J Sci Food Agric 1996, **71**, 141–144

Direct Enzymatic Analysis of Glycerol in Honey: a Simplified Method

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(Received 6 December 1994; revised version received 9 August 1995; accepted 3 November 1995)

Abstract: The glycerol content of honey has been determined using a Boehringer Mannheim enzymatic method simplified for this purpose. The solution of honey was employed directly, without clarification and without addition of NaOH solution. With these conditions, there were no observed interference effects. It is necessary to use a 1·5 ml micro-cuvette. The enzymatic determination was measured spectrophotometrically at 365 nm using the enzymes in quadruple the quantities recommended by the supplier. The method combines precision, good recovery, sensitivity, simplicity and low cost.

Key words: honey, glycerol, enzymatic analysis, direct enzymatic analysis.

INTRODUCTION

Glycerol is a minor constituent in honey and may also be considered a fermentation product because it is thought to be produced by microorganisms (Spencer and Sallans 1956; Spencer and Shu 1957; Peterson *et al* 1958; Hajny *et al* 1960; Laub and Marx 1987). As glycerol content is related with the number of microorganisms present in honey (Laub and Marx 1987), its determination is of considerable interest.

The number of references to glycerol determination in unfermented honey is small. Laub and Marx (1987) determined glycerol in honey by using a high-performance liquid chromatographic method, based on the technique of Pfeiffer and Radler (1985) for determining glycerol in wine. Laub and Marx (1987) checked their data by using gas chromatography as per Deifel's (1985) method for determining sugars.

Huidobro *et al* (1993) applied, for the first time, an enzymatic method for determining the glycerol content of honey. The method required previous clarification, by using Carrez I and II solutions (White 1979) follow by neutralization using 0·1 M NaOH solution. This method was precise, accurate, simple and not very expensive. The methodology was applied to 33 commercially purchased Galician (in north-western–Spain) honeys. All the samples bore the label 'Producto Galego de Calidade-Mel de Galicia' which guaranteed the origin. The samples represented the whole range of Galician manufacturers of labelled honeys. The glycerol values found in these 33 honeys ranged from 49·9 to 366·2 mg kg^{-1} (mean 137·6). These values are similar to the glycerol contents found by Laub and Marx (1987) in other honeys.

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J Sci Food Agric 0022-5142/96/$09.00

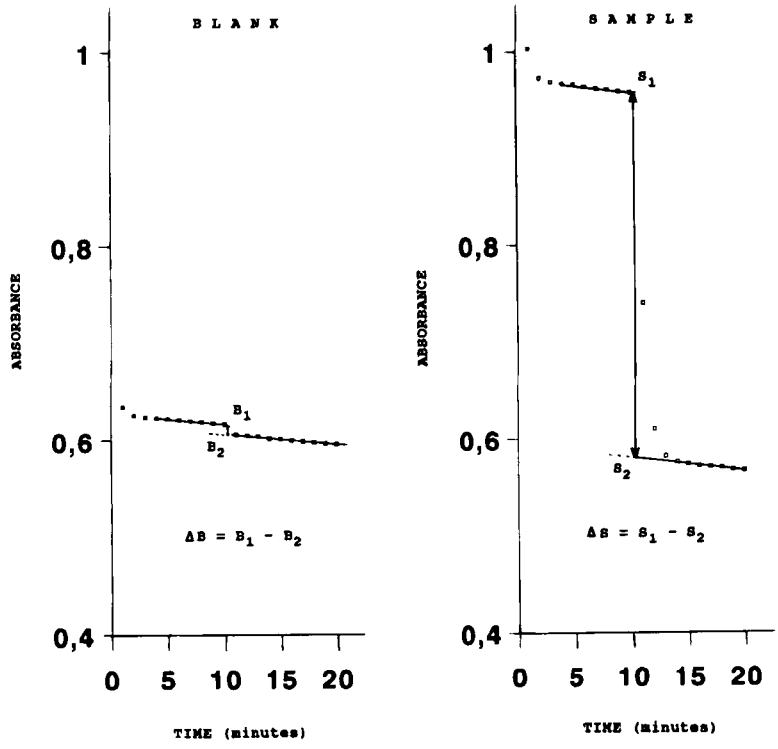

Fig 1. Absorbances at 365 nm measured to determine glycerol in honey using the direct enzymatic analysis.

Some months ago, Huidobro *et al* (1994) determined the ethanol content of honey using an enzymatic method without clarification and neutralization.

The purpose of this paper has been to try a simplification of the previous method for determining the glycerol content of honey, avoiding the use of clarification and neutralization. The fortification assays have been developed on the basis of the values of glycerol found in the honey samples previously analysed (Laub and Marx 1987; Huidobro *et al* 1993).

MATERIAL AND METHODS

Reagents and apparatus

(a) Boehringer-Mannheim (1989) enzymatic test for 3 × 10 determinations. Catalogue no 148 270. The test combination contains the following:

- (a1) coenzyme/buffer mixture, consisting of glycylglycine buffer, pH 7·4; reduced nicotinamide-adenine dinucleotide (NADH), 7 mg; adenosine-5′-triphosphate (ATP), 22 mg; phosphoenolpyruvate (PEP) 11 mg; magnesium sulfate and stabilizers;
- (a2) enzyme suspension, consisting of pyruvate kinase (PK), 240 U and lactate dehydrogenase (L-LDH), 220 U;
- (a3) glycerokinase suspension (GK), 34 U.

(b) Hitachi 100-60 UV–vis double-beam spectrophotometer.

Principle of the enzymatic method (Boehringer-Mannheim 1989)

Glycerol is phosphorylated by adenosine-5′-triphosphate (ATP) to L-glycerol-3-phosphate in the reaction catalysed by glycerokinase. The adenosine-5′-diphosphate formed in the above reaction is reconverted by phosphoenolpyruvate with the aid of pyruvate kinase into adenosine-5′-triphosphate with the formation of pyruvate. In the presence of the enzyme lactate dehydrogenase, pyruvate is reduced to L-lactate by reduced nicotinamide-adenine dinucleotide, this being oxidized back to nicotinamide-adenine dinucleotide. The amount of reduced nicotinamide-adenine dinucleotide oxidized in this reaction is stoichiometric with the amount of glycerol. The loss of reduced nicotinamide-adenine dinucleotide is finally calculated from the decrease in absorption at 365 nm.

Procedure

Weigh 5 g of honey and transfer with distilled water to a 100 ml volumetric flask. Into a 1·5 ml cuvette, pipette 0·25 ml (a1), 0·50 ml of sample solution and 0·010 ml of enzyme suspension (a2). Mix, wait for completion of the reaction (approx 5–10 min) when the incremental decrease in absorbance is constant and read, at room temperature, the absorbance at 365 nm vs distilled water (absorbance S_1).

Start the reaction by addition of 0·010 ml glycerokinase suspension (a3), mix, wait for completion of the

TABLE 1
Precision of the modified enzymatic method for measuring glycerol content (mg kg^{-1}) of honeys

	Honey			
	1	*2*	*3*	*4*
	37·1	56·0	121·0	306·2
	39·2	56·4	121·1	306·2
	37·0	55·5	121·6	305·8
	37·2	56·5	121·0	306·2
	38·4	55·2	120·3	306·6
	38·6	55·4	119·9	306·0
	39·1	55·8	121·0	306·2
	39·4	56·1	121·5	306·2
	38·5	55·8	121·0	306·8
	39·3	56·1	121·6	305·7
Mean	38·4	55·9	121·0	306·2
SD[a]	0·9473	0·4237	0·5457	0·3281
%CV[b]	2·47	0·76	0·45	0·11

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

reaction (approx 5–10 min) and read the absorbance immediately (S_2).

The blank is measured following the same procedure with 0·50 ml of distilled water instead of 0·50 ml of sample solution (B_1 and B_2).

After 5 min the absorbance decreases steadily with the same slope. In our case, a second and slower reaction occurs parallel with the main reaction. This causes a shift of the absorbance value that can be eliminated by graphical or mathematical extrapolation. Graphical determination of the true end-point of the main reaction is carried out as follows: (a) at intervals of 1 min take an absorption reading four to six times more than indicated in the general method; (b) plot absorption against time on 1 mm graph paper; (c) extrapolate the linear portion of the curve obtained to the time of the addition of suspension (a3) (B_1 and S_1). Then, the true value of the absorption at the end-point (B_2 and S_2) is the value at which the extrapolated line cuts the ordinate marking the beginning of the reaction (Fig. 1).

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

TABLE 2
Study of the recovery of the enzymatic method to determine directly glycerol (mg kg^{-1}) in honey avoiding the use of clarification and neutralisation

Present	*Added (mg kg^{-1})*	*Found (mg kg^{-1})*	*Recovery (%)*
	25	43·2	96·0
	25	42·8	94·4
	25	43·3	96·4
	125	141·2	97·6
	125	139·8	96·5
	125	140·2	96·8
19·2			
	225	234·1	95·5
	225	235·2	96·0
	225	236·1	96·4
	325	332·1	96·3
	325	331·2	96·0
	325	332·9	96·5
n			12
Mean			96·2
SD			0·7652
CV%			0·80

Calculations

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

mg glycerol per kg honey =

$$\frac{4336{\cdot}4}{\text{sample wt in g}} \times (A_{\text{sample}} - A_{\text{blank}})$$

The factor of 4336·4 =

$$\frac{0{\cdot}77 \times 92{\cdot}1}{3{\cdot}40 \times 1 \times 0{\cdot}50 \times 1000} \times \frac{100}{1000} \times 1000 \times 1000 \times \frac{100}{96{\cdot}2}$$

where A_{sample} is the absorption of sample; A_{blank} is the absorption of blank; 0·77 = final volume (ml); 92·1 = mol wt of glycerol; 3·40 = absorption coefficient of NADH at Hg 365 nm ($l \times \text{mmol}^{-1} \times \text{cm}^{-1}$); 1 is the light path (cm); 0·50 = sample volume (ml); 1000 = ml in 1 litre; (100/1000) = g glycerol in 100 ml final solution; 1000 = mg in 1 g; 1000 = g in 1 kg; and (100/96·2) = recovery factor.

RESULTS AND DISCUSSION

For the enzymatic analysis, the recommendation of Boehringer Mannheim (1989) is for an optimum quantity, 3–40 μg of glycerol per cuvette (in 0·1–2·0 ml sample for a total volume of 3·02 ml).

On the basis of Laub and Marx's (1987) study, 98% of honeys analysed contained less than 400 mg kg^{-1} glycerol. On the basis of the Huidobro *et al* (1993) study, 100% of the samples analyzed contained less than 367 mg kg^{-1} glycerol. It follows that 0·25 ml of a solution prepared from 5 g of honey in 100 ml of distilled water (as per the method given in the procedure below) contains 1·25–9·25 μg of glycerol in a total volume of 0·77 ml, meeting the conditions of the enzymatic test because this is equal to the 5–37 μg by using 2 ml of sample (Huidobro *et al* 1993).

Previous neutralization is not necessary because the buffer of the enzymatic test is sufficient to allow the reaction to proceed.

The enzymatic reactions proceeded very slowly, however, taking 10–15 min to complete, compared with the previous method (Huidobro *et al* 1993) which used 0·020 ml of suspension (a2) (reference no 109 096 of pyruvate kinase and lactate dehydrogenase) and 0·020 ml of suspension (a3) (reference no 127 159 of glycerokinase suspension). The reaction rate was therefore increased by using the enzymes in quadruple the quantities recommended by the supplier.

The cost of the enzymatic analysis can be reduced by using, for each determination, 0·010 ml of the suspensions (a2) and 0·010 ml of the suspension (a3) and the fourth part of both the solution (a1) and the sample. In this way, the suspensions (a2) and (a3) of the enzymatic test, are enough for the 30 determinations. Furthermore, in the bottle (a1), there would be extra coenzyme/buffer mixture which could give rise to develop other analysis, together with more suspensions (a2) and (a3).

Repeatability

Precision was satisfactory. It was established by measuring the glycerol content of 10 solutions of each of four samples having very low, low, medium and high glycerol levels. Coefficients of variation of 2·47%, 0·76%, 0·45% and 0·11% were obtained (Table 1). The previous method (Huidobro *et al* 1993) provided coefficients of variation similar to these (1·09%, 0·37% and 0·14% for low, medium and high glycerol levels, respectively).

Recovery of added glycerol

The accuracy was established by adding increasing amounts of glycerol covering the concentration range present in the samples analyzed (50–370 mg kg^{-1}) to a honey sample containing 19·2 mg kg^{-1} of glycerol and using the method to determine the total glycerol content (Table 2). The glycerol reference solution (Boehringer Mannheim no 148270) included in the enzymatic test kit was used. The mean recovery was 96·2% and the coefficient of variation (CV%) was 0·80%. If compared with the mean recovery obtained in the method set up by Huidobro *et al* in 1993 (102·2%, CV% of 0·59), the mean recovery in this simplified method is slightly lower but still satisfactory.

CONCLUSION

The proposed method for glycerol determination in honey is quick and simple, since it avoids clarification and neutralization steps. It supposes an important saving of time and reagents, with a good precision and accuracy and can be therefore easily adapted to routine operations with large numbers of samples.

ACKNOWLEDGEMENT

The authors thank Dr Juan Carlos García Monteagudo, of the Departamento de Fisico-Química; Profs Oscar García Martin and José Luis Sánchez López, of the Departamento de Bioquímica; Profs José Sordo Rodríguez and Alfonso Castiñeiras Campos, of the Departamento de Quimica Inorgánica for their comments and for providing material. Thanks to Fina Guasch Farrás, Product Manager of Boehringer Mannheim of Barcelona, Spain, for providing material for this study. Thanks also to all the honey producers who provided the samples for this study. Specially, the authors thank the Consellería de Agricultura, Ganadeiría e Montes of the Xunta de Galicia, who funded this study through the 'Producto Galego de Calidade-Mel de Galicia' scheme.

REFERENCES

Boehringer Mannheim Gmbh 1989 *Biochemical Analysis Food Analysis.* Glycerol Cat. No. 148 270. PO Box 310120, Mannheim, Germany.

Deifel A 1985 Gas chromatography determination of sugars in honey. *Deutsch Lebensm Tdsch* **81** 209–212.

Hajny G J, Hendershot W F, Peterson W H 1960 Factors affecting glycerol production by a newly isolated osmophylic yeast. *Appl Microbiol* **8** 5–11.

Huidobro J F, Rea M E, Branquinho de Andrade P C, Sancho M T, Muniategui S, Simal-Lozano J 1993 Enzymatic determination of glycerol in honey. *J Agric Food Chem* **41** 557–559.

Huidobro J F, Rea M E, Branquinho de Andrade P C, Sánchez M P, Sancho M T, Muniategui S, Simal-Lozano J 1994 Enzymatic determination of primary normal alcohols as apparent ethanol content in honey. *J Agric Food Chem* **42** 1975–1978.

Laub E, Marx R 1987 Glycerin, a natural ingredient of honey. *Lebensmittelchem. Gerichtl Chem* **41** 110.

Pfeiffer P, Radler F 1985 Determination of organic acids, sugar glycerol and ethanol in wine by HPLC with a cation exchange resin. *Weinwirtsch Technol* **8** 5234–5235.

Peterson W H, Hendershot W F, Hajny G J (1985) Factors affecting production of glycerol and D-arabitol by representative yeasts of the genus *Zygosaccharomyces Appl Microbiol* **6** 349–357.

Spencer J F T, Sallans H R 1956 Production of polyhydric alcohols by osmophilic yeasts. *Can J Microbiol* **2** 72–79.

Spencer J F T, Shu P 1957 Polyhydric alcohol production by osmophilic yeasts: effect of oxygen tension and inorganic phosphate concentration. *Can J Microbiol* **3** 559–567.

White J W Jr 1979 Spectrophotometric method for hydroxymethylfurfural in honey. *J A O A C* **62** 509–514.