Enzymatic Determination of Primary Normal Alcohols as Apparent Ethanol Content in Honey

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Primary normal alcohols of 33 unpasteurized Galician (northwestern Spain) honeys have been determined as apparent ethanol contents. A modification of the Boehringer-Mannheim enzymatic method has been used. The solution of honey has been employed directly, neither clarified nor neutralized. Removal of interferences required absorbance measurements at 340 nm, by using the solution of honey with the solution of aldehyde dehydrogenase into the reference cuvette. Water is added to the reference cuvette and alcohol dehydrogenase suspension to the sample cuvette. The blanks are measured following the same procedure with redistilled water instead of sample solution. Ten replicate analyses of each of four samples with apparent ethanol levels of 13.5, 35.3, 50.1, and 141.8 mg/kg gave coefficient of variations of 1.74%, 0.48%, 0.34%, and 0.22%, respectively. The modified enzymatic method performed well in recovery experiments (recovery 100.1%). The apparent ethanol contents of 32 of the 33 honeys studied lay in the range 13.5–50.1 mg/kg (mean 27.8 mg/kg); the remaining unspoiled honey had an apparent ethanol content of 141.8 mg/kg.

Keywords: *Honey; ethanol; alcohols; enzymatic analysis*

INTRODUCTION

It has long been recognized that honey can be spoiled by unintentional fermentation, producing ethanol, carbon dioxide, and volatile and nonvolatile acids (Fabian and Quinet, 1928; Marvin, 1928, 1930; Lochhead and Heron, 1929; Wilson and Marvin, 1929; Lochhead and Farrell, 1930, 1931; Dyce, 1931; Marvin et al., 1931; Wilson and Marvin, 1931, 1932; Lochhead, 1933). The yeasts responsible may come from the body of the bee, from the floor of the hive or the processing room, or from processing equipment (Fabian and Quinet, 1928; Crane, 1975; Comi et al., 1982; Crane, 1990). Small quantities of ethanol are also, however, a natural component of unspoiled honey (Duisberg, 1967), which makes it desirable to know how high such nonpathological levels may be. Surprisingly, the only published information in this area appears to be Borries' (1934) finding that naturally occurring ethanol is equivalent to less than 1% of the sugar content of the honey.

The first gas chromatographic (GC) separation of ethanol from honey was performed using Carbowax 1500 or polyphenyl ether as stationary phase (Cremer and Riedmann, 1964, 1965). Subsequently, an improved GC method for the determination of volatile honey components was developed which uses acetone for extraction, an OV-1 capillary column, and mass spectrometry (MS) for detection (Bicchi et al., 1983).

Alternatively, kits for the determination of ethanol in various foods, including honey, have been developed by Boehringer-Mannheim (1989) on the basis of enzyme–substrate reactions. However, the details of the enzymatic method for honey were not optimized by Boehringer-Mannheim, and their validation has not been described in the literature (personal communication).

The purpose of this work has been to determine the apparent ethanol (ethanol, 1-propanol, butanol, and pentanol, among other primary normal alcohols) contents of several unspoiled honeys by applying the Boehringer-Mannheim (1989) enzymatic method. In the literature, we have not found data about apparent ethanol contents of honeys determined by using this method.

MATERIALS AND METHODS

Samples. We used 33 samples honeys of Galicia (northwestern Spain); 31 honeys were floral honeys, and 2 honeys were a mix of floral and honeydew sources (Huidobro et al., 1993). Microscopic analysis showed that the honeys were unspoiled by yeasts.

Reagents and Apparatus. *(a) Boehringer-Mannheim (1989) Enzymatic Test for 3 × 10 Determinations (Catalog No. 176 290).* The test combination contains the following.

(a1) Potassium Diphosphate Buffer (pH 9.0) Containing Stabilizers. This solution is used without dilution. It is stable for 1 year at 4 °C. Before use, allow solution to come to room temperature.

(a2) Tablets Containing 4 mg of Nicotinamide Adenine Dinucleotide (NAD), 0.8 IU of Aldehyde Dehydrogenase (Al-DH), and Stabilizers. Dissolve with 3 mL of (a1) one tablet of (a2). This solution is stable for 1 day at 4 °C. Before use, allow solution to come to room temperature.

(a3) 7000 IU of Alcohol Dehydrogenase (ADH) in Suspension with Stabilizers. This solution is used without dilution. It is stable for 1 year at 4 °C.

(a4) 0.058 g/L Ethanol Standard Solution.

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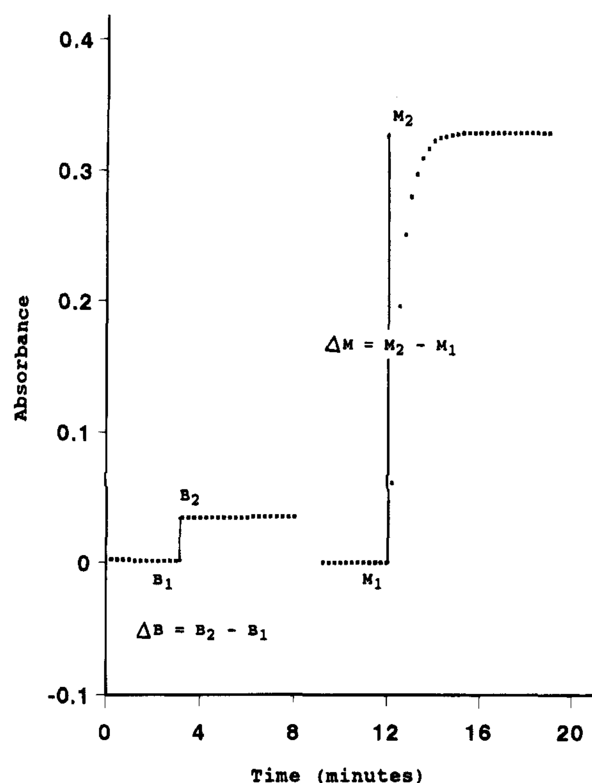

Figure 1. Absorbances at 340 nm measured to determine ethanol in honey using the enzymatic method.

(*b*) A Hitachi 100-60 UV–vis double-beam spectrophotometer was used.

Procedure. *Sample Solution.* Dissolve 5 g of honey with water and transfer to a 50-mL flask and make up to mark with water.

Spectrophotometry Measurements at 340 nm. Dissolve 1 NAD tablet (a2) in 3 mL of potassium diphosphate buffer (a1), divide equally (1.5 mL) between two quartz spectrophotometer cuvettes (2 mL and 1 cm light path). Then add 0.250 mL of sample solution to each cuvette, stir, and obtain the absorbance difference at 340 nm (M_1) when stable (after approximately 3 min). Then add 0.025 mL of water to the reference cuvette and 0.025 mL of enzyme suspension (a3) to the sample cuvette, stir, and obtain the absorbance difference at 340 nm (M_2) when stable (after approximately 5–10 min). Repeat the whole procedure with blanks in which sample solution has been replaced by the same volume of double-distilled water; obtain absorbance differences B_1, corresponding to M_1, and B_2, corresponding to M_2 (Figure 1).

Calculations. For honey, following our procedure, the apparent ethanol content is calculated as follows (Boehringer-Mannheim, 1989):

mg of apparent ethanol/kg of honey =

$$\frac{1298}{\text{sample wt in g}} \times (A_{\text{sample}} - A_{\text{blank}})$$

The factor of

$$1298 = \frac{1.775 \times 46.07}{6.30 \times 1 \times 0.250 \times 2 \times 1000} \times \frac{50}{1000} \times 1000 \times 1000$$

In these equations A_{sample} is the absorption of the sample, A_{blank} is the absorption of the blank, 1.775 = final volume (mL), 46.07 = mol wt of ethanol, 6.30 = absorption coefficient of NADH at Hg 340 nm (L mmol^{-1} cm^{-1}), 1 = light path (cm), 0.250 = sample volume (mL), 2 = the quantity of NADH obtained is equivalent to the half of the ethanol quantity, 1000 = mL in 1 L, (50/1000) = g of apparent ethanol in 50 mL of final solution, 1000 = mg in 1 g, and 1000 = g in 1 kg.

Table 1. Study of the Precision of the Enzymatic Method To Determine Apparent Ethanol (Milligrams per Kilogram) in Honey

	sample 8	sample 7	sample 1	sample 25
mean	13.5	35.3	50.1	141.8
SD[a]	0.2348	0.1729	0.1703	0.3127
CV%[b]	1.74	0.48	0.34	0.22

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

Table 2. Study of the Recovery of the Enzymatic Method To Determine Apparent Ethanol (Milligrams per Kilogram) in Honey

present	added	found	recovery, %
	10	16.7	99.0
	10	16.8	100.0
	10	16.7	99.0
	30	36.8	100.0
	30	36.7	99.7
	30	36.8	100.0
6.8			
	50	56.9	100.2
	50	56.9	100.2
	50	56.7	99.8
	150	157.4	100.4
	150	157.9	100.7
	150	159.7	101.9
n			12
mean			100.1
SD[a]			0.763
CV%[b]			0.76

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

RESULTS

Repeatability. From each of 4 (samples 8, 7, 1, and 25) unspoiled honeys with low (13.5 mg/kg), medium (35.3 mg/kg), high (50.1 mg/kg), and very high (141.8 mg/kg) apparent ethanol contents, 10 samples were taken for apparent ethanol determination as above. The greatest coefficient of variation (that of the low apparent ethanol honey) was 1.74% (Table 1).

Recovery. Samples of a honey containing 6.8 mg/kg of apparent ethanol were fortified with various amounts of apparent ethanol (the reference solution of the Boehringer-Mannheim kit) to cover the concentration range present in the samples analyzed (approximately 10–150 mg/kg), and the apparent ethanol contents of the fortified samples were determined. Mean recovery was 100.1%, with a coefficient of variation of 0.76% (Table 2).

Specificity. Included in the "apparent ethanol concentration" determined as above are the concentrations of all the other linear primary alcohols except methanol, though ethanol is the major contributor (Cremer and Riedmann, 1964, 1965). Nonlinear alcohols do not contribute significantly, and secondary, tertiary, and aromatic alcohols are not susceptible to the enzymes used in this method. Even high concentrations of glycerol (more than 400 mg/kg) in honey (Laub and Marx, 1987) do not cause significant interferences (Boehringer-Mannheim, 1989).

Apparent Ethanol Contents of the Galician Honeys Analyzed. The apparent ethanol contents of the 33 Galician honeys analyzed ranged from 13.5 to 141.8 mg/kg. If the sample containing 141.8 mg/kg is excluded, the sample with the highest apparent ethanol

Table 3. Apparent Ethanol Contents of the Honeys Analyzed

sample	apparent ethanol, mg/kg	sample	apparent ethanol, mg/kg
1	50.1	20	24.9
2	44.2	21	25.6
3	41.9	22	36.7
4	31.7	23	15.0
5	30.5	24	26.1
6	26.5	25	141.8
7	35.3	26	42.4
8	13.5	27	23.1
9	25.5	28	13.6
10	23.2	29	15.4
11	35.3	30	18.7
12	13.5	31	16.4
13	29.2	32	23.7
14	32.2	33	41.0
15	35.8		
16	32.0	mean	31.3
17	31.9	SD[a]	22.2
18	15.7	V_{min}	13.5
19	19.0	V_{max}	141.8

[a] SD, standard deviation.

content had 50.1 mg/kg of apparent ethanol (Table 3). Mean concentration was 31.3 mg/kg (27.8 mg/kg if the high outlier is excluded).

DISCUSSION

The procedure laid down by Boehringer-Mannheim (1989) for preparation of honey samples prior to determination of apparent ethanol with their enzymatic kit is as follows: dissolve 20 g of honey with water, make up to 100 mL of water, transfer 10 mL of this solution to a 25-mL volumetric flask, add Carrez solutions I [3.60 g of $K_4Fe(CN)_6{\cdot}3H_2O$/100 mL of water] and II (7.20 g of $ZnSO_4{\cdot}7H_2O$/100 mL of water) to clarify, neutralize with NaOH solution, make up to 25 mL with water, and stir.

When applied to our samples of unfermented honey, which had apparent ethanol contents in the range 13.5–141.8 mg/kg, the quantity of apparent ethanol in 0.5 mL of final solution is 0.54–5.67 μg, well within the range recommended by Boehringer-Mannheim (0.5–12.0 μg in 0.1–0.5 mL). In spite of this, determination of apparent ethanol according to the Boehringer-Mannheim (1989) method proved to be impossible because absorbance differences–time (the absorbance differences are with respect to blank cuvettes containing water) failed to recover the same slope after addition of the alcohol dehydrogenase as before.

Attempts to overcome this problem by reversing the order of clarification and neutralization or by changing the concentrations of NaOH and/or Carrez solutions all met with failure.

It was therefore decided to use a pair of controls in which alcohol dehydrogenase was replaced by water. The absorbance differences obtained when sample plus enzyme was run against sample plus water and water plus enzyme against water plus water (the procedure described under Material and Methods) stabilized satisfactorily, allowing unequivocal readings to be taken.

In addition, we observed that previous clarification and neutralization were not necessary. The buffer of the enzymatic test provides the appropiate pH for the determination.

In conclusion, the Boehringer-Mannheim method for enzymatic determination of ethanol in foods was modified to allow precise determination of the apparent ethanol content of unspoiled honey. In the modified method interferences are taken into account by running ADH-free control solutions against sample and blank; neither clarification nor neutralization of the sample is required. The method is sufficiently precise, interference-free, simple, and inexpensive for practical application.

The apparent ethanol contents of 33 honeys of Galicia (northwestern Spain) lay in the range 13.5–141.8 mg/kg (mean 31.3 mg/kg), with 32 of them in the range 13.5–50.1 mg/kg (mean 27.8 mg/kg).

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