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LACTOSE & D-GALACTOSE (*Rapid*)

ASSAY PROCEDURE

K-LACGAR 03/20

Incorporating A Procedure For The Analysis Of "Low-Lactose" Or "Lactose-Free" Samples Containing High Levels Of Monosaccharides

(Improved *Rapid* Format)

(*115 Assays per Kit)

** The number of tests per kit can be doubled if all volumes are halved*

The reagents provided in this kit are also suitable for use with AOAC method 2006.06 – Lactose in milk.

Megazyme

Patented: US 7,785,771 B2 and EP1 828 407 (GB, FR, IE, DE)

INTRODUCTION:

Lactose, or milk sugar, is a white crystalline disaccharide. It is formed in the mammary glands of all lactating animals and is present in their milk. Lactose yields D-galactose and D-glucose on hydrolysis by lactase (β-galactosidase), an enzyme found in gastric juice. People who lack this enzyme after childhood cannot digest milk and are said to be lactose intolerant. Common symptoms of lactose intolerance include nausea, cramps, gas and diarrhoea, which begin about 30 minutes to 2 hours after eating or drinking foods containing lactose. Between 30 and 50 million Americans are lactose intolerant, with certain ethnic and racial populations being more widely affected than others; as many as 75 percent of all African-Americans and Native Americans and 90 percent of Asian-Americans are lactose intolerant. The condition is least common among persons of northern European descent.

Enzymic methods for the measurement of lactose are well known and are generally based on the hydrolysis of lactose to D-galactose and D-glucose with β-galactosidase, followed by determination of either D-galactose or D-glucose. In the International Dairy Federation Method (79B:1991) for the measurement of lactose in "dried milk, dried ice-mixes & processed cheese", details are given for deproteinisation of samples, hydrolysis of lactose with β-galactosidase and measurement of either released D-galactose or D-glucose. The measurement of lactose as D-galactose liberated is more generally reliable than measurement as D-glucose liberated because preparations generally contain more free D-glucose than free D-galactose.

Enzymic kits for the determination of D-galactose are very slow. This is due to the low rate of natural chemical "mutarotation" between the α- and β-anomeric forms of D-galactose. Only the β-form is recognised by β-galactose dehydrogenase. In incubations containing NAD^+, D-galactose and β-galactose dehydrogenase, there is a very rapid initial increase in absorbance due to the consumption of β-D-galactose, and this is followed by a very slow approach to the end-point. This very slow approach results from the very low rate of chemical "mutarotation" of α-D-galactose into β-D-galactose. Using technology developed by Megazyme (patented), a galactose mutarotase has now been incorporated into the assay format to rapidly catalyse this rate-limiting mutarotation step. The result is very rapid analysis times of approx. 5 min at room temperature (Figure 1, page 10).

In addition to analysis of lactose in "normal" samples this kit (K-LACGAR) is also suitable for the analysis of lactose in "low-lactose" or "lactose-free" samples which contain high levels of monosaccharides (see Procedure B, page 7).

PRINCIPLE:

In the currently described procedure (a modification of AOAC Official Method 984.15; lactose in milk) lactose is hydrolysed to D-galactose and D-glucose by *Aspergillus niger* β-galactosidase at pH 5.0 (1).

$$\text{(1)}\quad \text{Lactose} + H_2O \xrightarrow{(\beta\text{-galactosidase})} \text{D-galactose} + \text{D-glucose}$$

Interconversion of the α- and β-anomeric forms of D-galactose is catalysed by galactose mutarotase (GalM) (2).

$$\text{(2)}\quad \alpha\text{-D-Galactose} \xrightleftharpoons{\text{(GalM)}} \beta\text{-D-galactose}$$

The β-D-galactose is oxidised by NAD^+ to D-galactonic acid in the presence of β-galactose dehydrogenase (β-GalDH) at pH 8.6 (3).

$$\text{(3)}\quad \beta\text{-D-Galactose} + NAD^+ \xrightarrow{(\beta\text{-GalDH})} \text{D-galactonic acid} + NADH + H^+$$

The amount of NADH formed in this reaction is stoichiometric with the amount of lactose. It is the NADH which is measured by the increase in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assays are specific for lactose and D-galactose. The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 1.48 mg of lactose (or 0.74 mg of D-galactose)/L of sample solution at the maximum sample volume of 1.00 mL. The detection limit is 2.96 mg of lactose/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 1.00 mL.

The assay is linear over the range of 4 to 80 µg of D-galactose (or 8 to 160 µg of lactose) per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 1.00 mL, this corresponds to a lactose concentration of approx. 0.74 to 1.48 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of D-galactose has been completed within 10 min at room temperature, it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-galactose (approx. 40 µg in 0.1 mL) to the cuvette on completion of the reaction.

A significant increase in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding lactose or D-galactose to the sample in the initial extraction steps.

To confirm that lactose is completely hydrolysed by β-galactosidase, perform the incubation for the recommended time and for twice the recommended incubation time. The final determined values for lactose should be the same.

Since divalent metal ions inhibit the β-galactose dehydrogenase employed in this assay, EDTA is included in buffer 2.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 115 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (2.5 mL, pH 5.0).
Stable for > 2 years at 4°C.

Bottle 2: Buffer (25 mL, pH 8.6) plus EDTA and sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 3: NAD^+.
Stable for > 5 years below -10°C.

Bottle 4: β-Galactosidase suspension (1.2 mL).
Stable for > 4 years at 4°C.

Bottle 5: β-Galactose dehydrogenase plus galactose mutarotase suspension, 2.4 mL.
Stable for > 2 years at 4°C.

Bottle 6: D-Galactose standard solution (5 mL, 0.4 mg/mL in 0.02% w/v sodium azide).
Stable for > 2 years; store sealed at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Dilute the contents of bottle 1 to 24 mL with distilled water. Use this solution to dilute the entire contents of bottle 4. Use immediately.
2. Use the contents of bottle 2 as supplied. Stable for > 2 years at 4°C.
3. Dissolve the contents of bottle 3 in 12 mL of distilled water. **Stable for ~ 4 weeks at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).
4. Transfer the contents of bottle 4 (1.2 mL) to the 24 mL of diluted buffer in bottle 1. This is now Solution 4. Divide into appropriately sized aliquots and store in polypropylene containers below -10°C between use and cool during use if possible. Stable for > 2 years below -10°C.
5. Use the contents of bottle 5 as supplied. Before opening for for the first time, shake the bottle to remove any protein that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position. **Swirl the bottle to mix contents before use.** Stable for > 2 years at 4°C.
6. Use the contents of bottle 6 as supplied. Stable for > 2 years; store sealed at 4°C.

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL, 100 mL and 500 mL).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 200 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of diluted β-galactosidase, buffer 1 and 0.1 mL aliquots of NAD^+ solution).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No. 1 (9 cm) filter papers.

PROCEDURE A: (Standard Assay Procedure)

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.72 mL
Sample solution: 4-80 µg of D-galactose (or 8-160 µg of lactose) per cuvette (in 0.20-1.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Lactose		Galactose	
	Blank	Sample	Blank	Sample
sample solution	-	0.20 mL	-	0.20 mL
solution 4 (β-Galactosidase)	0.20 mL	0.20 mL	-	-
Ensure that all of the solutions are delivered to the bottom of the cuvette. Mix the contents by gentle swirling, cap the cuvettes and incubate them for 10 min at ~ 25°C. **Add:**				
distilled water (at ~ 25°C)	2.20 mL	2.00 mL	2.40 mL	2.20 mL
solution 2 (buffer)	0.20 mL	0.20 mL	0.20 mL	0.20 mL
solution 3 (NAD^+)	0.10 mL	0.10 mL	0.10 mL	0.10 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:				
suspension 5 (β-GalDH/GalM)	0.02 mL	0.02 mL	0.02 mL	0.02 mL
Mix*, read the absorbance of the solutions (A_2) at the end of the reaction (< 5 min). If the reaction has not stopped after 6 min, continue to read the absorbances at 1 min intervals until the absorbances remain the same over 1 min**.				

* for example with a plastic spatula or by gentle inversion after closing the cuvette with a cuvette cap or Parafilm®.

** if the absorbance A_2 increases constantly, extrapolate the absorbance to the time of addition of suspension 5 (β-GalDH/GalM).

CALCULATION:

Determine the absorbance differences (A_2-A_1) for blanks and samples. Subtract the absorbance difference of the blank from the absorbance difference of the corresponding sample, thereby obtaining the change in absorbance (ΔA) resulting from the sample, as follows:

Determination of D-galactose:

$$\Delta A_{\text{D-galactose}} = (A_2\text{-}A_1)_{\text{galactose sample}} - (A_2\text{-}A_1)_{\text{galactose blank}}$$

Determination of lactose + D-galactose:

$$\Delta A_{\text{lactose + D-galactose}} = (A_2\text{-}A_1)_{\text{lactose sample}} - (A_2\text{-}A_1)_{\text{lactose blank}}$$

Determination of lactose:

$$\Delta A_{\text{lactose}} = \Delta A_{\text{lactose + D-galactose}} - \Delta A_{\text{D-galactose}}$$

The values of $\Delta A_{\text{D-galactose}}$ and $\Delta A_{\text{lactose + D-galactose}}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results. However, with milk, the D-galactose content is very low.

The concentration of D-galactose and lactose can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of the substance assayed [g/mol]
ε = extinction coefficient of NADH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for D-galactose:

$$c = \frac{2.72 \times 180.16}{6300 \times 1 \times 0.2} \times \Delta A_{\text{D-galactose}} \quad [g/L]$$

$$= 0.3889 \times \Delta A_{\text{D-galactose}} \quad [g/L]$$

for lactose:

$$c = \frac{2.72 \times 342.3}{6300 \times 1 \times 0.2} \times \Delta A_{\text{lactose}} \quad [g/L]$$

$$= 0.7389 \times \Delta A_{\text{lactose}} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F. When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) D-galactose or lactose is calculated from the amount weighed as follows:

$$= \frac{c\ [g/L\ \text{sample solution}]}{\text{weight}_{\text{sample}}\ [g/L\ \text{sample solution}]} \times 100 \quad [g/100\ g]$$

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

PROCEDURE B: (For "low-lactose" or "lactose-free" samples containing high levels of monosaccharides)

Step 1:

Add 1 mL of milk (or homogenised sample) to 4 mL of water, mix then add 1 mL of 10 mg/mL sodium borohydride (dissolved in 50 mM NaOH and less than 5 h old). Incubate this solution in a sealed plastic container at 40°C for 30 min then neutralise by the addition of 2.5 mL of 0.2 M acetic acid. Filter through Whatman No. 1 filter paper or centrifuge in a microfuge at 13,000 x *g* and use the filtrate or supernatant directly in the assay or with an appropriate dilution in distilled water (if required). The filtrate will be hazy but this is stable in the assay and contributes very little to the absorbance. *Typically use a sample volume of 0.2 mL. in* ***Step 2***.

Step 2:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.72 mL
Sample solution: 4-80 µg of D-galactose (or 8-160 µg of lactose) per cuvette (in 0.20-1.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Lactose		Galactose	
	Blank	Sample	Blank	Sample
sample solution	-	0.20 mL	-	0.20 mL
solution 4 (β-Galactosidase)	0.20 mL	0.20 mL	-	-
Ensure that all of the solutions are delivered to the bottom of the cuvette. Mix the contents by gentle swirling, cap the cuvettes and incubate them for **2 h** at ~ 25°C. **Add:**				
distilled water (at ~ 25°C)	2.20 mL	2.00 mL	2.40 mL	2.20 mL
solution 2 (buffer)	0.20 mL	0.20 mL	0.20 mL	0.20 mL
solution 3 (NAD^+)	0.10 mL	0.10 mL	0.10 mL	0.10 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:				
suspension 5 (β-GalDH/GalM)	0.02 mL	0.02 mL	0.02 mL	0.02 mL
Mix*, read the absorbance of the solutions (A_2) at the end of the reaction (< 5 min). If the reaction has not stopped after 6 min, continue to read the absorbances at 1 min intervals until the absorbances remain the same over 1 min**.				

* for example with a plastic spatula or by gentle inversion after closing the cuvette with a cuvette cap or Parafilm®.

** if the absorbance A_2 increases constantly, extrapolate the absorbance to the time of addition of suspension 5 (β-GalDH/GalM).

CALCULATION: (Procedure B)

The calculation of results for Procedure B is the same as given for Procedure A (see page 5 and 6). However, the dilution factor of **8.5** from Step 1 of Procedure B must be accounted for, plus any further dilution of the sample prior to Step 2.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of D-galactose present in the cuvette (i.e. in the 0.2 mL of sample being analysed) should range between 4 and 80 µg (i.e. lactose in the sample solution used should range between approx. 8 and 160 µg). The sample solution must therefore be diluted sufficiently to yield a lactose concentration between 0.04 and 0.80 g/L.

Dilution Table

Estimated concentration of lactose (g/L)	Dilution with water	Dilution factor (F)
< 0.8	No dilution required	1
0.8-8.0	1 + 9	10
8.0-80	1 + 99	100
> 80	1 + 999	1000

If the value of $\Delta A_{D\text{-galactose}}$ or $\Delta A_{lactose + D\text{-galactose}}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 1.00 mL making sure that the sum of the sample, distilled water and solution 4 components in the reaction is 2.40 mL, and using the new sample volume in the equation.

2. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette 1 mL of the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 2 mL of Carrez I solution, 2 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

3. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if > 0.1 mL of an acidic sample is to be used undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 8.6 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing a significant amount of carbon dioxide, such as beer, should be degassed by increasing the pH to approx. 8.6 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no β-GalDH/GalM may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of polyvinylpolypyrrolidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask at 60°C. Adjust to room temperature and fill the volumetric flask to the mark with water. Store on ice or in a refrigerator for 15-30 min to allow separation of the fat and then filter. Discard the first few mL of filtrate and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge at 1,500 *g* for 10 min and neutralise the supernatant with 1 M KOH. Alternatively, clarify with Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of lactose in milk, cream or yogurt.

Accurately weigh approx. 1 g of milk, cream, yogurt or condensed milk into a 100 mL volumetric flask, add approx. 60 mL of distilled water, mix and store at 50°C for 15 min with occasional swirling. Add 2 mL of Carrez I solution and mix. Add 2 mL of Carrez II solution and mix. Add 4 mL of 100 mM NaOH solution and mix vigorously. Dilute to volume with distilled water and mix thoroughly.

Filter an aliquot of the solution through Whatman No. 1 filter paper. Discard the first few mL of filtrate. Use the clear filtrate (sample solution) in the assay. *Typically, for milk, cream and yogurt, no dilution is required and a sample volume of 0.2 mL is satisfactory; for condensed milk, a dilution of 1:3 and sample volume of 0.2 mL are satisfactory.*

(b) Determination of lactose in cheese and chocolate.

Add 10 g of grated cheese or 0.5 g of grated chocolate to a 200 mL beaker. Add approx. 60 mL of distilled water and a stirrer bar, and mix on a magnetic stirrer at 50°C for approx. 15 min. Add 2 mL of Carrez I solution and mix. Add 2 mL of Carrez II solution and mix. Add 4 mL of 100 mM NaOH solution and mix vigorously. Quantitatively transfer the solution to a 100 mL volumetric flask and dilute to volume with distilled water. Mix thoroughly and filter an aliquot of the solution through Whatman No. 1 filter paper. Discard the first few mL of filtrate. Use the clear filtrate (sample solution) in the assay. *Typically, for chocolate and most cheeses, no dilution is required and a sample volume of 0.2 mL is satisfactory; however for red cheddar cheese, a dilution of 1:10 and sample volume of 0.2 mL are satisfactory.*

REFERENCE:

Beutler, H. O. (1988). Lactose and D-Galactose. *"Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VI**, pp. 104-112, VCH Publishers (UK) Ltd, Cambridge, UK.

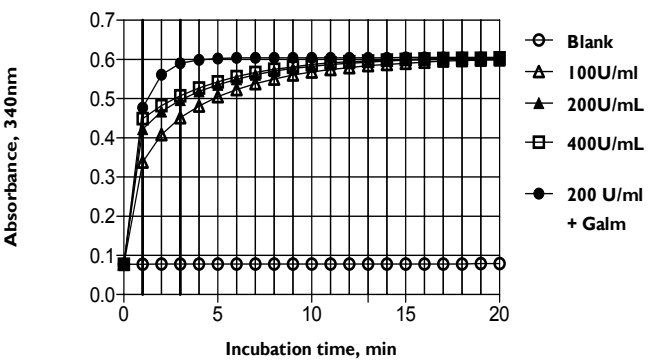

Figure 1. Effect of galactose mutarotase on the rate of oxidation of D-galactose by β-D-galactose dehydrogenase. Incubations were performed with 20 µL of β-D-galactose dehydrogenase at 100, 200 and 400 U/mL and with β-D-galactose dehydrogenase (200 U/mL) plus galactose mutarotase, as indicated.

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