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Enzymatic determination of L-malic acid in honey

Inés Mato,[a] José F. Huidobro,[a]* M. Pilar Sánchez,[a] Soledad Muniategui,[b] Miguel A. Fernández-Muiño[c] & M. Teresa Sancho[c]

[a]*Facultad de Farmacia, Área de Nutrición y Bromatología, Universidad de Santiago, Campus Universitario Sur, 15706 Santiago de Compostela (Galicia), Spain*
[b]*Facultad de Ciencias, Área de Química Analítica, Universidad de La Coruña, Campus de la Zapateira s/n, 15071 La Coruña (Galicia), Spain*
[c]*Facultad de Ciencia y Tecnología de los Alimentos y Ciencias Químicas, Área de Nutrición y Bromatología, Universidad de Burgos, Plaza de Misael Bañuelos s/n, 09001 Burgos (Castilla y León), Spain*

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L-Malic acid determination has been carried out in honey using a direct enzymatic method. The sample solution was prepared from 2.5 g honey in 100 ml Milli-Q water. The enzymatic determination was measured spectrophotometrically at 340 nm, using glutamate-oxaloacetate transaminase and L-malate dehydrogenase. The direct method combines precision (CV was 3.5%, at worst), good recovery (100 ± 3.5%), zero interference, simplicity, and low cost (cost was reduced by 50% using a microtest). This direct enzymatic method was applied to 20 floral honeys of Galicia (northwestern Spain) and the results ranged between 94 and 596 mg kg^{-1} (mean 246 mg kg^{-1}) of L-malic acid, which is in keeping with value ranges obtained by other authors. Different clarifications [as polyvinylpolypyrrolidone (PVPP), Carrez, Carrez with NaOH, Carrez with KOH, Carrez together with PVPP and activated charcoal] and a pair of controls have also been used but the precision and the recovery of direct enzymatic method of L-malic acid in honey did not improve.

INTRODUCTION

Honey is a complex product with many constituents. Although organic acids are present in honey in small concentrations (< 0.5%), they are an important group of constituents that contribute to flavour (White, 1979*a*) and to stability towards microorganisms (White, 1979*b*).

In honey, 19 organic acids, certainly or probably present, have been identified (Crane, 1990). Malic acid, which is usually the second acid after D-gluconic acid (Cherchi *et al.*, 1994), was one of the first acids identified in honey (Hilger, 1904). The origin of this acid, as well as other organic acids, is not very well known. It could originate from glucose, fructose or sucrose of the nectar by the action of enzymes which the bee adds at ripening (Echigo and Takenaka, 1974). Many of the honey acids are intermediates in Krebs' cycle of biological oxidation and may already be present in the nectar (White, 1979*b*). Honeydew also contains organic acids, especially citric, malic, succinic and fumaric acids (Maurizio, 1979).

There are two isomers of malic acid: L-malic and D-malic. L-Malic is the natural isomer and D-malic is not known to occur in natural products, although small amounts of this D-isomer may be present in some products because the racemate (produced by chemical synthesis) is used as an acidulant (Bergmeyer, 1985).

Malic acid has been isolated, with other acids, in honey by paper chromatography, ion-exchange chromatography and silicic acid partition chromatography (Stinson *et al.*, 1960). Malic acid and other acids were also determined by Cherchi *et al.* (1994), using a high-performance liquid chromatographic method with two columns connected in series after sample purification by solid-phase extraction. The values of malic acid found by Cherchi *et al.* (1994) in 48 floral honeys ranged between 49.7 and 178.1 mg kg^{-1}. This HPLC method, apart from being nonspecific for the determination of D- and L-isomers, is also expensive and laborious.

*To whom correspondence should be addressed.

The enzymatic method allows the straightforward specific determination of D- and L-isomers. This method has been developed by several authors in various foods. In honey, Tourn *et al.* (1980) determined the L-malic acid content of only four samples (floral and honeydew honeys) using a direct enzymatic method, but data about precision and recovery have not been studied or described. The values of L-malic acid found for honeydew honeys were 1300 and 7400 mg kg^{-1} and, for floral honeys, were 150 and 640 mg kg^{-1}. So the content of L-malic acid in honey may be an analytical property for the differentiation of two maintypes: floral honey and honeydew honey. This could be important because there are few chemical procedures available to determine the botanical origin of honey.

The purpose of the present study has been to optimise the direct enzymatic method for determining L-malic acid in honey by studying precision and recovery and by using different clarifications.

MATERIALS AND METHODS

Samples

The work was carried out on 20 floral samples from Galicia (in northwest Spain). The samples were harvested in autumn 1994 and stored in the darkness at room temperature till analysis four months later. The botanical origin of the samples was determined by the procedure of Louveaux *et al.* (1978) after treating and dyeing the sediment in the honeys using the method of Terradillos *et al.* (1994). One sample was *Castanea sativa* honey, seven samples were *Eucalyptus* sp. honeys, one sample was *Rubus* sp. honey, and eleven samples were multifloral samples.

Reagents and apparatus

A Boehringer-Mannheim (1995) enzymatic test kit was used for approximately 25 determinations (Catalogue No. 139 068). The test combination contained the following: (a1) solution consisting of glycylglycine buffer, pH 10.0; L-glutamic acid, 440 mg; stabilisers; (a2) NAD lyophilisate, 210 mg; (a3) glutamate-oxaloacetate transaminase suspension (GOT), 160 U; and (a4) L-malate dehydrogenase solution (L-MDH), 2400 U. A Kontron Uvikon 810 P UV–vis double-beam spectrophotometer was used.

Procedure

Approximately 2.5 g honey was dissolved in 20 ml Milli-Q water, transferred to a 100 ml volumetric flask, and made to the mark with water. Into a 1.5 ml cuvette, the following were pipetted: 0.50 ml of solution (a1), 0.10 ml of NAD solution (a2), 0.50 ml of sample solution and 0.005 ml of glutamate-oxaloacetate transaminase suspension (GOT) (a3). The content of the cuvette was mixed thoroughly and completion of the reaction awaited (after approximately 5 min). When the incremental increase in absorbance was constant the absorbance at 340 nm vs Milli-Q water (S_1) was read. The reaction was started by the addition of 0.005 ml L-malate dehydrogenase solution (L-MDH) (a4). The content of the cuvette was mixed, and after approximately 5 min the absorbance of the solutions (S_2) was read.

After 5 min the absorbance increases steadily with a uniform slope. In our case, a second and slower reaction occurred in parallel with the main reaction. This caused 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 was carried out as follows: (i) at intervals of 1 min, an absorption reading five times more than indicated in the general method was taken; (ii) absorption against time was plotted on 1 mm graph paper; (iii) the linear portion of the curve obtained to the time of the addition of solution (a4) (B_1 and S_1) was extrapolated. 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). The blank was measured following the same procedure with 0.50 ml Milli-Q water instead of 0.50 ml sample solution (B_1 and B_2). The absorbance difference was determined for both blank and sample and the absorbance difference of the blank was subtracted from the absorbance difference of the sample:

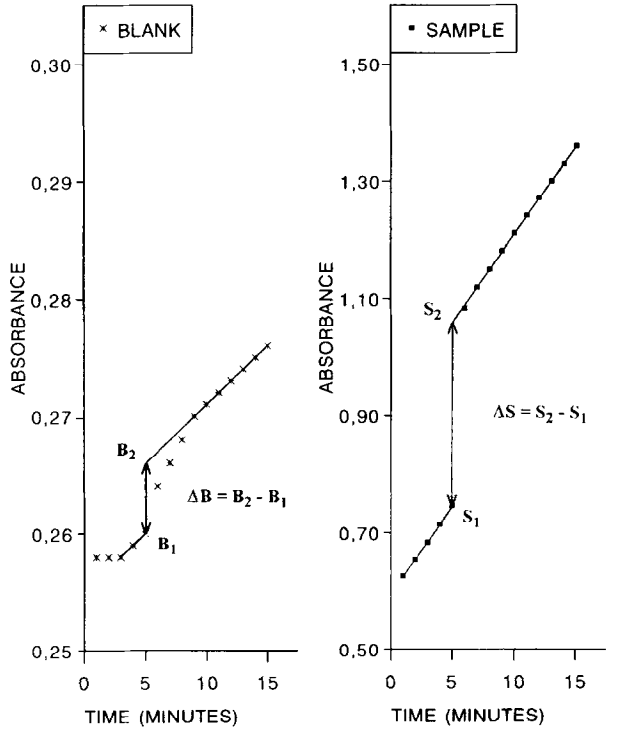

Fig. 1. Absorbances at 340 nm measured to determine L-malic acid in honey using the direct enzymatic method.

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

Specificity of the enzymatic method

The enzymatic method is specific for L-malic acid with D-isomer not reacting. Also, L-lactic acid, D-lactic acid, L-asparaginic acid and fumaric acid are not converted (Boehringer-Mannheim, 1995).

Calculations

The calculations were carried out as specified by Boehringer-Mannheim (1995) for other foodstuffs. For honey, L-malic acid was calculated as follows:

$$\text{mg L - MALIC ACID/KG OF HONEY} = \frac{4725}{\text{sample wt in g}} \times \Delta A.$$

$$\left[4725 = \frac{1.11 \times 134.1}{6.30 \times 1 \times 0.50 \times 1000} \times \frac{100}{1000} \times 1000 \times 1000\right].$$

In these equations: $\Delta A = (S_2 - S_1) - (B_2 - B_1)$, where $(S_2 - S_1)$ is the absorption of the sample and $(B_2 - B_1)$ is the absorption of the blank; 1.11 = final volume (ml); 134.1 = mol wt of L-malic acid; 6.30 = absorption coefficient of NADH at $\lambda = 340$ nm (litre $\times$ mmol^{-1} $\times$ cm^{-1}); 1 is the light path (cm); 0.50 = sample volume (ml); 1000 = ml in 1 litre; 100/1000 = g L-malic acid in 100 ml final solution; 1000 = mg in 1 g; and 1000 = g in 1 kg.

Tests of clarification

Different clarifications were used with a standard of L-malic acid. The concentration of this standard solution was close to the mean of the samples (5 mg L-malic acid/litre sample solution equal to 200 mg L-malic acid/kg of honey).

Polyvinylpolypyrrolidone (PVPP) clarification
Ten millilitres of this standard solution (5 mg L-malic/litre) was stirred with 0.1 g PVPP for 1 min and filtered. The filtered solution was used for the enzymatic assay.

Carrez clarification
Standard solution was clarified with Carrez solutions, (White, 1979*c*). 0.5 ml of Carrez I solution [15 g $K_4Fe(CN)_6 \cdot 3H_2O$/100 ml water] was added and stirred. Then 0.5 ml Carrez II solution [30 g $ZnSO_4 \cdot 7H_2O$/100 ml water]) was added and stirred. It was made up to 50 ml (the final concentration of standard solution must be 5 mg L-malic/litre) and filtered, discarding the first 10 ml of filtrate. The filtered solution was used for carrying out the enzymatic test.

Carrez clarification with NaOH
The procedure was similar to 'Carrez clarification'. Besides adding the Carrez solutions, 4 ml of 0.1 N NaOH was added. It was then made up to 50 ml and filtered, discarding the first 10 ml of the filtrate. The filtered solution was used for the enzymatic assay.

Carrez clarification with KOH
The same procedure was used as for the 'Carrez clarification with NaOH'. It was added at 4 ml of 0.1 N KOH instead of 0.1 N NaOH.

Carrez together with PVPP
First the standard solution was clarified with Carrez solutions as described above. Then 10 ml of filtrate was treated with PVPP using the procedure described for 'PVPP clarification'.

Activated charcoal
Standard solution (5 ml) was stirred with 0.1 g of activated charcoal for 1 min and filtered. This filtered solution was used for carrying out the enzymatic assay.

When these different clarifications were applied to the honey sample, the same procedures were used as for the standard solution of L-malic acid. A honey solution (2.5 g honey dissolved in 100 ml Milli-Q water) was used instead of the standard solution.

RESULTS

Direct enzymatic method

Repeatability
The precision of the direct enzymatic method was satisfactory. It was established by measuring the L-malic acid content of 10 solutions from each of four floral samples (7, 11, 16 and 15) with low (94 mg kg^{-1}), medium (240 mg kg^{-1}), high (463 mg kg^{-1}), and very high (596 mg kg^{-1}), L-malic acid levels, respectively.

Table 1. Precision of the direct enzymatic method for measuring L-malic acid content (mg kg^{-1}) of honeys

	Honey samples			
	7	11	16	15
	99	243	461	588
	91	240	464	599
	95	247	453	595
	91	232	468	589
	95	237	466	599
	96	232	464	598
	99	243	455	601
	89	247	468	595
	95	240	461	601
	94	237	466	598
Mean	94	240	463	596
SD[a]	3.31	5.39	5.17	4.60
%CV[b]	3.5	2.2	1.1	0.8

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

Coefficients of variation of 3.5, 2.2, 1.1 and 0.8% were obtained, respectively (Table 1).

Recovery of added L-*malic acid*

The recovery was established by adding increasing amounts of L-malic acid, covering the concentration range present in the samples analysed (approximately 100–700 mg kg^{-1}), to a honey sample containing 47 mg kg^{-1} of total L-malic acid and using the proposed method to determine the L-malic content (Table 2). The L-malic acid reference solution (Boehringer-Mannheim, No. 139 068), included in the enzymatic test kit, was used. The mean recovery of the direct enzymatic method was 100 ± 3.5%.

Table 2. Study of the recovery of the direct enzymatic method to determine L-malic acid (mg kg^{-1}) in honey

Present (mg kg^{-1})	Added (mg kg^{-1})	Found (mg kg^{-1})	Recovery (%)
	50	99	104
	50	95	96
	50	99	104
	250	288	96
	250	300	101
	250	305	103
47			
	450	480	96
	450	511	103
	450	485	97
	650	681	98
	650	669	96
	650	713	102
n			12
Mean			100
SD[a]			3.45
%CV[b]			3.5

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

Table 3. L-Malic acid contents of the honeys analysed

Sample	Botanical origin	L-Malic acid (mg kg^{-1})
1	*Castanea sativa*	519
2	*Eucalyptus* sp.	103
3	*Eucalyptus* sp.	135
4	*Eucalyptus* sp.	131
5	*Eucalyptus* sp.	177
6	*Eucalyptus* sp.	189
7	*Eucalyptus* sp.	94
8	*Eucalyptus* sp.	126
9	*Rubus* sp.	216
10	Multifloral	114
11	Multifloral	240
12	Multifloral	475
13	Multifloral	235
14	Multifloral	148
15	Multifloral	596
16	Multifloral	463
17	Multifloral	141
18	Multifloral	545
19	Multifloral	135
20	Multifloral	137
Mean		246
SD[a]		169
V_{min}		94
V_{max}		596

[a] Standard deviation.

L-Malic acid contents of the Galician honeys analysed

The L-malic acid contents of the 20 Galician floral honeys analysed using the direct enzymatic method are shown in Table 3. The mean L-malic acid concentration was 246 ± 169 mg kg^{-1}. Most of the values were less than the values found by Tourn *et al.* (1980) but higher than Cherchi *et al.* (1994) for floral honeys.

Tests of clarification

The results of the different clarifications applied to a standard solution of L-malic acid are showed in Table 4. According to these results, 'Carrez clarification with NaOH', 'Carrez clarification with KOH' and 'Activated charcoal clarification' were rejected to investigate in samples. Other clarifications: 'Polyvinylpolypyrrolidone (PVPP) clarification', 'Carrez solutions' and 'Carrez with PVPP' were investigated in honey samples due to the best results occurring in the L-malic acid standard solution. The precision of the method using the 'PVPP clarification' and 'Carrzez solutions' did not improve the precision of the direct enzymatic method because the coefficients of variation were 4.5 and 5.3%, respectively. The last clarification investigated was 'Carrez with PVPP'. In this case the precision improved because the coefficient of variation was 2.8%. The recovery was studied due to this high precision. It was established by adding increasing amounts of L-malic acid to a honey sample, but the results obtained were a mean value of 93 ± 3.8%. Precision improved only slightly with this sample treatment but recovery was worse than that for direct determination.

DISCUSSION

Before this work, Tourn *et al.* (1980) applied the direct enzymatic method to determine L-malic acid in honey.

Table 4. Comparison of the values obtained for L-malic acid using the enzymatic method after applying different clarifications to standard solution of L-malic acid

Clarifications	Present before clarification (mg $litre^{-1}$)	Found after clarification (mg $litre^{-1}$)	%
PVPP	5.0	5.1	102
Carrez	5.0	4.8	96
Carrez + NaOH	5.0	3.5	70
Carrez + KOH	5.0	2.4	48
Carrez + PVPP	5.0	5.0	100
Activated charcoal	5.0	5.4	108

However, we thought that some points were unclear. Firstly, any data about precision and recovery were not described. Moreover, the range of honey solutions given was too indeterminate (1–7 g honey/100 ml) and the content of L-malic acid of each sample must be guessed at.

We have done a study of precision and recovery to apply the direct enzymatic method as an analytical method for honey or for other sugar foods (Tables 1, 2). Our proposed amount of honey was 2.5 g in 100 ml Milli-Q water, and 0.5 ml of this solution (in a total volume of 1.11 ml) was used for carrying out the test. In these conditions all the floral honeys with L-malic acid content between 20 and 800 mg kg^{-1} could be analysed [according to the recommendation of Boehringer-Mannheim (1995)]. If a sample had a higher concentration of L-malic acid (honeydew honeys), only 10 ml of the previous sample solution was pipetted and transferred to a 100 ml volumetric flask to make to the mark. In this case, honeys with a content of L-malic acid between 200 and 8000 mg kg^{-1} could be analysed. Futhermore, the cost of the enzymatic analysis was reduced by 50%, because only 50% of the sample volume and reagent volumes specified by the supplier Boehringer-Mannheim (1995), were used.

Although we thought the method was acceptable for an analytical determination of L-malic acid in honey, we attempted to improve the precision and recovery of the direct method by applying, firstly, different clarifications to the sample and then a pair of controls.

The clarification could have allowed elimination of the slope before and after addition of L-malate dehydrogenase in the determination. 'PVPP clarification' was first used because it was recommended by Boehringer-Mannheim (1995) for L-malic acid in other foodstuffs. We then used 'Carrez solutions', 'Carrez clarification with NaOH', 'Carrez clarification with KOH' and 'Carrez together with PVPP' because they have been applied previously to determine other honey components using an enzymatic method (Huidobro *et al.*, 1993; Val *et al.*, 1998). And finally, 'Activated charcoal clarification' was used because it had been applied to determine acids in other foodstuffs (Boehringer-Mannheim, 1995). Nevertheless, with these sample treatments, neither the precision nor the recovery improved the results of the direct determination.

Finally, it was decided to use a pair of controls. Solution (a1), NAD solution (a2), sample solution and glutamate-oxaloacetate transaminase suspension (GOT) (a3) were added to both sample and reference cuvettes. Then L-malate dehydrogenase solution (L-MDH) (a4) was added into the sample cuvette and Milli-Q water to the reference cuvette, respectively. Milli-Q water was added instead of sample solution for measuring the blank. However, the pair of controls failed too, because the absorbance differences obtained did not stabilise and unusually decreased (Huidobro *et al.*, 1994).

In summary, the direct enzymatic method of L-malic acid in honey has been optimised for precision and recovery with the amount of honey used. Moreover the cost was reduced by 50% using a microtest. This direct method could give information on the content of L-malic acid in honey, as well as other foods with a high sugar content. Although different clarifications (as PVPP, Carrez, Carrez with NaOH, Carrez with KOH, Carrez together with PVPP and Activated charcoal) and a pair of controls have been investigated, the precision together with the recovery of the direct method did not improve.

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