glucose solution into polyols such as glycerol, D-arabitol, erythritol, and manitol. Glycerol may therefore be considered a fermentation product. In the fermentation of a 20% glucose solution, aeration and low phosphate content favor the production of polyols such as glycerol, whereas anaerobic fermentation produces mainly ethanol, to the detriment of the polyols.

Although several authors have determined the glycerol content of mead, the literature on glycerol determination in unfermented honey is small, to say the least. Laub and Marx (1987) have made the only determination of glycerol in honey, using a high-resolution liquid chromatography method with a cation-exchange resin and a refractive index detector. This method is based on Pfeiffer and Radler's (1985) determination of glycerol in wine. Laub and Marx (1987) checked their data using gas chromatography, following Deifel's (1985) work to determine sugars in honey.

Boehringer-Mannheim (1989) has determined glycerol using an enzymatic method in various foods such as fruits, wine, beer, and marzipans. It struck us as surprising that nobody had hitherto applied this simple method to honey. Hence, in the work described here, a modified enzymatic method is used to determine glycerol in honey for the first time.

MATERIALS AND METHODS

Samples. The work was carried out on 33 honey samples harvested in 1991 and furnished by Galician manufacturers (northwestern Spain). All of the honeys bore the Producto Galego de Calidade–Mel de Galicia guarantee of origin. Thirty-one samples were unpasteurized.

The pollen analyses of these samples showed that 31 samples were floral honeys and 2 samples were of floral and honeydew origin.

Reagents and Apparatus. (*a*) *Boehringer-Mannheim (1989) Enzymatic Test for 3 × 10 Determinations* (Catalog No. 148 270). The test combination contains the following.

(*a1*) *Coenzyme/Buffer Mixture*: glycylglycine buffer, pH 7.4; 7 mg of reduced nicotinamide adenine dinucleotide (NADH); 22 mg of adenosine 5′-triphosphate (ATP); 11 mg of phosphoenolpyruvate (PEP); magnesium sulfate, and stabilizers. Dissolve with 11 mL of redistilled water. Before use, allow the solution to stand for approximately 10 min at room temperature. This solution is stable for 4 days at 4 °C.

(*a2*) *Enzyme Suspension*: 240 units of pyruvate kinase (PK) and 220 units of lactate dehydrogenase (L-LDH). This suspension is stable for 1 year at 4 °C.

(*a3*) *34 Units of Glycerokinase Suspension (GK)*. This suspension is stable for 1 year at 4 °C.

(*b*) *Carrez I Solution*: 15 g of potassium hexacyanoferrate-(II)–trihydrate (Merck Art. 4984) diluted to 100 mL with water.

(*c*) *Carrez II Solution*: 30 g of zinc acetate–dihydrate (Merck Art. 8802) diluted to 100 mL with water.

(*d*) *Crison microPH 2002 pHmeter, EA-120*: combined glass electrode.

(*e*) *Hitachi 100-60*: UV–vis double-beam spectrophotometer.

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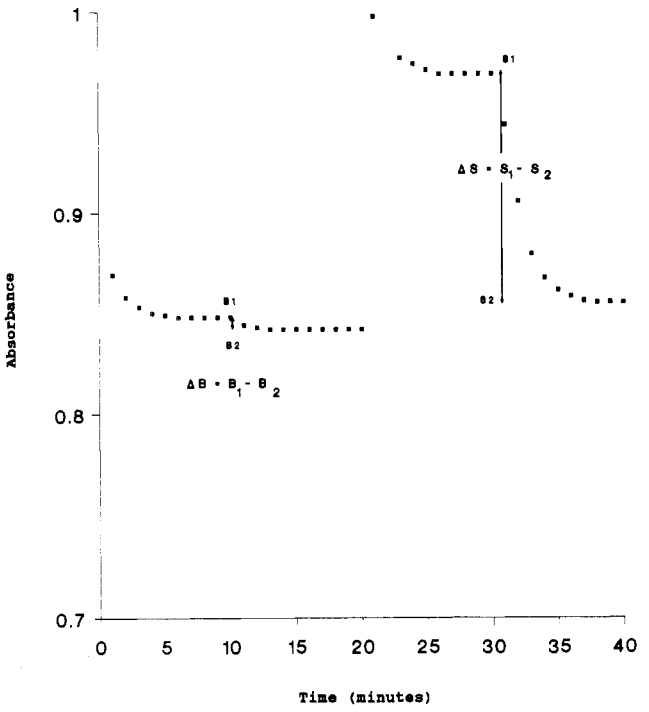

Figure 1. Absorbances at 365 nm measured to determine glycerol in honey using the enzymatic method.

Procedure. *Preparation of the Sample.* For the enzymatic analysis, the recommendation of Boehringer-Mannheim (1989) is 3–40 μg of glycerol/cuvette (in 0.1–2.0-mL sample volume).

On the basis of Laub and Marx's (1987) study, 98% of honeys analyzed contained less than 400 mg/kg glycerol. It follows that 2 mL of a solution prepared from 5 g of honey as per the method given in the procedure below contains 5–37 μg of glycerol, meeting the conditions of the enzymatic test.

Method. Weigh 5 g of honey and dissolve in 20 mL of redistilled water. Transfer to a 50-mL volumetric flask. Wash the beaker with two 5-mL portions of redistilled water and add washings to volumetric flask. Add 0.5 mL of Carrez I solution and stir. Add 0.5 mL of Carrez II solution and stir (White, 1979). Add about 4 mL of 0.1 N sodium hydroxide and stir. Make up to 50 mL. Stir. Filter using a Whatman No. 40 11-cm filter paper, discarding first 10 mL of filtrate.

Pipet 25 mL of the filtrate and adjust to pH 7.4, using the necessary quantity of 0.1 N NaOH and 0.1 N HCl. Transfer quantitatively to a 50-mL volumetric flask, make up to the mark, and stir.

Pipet into cuvette 1.00 mL of solution a1, 2.00 mL of sample solution, and 0.020 mL of enzyme suspension a2. Mix, wait for completion of the reaction (approximately 5–10 min), and read the absorbance at 365 nm vs redistilled water (absorbance S_1).

The measure of absorbance was made at 365 nm because at this wavelength the higher absorbance (S_1) was less than unity, in all samples analyzed, and the absorbance is also suitably sensitive and stable.

Start reaction by addition of 0.020 mL of glycerokinase suspension (a3). Mix, wait for completion of the reaction (approximately 5–10 min), and read the absorbance immediately (S_2).

The blanks were measured following the same procedure with 2.00 mL of redistilled water instead of 2.00 mL of sample solution (values $B_1 - B_2$).

After 10 min, the values of S_1 and S_2 were constant, whereas it is not necessary to extrapolate the absorbances to the time of the addition of suspension a3 (Figure 1).

Determine the absorbance differences ($S_1 - S_2$) for both sample and blank ($B_1 - B_2$).

For the determination of glycerol in the first few honeys, 6.0 units of pyruvate kinase, 5.5 units of lactate dehydrogenase, and 0.85 unit of glycerokinase were used (Boehringer-Mannheim, 1989). The reactions proceeded very slowly, however, taking 10–15 min to complete. The reaction rate was therefore increased by doubling these quantities (taking 0.020 mL of a2 and a3 suspensions). Since the kit does not contain enough enzyme for 30 determinations, additional enzymes have to be obtained from Boehringer-Mannheim [quoting reference no. 0109096 (pyruvate kinase and lactate dehydrogenase) and 0127159 (glycerokinase)].

The determination of glycerol in honey according to this method can also be carried out using 1-cm sample cells with 1.8-mL capacity, in which case the quantities of honey solution and of the reagents in the enzymatic test are halved. This, naturally, makes the analysis cheaper.

Table I. Study of the Precision of the Enzymatic Method To Determine Glycerol in Honey

	50.0	154.6	366.2
	49.7	154.2	366.2
	50.0	154.0	366.5
	50.6	154.0	366.9
	49.4	154.5	366.0
	49.2	154.9	365.9
	49.5	155.6	366.8
	50.8	154.8	366.4
	50.3	155.6	365.5
	50.5	154.8	365.4
mean	50.0	154.7	366.2
SD[a]	0.5457	0.5724	0.4984
CV%[b]	1.09	0.37	0.14

[a] SD, standard deviation. [b] CV%, coefficient of variation percent.

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

$$\text{mg of glycerol/kg of honey} = \frac{4117}{\text{sample wt in g}} \times (A_{\text{sample}} - A_{\text{blank}})$$

$$4117 = \frac{3.04 \times 92.1}{3.4 \times 1 \times 2 \times 1000} \times \frac{50}{1000} \times 1000 \times 1000 \times 2$$

where A_{sample} is absorption of sample ($S_1 - S_2$); A_{blank} is absorption of blank ($B_1 - B_2$); 3.04 is the final volume (mL); 92.1 is the molecular weight of glycerol; 3.4 is the absorption coefficient of NADH at Hg 365 nm (L × mmol^{-1} cm^{-1}); 1 is the light path (cm); 2 is the sample volume (mL); 1000 is mL/L; (50/1000) is g of glycerol in 50 mL of final solution; 1000 = mg/g; 1000 = g/kg; and 2 is the ratio of volumes of final solution and clarified solution (50 mL/25 mL).

RESULTS AND DISCUSSION

Repeatability. Measurement of the glycerol content of 10 solutions of the 3 honey samples with a low, medium, and high content of glycerol returned a coefficient of variation of less than 1.1% in all cases (Table I).

Recovery of Added Glycerol. The recovery was established by adding increasing amounts of glycerol covering the concentration range present in the samples analyzed (50–370 mg/kg) to a honey sample containing 22.7 mg/kg glycerol and using the method to determine the total glycerol content (Table II). The glycerol reference solution (Boehringer-Mannheim No. 148 270) included in the enzymatic test kit was used. The mean recovery was 102.2%, and the coefficient of variation (CV%) was 0.59%.

Values of Glycerol Content. The glycerol contents of the 33 honey samples analyzed are shown in Table III. The mean glycerol concentration was 137.6 mg/kg, with a spread of values from 50.0 to 366.2 mg/kg. These results lie within the range of glycerol contents found by Laub and Marx (1987) in other honeys using high-resolution liquid chromatography.

Conclusions. Enzymatic determination of glycerol in honey has been carried out for the first time. The method meets the conditions of precision, recovery, sensitivity,

Table II. Study of the Recovery of the Enzymatic Method To Determine Glycerol in Honey

present	added, mg/kg	found, mg/kg	recovery, %
	25	48.3	102.4
	25	47.9	100.8
	125	150.2	102.0
	125	150.9	102.6
	125	150.1	101.9
22.7			
	225	253.3	102.5
	225	254.7	103.1
	225	253.0	102.4
	325	354.2	101.9
	325	354.4	102.1
n			10
mean			102.2
SD[a]			0.607
CV%[b]			0.59

[a] SD, standard deviation. [b] CV%, coefficient of variation percent.

Table III. Glycerol Contents of the Honeys Analyzed

sample	glycerol, mg/kg	sample	glycerol, mg/kg
1	154.7	20	74.5
2	173.0	21	168.1
3	183.6	22	128.5
4	166.1	23	120.6
5	119.8	24	104.6
6	82.9	25	338.7
7	366.2	26	89.6
8	64.1	27	115.5
9	121.7	28	81.5
10	105.5	29	162.3
11	175.1	30	113.3
12	50.0	31	113.0
13	80.4	32	70.0
14	322.0	33	54.3
15	265.9		
16	155.0	mean	137.6
17	75.5	SD[a]	80.7
18	72.2	V_{min}	49.9
19	73.4	V_{max}	366.2

[a] SD, standard deviation.

simplicity, and low cost required for an analytical method to be useable.

The results obtained by the enzymatic method are similar to those obtained by other authors using other methods (HPLC, GC).

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