# Enzymatic Determination of Galactose and Lactose in Honey

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Even when galactose and lactose are present in extraordinarily low concentrations in honey, both sugars are important constituents of this foodstuff because they might be useful in its characterization. Lactose and galactose determination in honey is also important because of the toxic effect of both sugars on honey bees. For the first time, a modification of the Boehringer-Mannheim GmbH enzymatic method is applied to 46 floral unpasteurized honeys for analyzing these sugars accurately, specifically, and quickly, with very low detection limits. Galactose is quantified directly without previous elution. The method avoids both previous chromatographic elution of mono- and disaccharides and removal of glucose. For galactose analysis pretreatment of honey solution with 0.5 mL of Carrez I, the same quantity of Carrez II, and 4 mL of 0.1 N NaOH is necessary; after filtration, 0.100 mL is used as sample solution, and galactose dehydrogenase is employed as enzyme. For simultaneous determination of galactose and lactose, not only is the previous clarification with Carrez solutions necessary, but neutralization with 1 mL of 0.1 N NaOH and 3 mL of 0.1 N KOH is also required. After the filtration, it is necessary to use 0.050 mL as sample solution and galactose dehydrogenase and $\beta$-galactosidase as enzymes. For a microtest, 0.99 mL final volume is required. Precision (%CV < 2.40 for galactose and <2.01 for lactose) and recovery (99.9% for galactose and 100.3% for lactose) were good. The galactose content of the honeys analyzed ranged between 0.0052 and 0.0151%. The lactose content of the honeys analyzed ranged between 0.0062 and 0.0383%.

**Keywords:** *Honey; galactose; lactose; enzymatic analysis*

## INTRODUCTION

Galactose is one of the nectar disaccharides (Battaglini et al., 1973).

Galactose and lactose have been detected in most honeydew honeys, but lactose has not been quantified (Maurizio, 1985). Values of these sugars could be useful for grouping different floral and honeydew honeys, which is very important in most countries.

Both galactose and lactose (among other sugars such as mannose and raffinose) are toxic to honey bees because of their lack of proper enzymes for its digestion. The toxicity of lactose is due to the effect of galactose. Both sugars reduce the longevity of honey bees even when fed in sucrose syrup (Barker and Lehner, 1974a,b; Herbert, 1992).

In addition, knowledge of lactose and galactose values in honey is interesting because of their possible nutritional implications in people with enzymatic deficiencies related to metabolism of both sugars.

Avigad et al. (1962) used galactose oxidase for oxidizing terminal galactose molecules having a free 6-hydroxyl group, the reagent specific for free galactose, raffinose, stachyose, planteose, and other oligosaccharides with a terminal galactose. Several related compounds are also oxidized (D-talose, 2-deoxy-D-galactose, D-galactosamine, *N*-acetyl-D-galactosamine), but none of these would be found in the higher sugar fraction of honey. Full details appear elsewhere in which a 1 g sample of honey is placed on a charcoal column, washed with 7% ethanol to remove other mono- and disaccharides, and eluted with 50% ethanol; the eluate is concentrated and analyzed with the galactose oxidase reagent of Fisher and Zapf (1964). Values are extremately variable, ranging from 0.0003 and 0.786% of bound galactose (White et al., 1986).

Paper chromatography has been the method employed for detecting galactose and lactose in honey (Maurizio, 1985), making it possible to distinguish between floral and honeydew honeys but only from the qualitative point of view.

Even when enzymatic methods are usually very accurate for analyzing several minority components of honey (Huidobro et al., 1993, 1994; Fernández-Muiño et al., 1996; and many other authors), it is surprising to find nothing about the development of an enzymatic analysis for determining lactose in honey. Furthermore, as chromatographic methods usually require very precise conditions and equipment, when only galactose and lactose analysis is necessary, it is very important to look for or develop the simplest and most accurate method with the lowest detection limit and the lowest cost.

The purpose of this work has been to apply the Boehringer-Mannheim GmbH (1995) enzymatic method for determining galactose and lactose in honey, looking

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for the best simplification to achieve accuracy and to save time and money. In a first assay we need to determine the galactose content in the honey.

## MATERIALS AND METHODS

**Samples.** The study involved 46 floral unheated, commercially purchased honeys from Galicia (in northwestern Spain). All samples bore the label "Producto Galego de Calidade-Mel de Galicia" (Diario Oficial de Galicia, 1989), which guaranteed their origin. The samples, which represented all Galician producers of honeys labeled in this way, were harvested in autumn 1995. 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). Samples included 1 *Castanea sativa* unifloral honey, 23 *Eucalyptus* sp. unifloral honeys, 2 *Rubus* sp. unifloral honeys, and 20 multifloral honeys.

**Principle.** The enzyme $\beta$-galactose dehydrogenase (Gal-DH) catalyzes the oxidation of D-galactose with the formation of reduced nicotinamide adenine dinucleotide (NADH). The NADH formed in this reaction is stoichiometric to the amount of D-galactose and is measured by means of its light absorbance at 340 nm. Lactose is hydrolyzed to D-glucose and D-galactose in the presence of the enzyme $\beta$-galactosidase and water. The determination of D-galactose after inversion is carried out simultaneously acccording to the principle outlined above. The lactose content is calculated from the difference of the absorbances of NADH at 340 nm before and after enzymatic inversion (Boehringer-Mannheim GmbH, 1995).

**Reagents and Apparatus.** (*a*) The method of Boehringer-Mannheim GmbH (1995) was used for the determination of lactose and D-galactose (enzymatic test for 30 determinations; Catalog No. 176 303). The test combination contains the following:

(*a1*) Lyophilysate (600 mg) consisting of citrate buffer, pH 6.6, ≈35 mg of NAD, magnesium sulfate, and stabilizers. The contents of bottle 1 are stable for 1 year at 4 °C. Dissolve with 7.0 mL of redistilled water. This solution is stable for 3 months at 4 °C. Bring solution 1 to 20−25 °C before use.

(*a2*) $\beta$-Galactosidase suspension (1.7 mL), ≈100 units. The contents of the bottle are stable for 1 year at 4 °C.

(*a3*) Approximately 34 mL of solution consisting of potassium diphosphate buffer, pH 8.6, and stabilizers. The contents of the bottle are stable for 1 year at 4 °C. Bring solution 3 to 20−25 °C before use.

(*a4*) Approximately 1.7 mL of galactose dehydrogenase suspension, ≈40 units. The contents of the bottle are stable for 1 year at 4 °C.

(*b*) Carrez I solution contained 15 g of potassium hexacyanoferrate(II) trihydrate (Merck Art. 4984) diluted to 100 mL with water.

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

(*d*) NaOH (0.1 N) was 4 g of sodium hydroxide (Merck Art. 6498) diluted to 100 mL with water.

(*e*) KOH (0.1 N) was 5.6 g of potassium hydroxide (Merck Art. 5033) diluted to 100 mL with water.

(*f*) A Kontron Uvikon 810P UV−vis double-beam spectrophotometer was used.

**Procedure.** *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 a mixture of 1 mL of 0.1 N NaOH and 3 mL of 0.1 N KOH and stir. Make up to 50 mL and stir. Filter using a Whatman no. 41 filter paper, discarding the first 10 mL of fitrate.

*Galactose.* Pipet into a cuvette 0.050 mL of solution 1 (a1), 0.100 mL of sample solution, and 0.020 mL of redistilled water. Mix and wait ≈15 min. Pipet 0.300 mL of solution 3 (a3) and 0.500 mL of redistilled water. Mix, wait for completion of the reaction (≈5 min), and read the absorbance at 340 nm versus redistilled water (absorbance $S_1$).

Start reaction by addition of 0.020 mL of galactose dehydrogenase (a4). Mix, wait for completion of the reaction (≈30 min), and read the absorbance immediately ($S_2$).

Blanks were measured following the same procedure with 0.100 mL of redistilled water instead of 0.100 mL of sample solution (values $B_2 - B_1$).

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

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

*Lactose.* Pipet into a cuvette 0.050 mL of solution 1 (a1), 0.050 mL of sample solution, 0.020 mL of enzyme suspension (a2), and 0.050 mL of redistilled water. Mix and wait ≈15 min. Pipet 0.300 mL of solution 3 (a3) and 0.500 mL of redistilled water. Mix, wait for completion of the reaction (≈5 min), and read the absorbance at 340 nm versus redistilled water (absorbance $S'_1$).

Start reaction by addition of 0.020 mL of galactose dehydrogenase (a4). Mix, wait for completion of the reaction (≈30 min), and read the absorbance immediately ($S'_2$).

Blanks were measured following the same procedure with 0.100 mL of redistilled water instead of 0.050 mL of sample solution and 0.050 mL of redistilled water (values $B'_2 - B'_1$).

After 10 min, the values of $S'_1$ and $S'_2$ were not constant, whereas it is necessary to extrapolate the absorbances to the time of the addition of solution a3.

Determine the absorbance differences ($S'_2 - S'_1$) for both sample and blank ($B'_2 - B'_1$).

Lactose and galactose enzymatic graphs are similar because the former is determined on the basis of the second one.

**Calculations.** The calculations were carried out as specified by the supplier, Boehringer-Mannheim GmbH (1995), for other foodstuffs. For honey, following our procedure, the galactose and lactose contents are calculated as follows:

galactose

$$\%\ \text{galactose} = \frac{1.416}{\text{sample wt in g}} \times \Delta A_{\text{galactose}}$$

$$1.416 = \frac{0.99 \times 180.16}{6.30 \times 1 \times 0.100 \times 1000} \times \frac{50}{1000} \times 100$$

where $\Delta A_{\text{galactose}} = (S_2 - S_1) - (B_2 - B_1)$, $(S_2 - S_1)$ is the absorption of sample in the assay without enzymatic hydrolysis and 0.100 mL of sample solution, $(B_2 - B_1)$ is the absorption of blank in the assay without enzymatic hydrolysis and 0.100 mL of redistilled water, 0.99 is the final volume (mL), 180.16 is the molecular weight of galactose (g/mol), 6.30 is the absorption coefficient of NADH at 340 nm (L × mmol$^{-1}$ × cm$^{-1}$), 1 is the light path (cm), 0.100 is the volume sample (mL), 1000 = mL in 1 L, (50/1000) = g of galactose in 50 mL of final solution, and 100 = g in 100 g.

lactose

$$\%\ \text{lactose} = \frac{5.379}{\text{sample wt in g}} \times \Delta A_{\text{lactose}}$$

$$5.379 = \frac{0.99 \times 342.3}{6.30 \times 1 \times 0.050 \times 1000} \times \frac{50}{1000} \times 100$$

where $\Delta A_{\text{lactose}} = [(S'_2 - S'_1) - (B'_2 - B'_1)] - 0.5[(S_2 - S_1) - (B_2 - B_1)]$, $(S'_2 - S'_1)$ is the absorption of sample in the assay with enzymatic hydrolysis and 0.050 mL of sample solution, $(B'_2 - B'_1)$ is the absorption of blank in the assay with enzymatic hydrolysis, $(S_2 - S_1)$ is the absorption of sample in the assay without enzymatic hydrolysis and 0.100 mL of sample solution, $(B_2 - B_1)$ is the absorption of blank in the assay without enzymatic hydrolysis, 0.5 is the relation between volume in determination of lactose (0.050 mL) and volume in determination of galactose (0.100 mL), 0.99 is the final volume

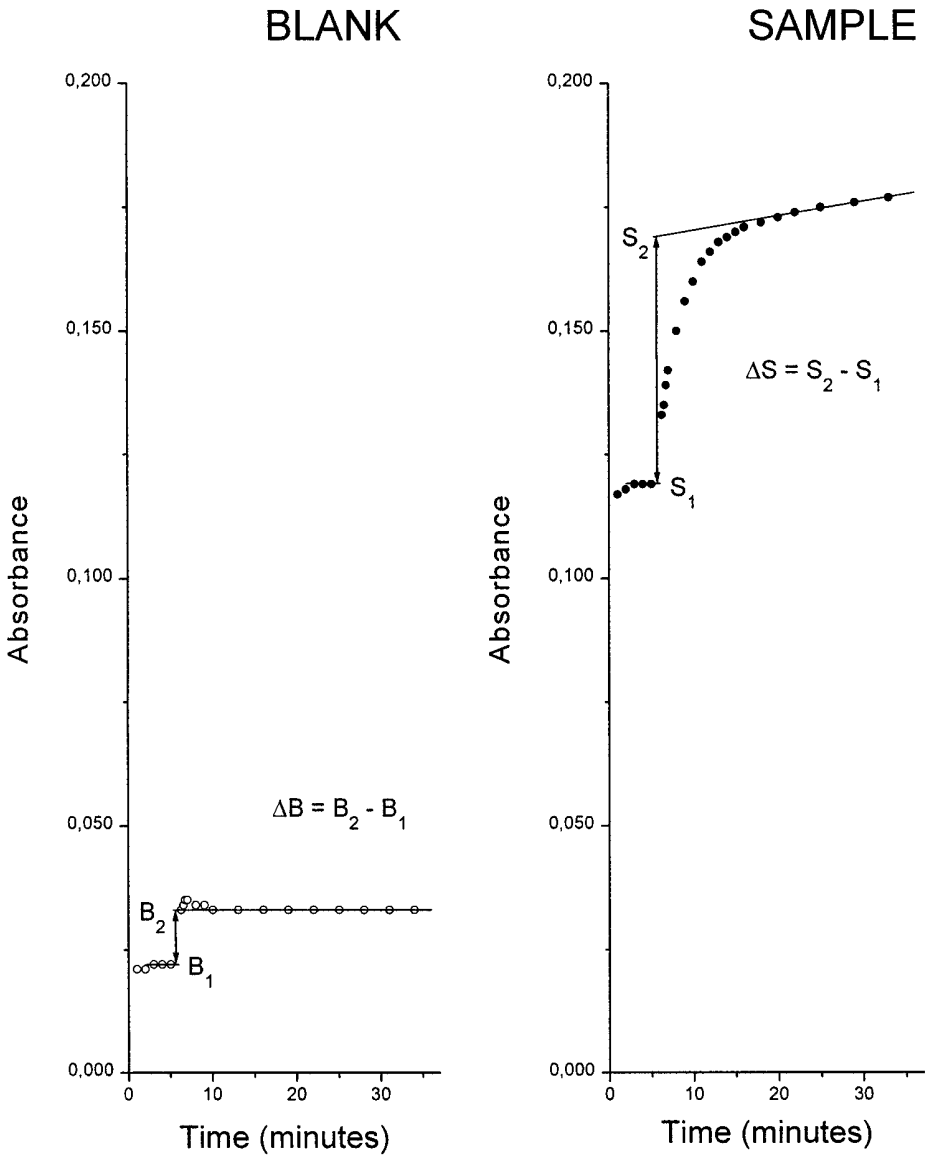


**Figure 1.** Absorbances at 340 nm measured to determine galactose in honey using the enzymatic analysis.

(mL), 342.3 is the molecular weight of lactose (g/mol), 6.30 is the absorption coefficient of NADH at 340 nm (L × mmol$^{-1}$ × cm$^{-1}$), 1 is the light path (cm), 0.050 is the volume sample (mL), 1000 = mL in 1 L, (50/1000) = g of lactose in 50 mL of final solution, and 100 = g in 100 g.

## RESULTS

**Repeatability.** Measurement of the galactose content of 10 solutions of the 3 honey samples (43, 5, and 37) with low, medium, and high contents of galactose returned a coefficient of variation (%CV) of <2.40% in all cases (Table 1).

Measurement of the lactose content of 10 solutions of the 3 honey samples (35, 46, and 3) with low, medium, and high contents of lactose returned a %CV of <2.01% in all cases (Table 2).

**Recovery of Added Galactose and Lactose.** The recovery was established by adding increasing amounts of galactose covering the concentration range present in the sample analyzed (0.0052−0.0151%) to honey sample 43 containing 0.0052% galactose and using the method to determine the galactose (Table 3). The galactose reference (Merck Art. 4061) was used. The mean recovery was 99.9%, and the %CV was 1.34%.

**Table 1. Study of the Precision of the Enzymatic Method To Determine Galactose in Honey**

| | sample 43 | sample 5 | sample 37 |
|---|---|---|---|
| | 0.0052 | 0.0107 | 0.0151 |
| | 0.0052 | 0.0111 | 0.0151 |
| | 0.0055 | 0.0111 | 0.0151 |
| | 0.0052 | 0.0111 | 0.0151 |
| | 0.0052 | 0.0111 | 0.0155 |
| | 0.0052 | 0.0111 | 0.0151 |
| | 0.0052 | 0.0111 | 0.0151 |
| | 0.0055 | 0.0107 | 0.0151 |
| | 0.0052 | 0.0111 | 0.0151 |
| | 0.0052 | 0.0107 | 0.0151 |
| mean | 0.0053 | 0.0110 | 0.0151 |
| SD | $1.26 \times 10^{-4}$ | $1.93 \times 10^{-4}$ | $1.26 \times 10^{-4}$ |
| %CV | 2.40 | 1.76 | 0.84 |

The recovery was established by adding increasing amounts of lactose covering the concentration range present in the sample analyzed (0.0062−0.0383%) to honey sample 35 containing 0.0062% lactose and using the method to determine the lactose (Table 4). The lactose monohydrate reference (Merck Art. 7656) was used. The mean recovery was 100.3%, and the %CV was 1.64%.

**Table 2. Study of the Precision of the Enzymatic Method To Determine Lactose in Honey**

| | sample 35 | sample 46 | sample 3 |
|---|---|---|---|
| | 0.0062 | 0.0232 | 0.0380 |
| | 0.0062 | 0.0229 | 0.0383 |
| | 0.0062 | 0.0229 | 0.0383 |
| | 0.0062 | 0.0232 | 0.0383 |
| | 0.0062 | 0.0229 | 0.0383 |
| | 0.0065 | 0.0229 | 0.0383 |
| | 0.0062 | 0.0229 | 0.0383 |
| | 0.0062 | 0.0229 | 0.0383 |
| | 0.0065 | 0.0229 | 0.0380 |
| | 0.0062 | 0.0233 | 0.0383 |
| mean | 0.0063 | 0.0230 | 0.0382 |
| SD | $1.26 \times 10^{-4}$ | $1.63 \times 10^{-4}$ | $1.26 \times 10^{-4}$ |
| %CV | 2.01 | 0.71 | 0.33 |

**Table 3. Study of the Recovery of the Enzymatic Method To Determine Galactose in Honey**

| present | added | found | recovery |
|---|---|---|---|
| | 0.0121 | 0.0175 | 101.7 |
| | 0.0121 | 0.0171 | 98.3 |
| | 0.0121 | 0.0172 | 99.2 |
| | 0.0242 | 0.0299 | 102.1 |
| 0.0052 | 0.0242 | 0.0291 | 98.8 |
| | 0.0242 | 0.0295 | 100.4 |
| | 0.0363 | 0.0411 | 98.9 |
| | 0.0363 | 0.0416 | 100.3 |
| | 0.0363 | 0.0412 | 99.2 |
| mean | | | 99.9 |
| SD | | | 1.334 |
| %CV | | | 1.34 |

**Table 4. Study of the Recovery of the Enzymatic Method To Determine Lactose in Honey**

| present | added | found | recovery |
|---|---|---|---|
| | 0.0200 | 0.0258 | 98.0 |
| | 0.0200 | 0.0264 | 101.0 |
| | 0.0200 | 0.0265 | 101.5 |
| | 0.0400 | 0.0461 | 99.8 |
| 0.0062 | 0.0400 | 0.0470 | 102.0 |
| | 0.0400 | 0.0454 | 98.0 |
| | 0.0600 | 0.0677 | 102.5 |
| | 0.0600 | 0.0658 | 99.3 |
| | 0.0600 | 0.0667 | 100.8 |
| mean | | | 100.3 |
| SD | | | 1.648 |
| %CV | | | 1.64 |

**Specificity.** Apart from D-galactose, $\beta$-galactose dehydrogenase also oxidizes L-arabinose and D-fucose (Beutler, 1984). We have found no reference in the literature to the presence of L-arabinose and D-fucose in honey (Crane, 1976; White, 1992).

Besides lactose, $\beta$-galactosidase also splits lactulose (disaccharide of glucose and fructose), but this disaccharide cannot be hydrolyzed by galactose. Substances with α-galactosidic linkages, such as raffinose, stachyose, galactinol, and melibiose, are not hydrolyzed (Beutler, 1984).

**Values of Galactose and Lactose.** The galactose and lactose contents of the 46 honey samples analyzed are shown Table 5. The mean galactose concentration was 0.0086%, with a spread of values from 0.0052 to 0.0151%. None of the honeys analyzed in this work had galactose concentrations as high as those reported by White et al. (1986). The mean lactose concentration was 0.0140%, with a spread of values from 0.0062 to 0.0383%. As far as we know, this is the first time that lactose has been determined in honey.

**Table 5. Percent Galactose and Percent Lactose Contents of 46 Honey Samples**

| sample | botanical origin | % galactose | % lactose |
|---|---|---|---|
| 1 | *C. sativa* | 0.0068 | 0.0132 |
| 2 | *Eucalyptus* sp. | 0.0088 | 0.0186 |
| 3 | *Eucalyptus* sp. | 0.0088 | 0.0383 |
| 4 | *Eucalyptus* sp. | 0.0083 | 0.0102 |
| 5 | *Eucalyptus* sp. | 0.0110 | 0.0115 |
| 6 | *Eucalyptus* sp. | 0.0089 | 0.0142 |
| 7 | *Eucalyptus* sp. | 0.0088 | 0.0119 |
| 8 | *Eucalyptus* sp. | 0.0091 | 0.0080 |
| 9 | *Eucalyptus* sp. | 0.0092 | 0.0077 |
| 10 | *Eucalyptus* sp. | 0.0082 | 0.0166 |
| 11 | *Eucalyptus* sp. | 0.0091 | 0.0114 |
| 12 | *Eucalyptus* sp. | 0.0094 | 0.0112 |
| 13 | *Eucalyptus* sp. | 0.0080 | 0.0070 |
| 14 | *Eucalyptus* sp. | 0.0085 | 0.0110 |
| 15 | *Eucalyptus* sp. | 0.0088 | 0.0135 |
| 16 | *Eucalyptus* sp. | 0.0088 | 0.0165 |
| 17 | *Eucalyptus* sp. | 0.0100 | 0.0132 |
| 18 | *Eucalyptus* sp. | 0.0067 | 0.0160 |
| 19 | *Eucalyptus* sp. | 0.0085 | 0.0120 |
| 20 | *Eucalyptus* sp. | 0.0074 | 0.0144 |
| 21 | *Eucalyptus* sp. | 0.0071 | 0.0124 |
| 22 | *Eucalyptus* sp. | 0.0094 | 0.0095 |
| 23 | *Eucalyptus* sp. | 0.0085 | 0.0114 |
| 24 | *Eucalyptus* sp. | 0.0093 | 0.0113 |
| 25 | *Rubus* sp. | 0.0093 | 0.0158 |
| 26 | *Rubus* sp. | 0.0091 | 0.0133 |
| 27 | multifloral | 0.0083 | 0.0127 |
| 28 | multifloral | 0.0096 | 0.0123 |
| 29 | multifloral | 0.0078 | 0.0129 |
| 30 | multifloral | 0.0089 | 0.0155 |
| 31 | multifloral | 0.0082 | 0.0159 |
| 32 | multifloral | 0.0085 | 0.0098 |
| 33 | multifloral | 0.0064 | 0.0165 |
| 34 | multifloral | 0.0100 | 0.0131 |
| 35 | multifloral | 0.0090 | 0.0062 |
| 36 | multifloral | 0.0085 | 0.0137 |
| 37 | multifloral | 0.0151 | 0.0137 |
| 38 | multifloral | 0.0099 | 0.0099 |
| 39 | multifloral | 0.0085 | 0.0198 |
| 40 | multifloral | 0.0081 | 0.0113 |
| 41 | multifloral | 0.0077 | 0.0180 |
| 42 | multifloral | 0.0060 | 0.0104 |
| 43 | multifloral | 0.0052 | 0.0179 |
| 44 | multifloral | 0.0104 | 0.0220 |
| 45 | multifloral | 0.0079 | 0.0194 |
| 46 | multifloral | 0.0064 | 0.0229 |
| | mean | 0.0086 | 0.0140 |
| | SD | $1.51 \times 10^{-3}$ | $5.21 \times 10^{-3}$ |
| | $V_{min}$ | 0.0052 | 0.0062 |
| | $V_{max}$ | 0.0151 | 0.0383 |

## DISCUSSION

The Boehringer-Mannheim GmbH (1995) enzymatic test for the determination of galactose and lactose uses for the assay 0.20 mL of solution a1, 0.100 mL of test solution, 1 mL of solution a3, 1.95 mL of redistilled water, and 0.050 mL of suspension a4. The absorbance differences for both blank and sample must be at least 0.100 to achieve a result accurate enough and the quantity for lactose and D-galactose has to be between 4 and 200 $\mu$g per cuvette (in 0.100−0.500 mL of sample volume).

These requirements implied a highly concentrated test solution that produced inaccurate results in the galactose determination not only in its direct determination in the sample but also with Carrez clarification and addition of Carrez and NaOH solution for reaching the optimum pH for the test (pH 8.6).

We tried to reduce the quantities of sample, Carrez solutions, 0.1 N NaOH solutions, and volume of test solution in the assay and found a satisfactory result by using 5 g of honey sample, 0.5 mL of both Carrez I and Carrez II, 4 mL of 0.1 N NaOH, and 0.300 mL of test solution for determination of galactose.

The cost of the enzymatic analysis can be reduced by using for each determination 0.050 mL of solution a1, 0.100 mL of test solution previously clarified and neutralized, 0.300 mL of solution a3, 0.500 mL of redistilled water, and 0.020 mL of suspension a4.

It is surprising that the test results are satisfactory with absorbance differences for both blank and samples between 0.014 and 0.098 in the analyzed samples, less than the 0.100 in the absorbance differences recommended by Boehringer-Mannheim GmbH (1995). Furthermore, higher relative quantities of sample produced interferences with inaccurate results.

Previous conditions were good for galactose but not for lactose determination, so we had to modify conditions by reducing the sample volume in the test and changing the neutralizant agent after Carrez clarification.

In the assay with a lower sample volume interferences in the lactose determination were eliminated but the recoveries were not satisfactory enough.

We investigated the effect of changing the neutralizing agent by using 0.1 N KOH. When we used 0.1 N NaOH as neutralizing agent, high recoveries of sugars were observed, but low recoveries were observed when 0.1 N KOH was used; therefore, we investigated the effect of using different mixes of both neutralizing agents. The mixture of 0.1 N NaOH and 0.1 N KOH (1:3) gave satisfactory results in precision and accuracy for both galactose and lactose.

The test was satisfactory even with absorbance differences for both blank and samples between 0.015 and 0.039 (free galactose plus galactose obtained from lactose).

In conclusion, enzymatic determination of galactose and lactose in honey, without previous chromatographic separation and avoiding the use of galactose oxidase, has been carried out for the first time. The method meets the conditions of precision, recovery, sensitivity, simplicity, and low cost required for an analytical method to be usable.

As this is the first time lactose in honey has been determined, it is not possible to compare our results with any bibliographic references.

The proposed method resolves the galactose and lactose analysis in honey, a foodstuff with high complexity in sugar composition having galactose and lactose are minor components. We think that the method could be useful for determining galactose and lactose in other foodstufs for which both sugars can be more important or indicative of quality and original purity.

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