Megazyme,
Bray Business Park,
Bray, Co. Wicklow,
A98 YV29,
Ireland.
Tel: + 353 1 286 1220
Fax: + 353 1 286 1264

Single Lab Validation of Megazyme's Lactose Assay Kit (K-LOLAC) for the determination of lactose concentration in low-lactose and lactose-free milk, milk products, and products containing dairy ingredients.

Objective Evaluation of the Megazyme Lactose Assay Kit for the determination of lactose concentration in low-lactose and lactose-free milk, milk products, and products containing dairy ingredients.

Authors R. Ivory, E. Delaney, D. Mangan and B. V. McCleary

Conclusion The method developed is fit for purpose

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1. Abstract

Megazyme's Lactose Assay (termed K-LOLAC or LOLAC) is an enzymatic method used for the rapid measurement of lactose in foodstuff and beverages. The Lactose Assay is simple, accurate and sensitive, and suitable for the determination of lactose in low-lactose or lactose-free products, including infant formula and adult nutritional drinks, conventional dairy samples and a variety of food samples. The method offers a sequential assay procedure for measurement of free D-glucose followed by lactose in the same reaction cuvette. Quantification is based on the hydrolytic activity of β-galactosidase, which hydrolyses lactose to glucose and galactose. Free D-glucose is first measured using a Hexokinase (HK)/Glucose 6-phosphate dehydrogenase (G-6PDH)/6-phosphogluconate dehydrogenase (6-PGDH) based assay procedure, and then β-galactosidase is added to hydrolyse the lactose in the same reaction vessel with concurrent measurement of the released D-glucose. The β-galactosidase employed is selective for lactose and the determined lactose values are similar to those obtained by ion chromatography. The free glucose measurement is complete within 10 min and the subsequent lactose measurement is complete within 15 min. The method includes pre-treatment steps to clarify and deproteinate samples and also to remove the high levels of free D-glucose in the samples, allowing accurate measurement of lactose at very low levels. This method was developed in-house and measures lactose in mg/100 mL or g/100 mL (liquid samples) and mg/100 g or g/100 g (solid samples).

The Single Lab Validation (SLV) outlined in this document was performed on a sample set of 36 different commercial food and beverage products purchased in Ireland and a set of 10 certified reference materials provided by Muva Kempten. Parameters examined during the validation included Working Range and Linear range, Selectivity, Limit of Detection (LOD), Limit of Quantification (LOQ), Trueness (*bias*), Precision (repeatability and intermediate precision), Robustness and Stability.

The methods outlined within allow accurate enzymic measurement of lactose in the sample types shown within this document and the enzymatic detection step is automatable for high-throughput sample analysis. It is a robust, quick and easy method for analysis of lactose in foodstuffs and beverages, based on the commercial Lactose Assay Kit (K-LOLAC) as developed by Megazyme.

2. Introduction

Lactose is by far the most abundant sugar found in bovine milk, usually present at ~ 5 g/100 mL (5% w/v). As a result, it is also typically found in a wide range of commercial dairy products including cheese, yoghurt, cream, butter and whey. Following ingestion, lactose is normally hydrolysed by lactase-phlorizin hydrolase (commonly referred to as lactase, β-galactosidase, EC. 3.2.1.108) in the human small intestine, with absorption of the released D-galactose and D-glucose.[1] Humans lacking or deficient in this enzyme cannot digest lactose, which therefore passes undigested to the colon where it undergoes rapid microbial fermentation causing adverse gastrointestinal symptoms associated with lactose intolerance, such as nausea, cramps, bloating and diarrohea.[2]

Lactose intolerance or lactase non-persistence (LNP) affects approximately 65% of the global human population. The frequency of primary lactose intolerance varies greatly between ethnic and racial populations, with approximately 5% of northern European and greater than 90% of Southeast Asian populations being affected. [3,4]

To address the prevalence of lactose intolerance, dairy manufacturers have introduced low-lactose and lactose-free dairy products, which are typically manufactured by the use of a commercial β-galactosidase (EC 3.2.1.23) to hydrolyse the lactose present into glucose and galactose. While the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) have not issued overarching regulations on the allowable lactose thresholds in all products, a number of countries have defined “low-lactose” as containing less than 1 g lactose per 100 g product and “lactose-free” as containing less than 10-100 mg lactose per 100 g product. In EU legislation, the term “lactose-free” has been defined only for infant and follow-on formula as < 10 mg per 100 kcal.[5]

3. Definitions

i. *Lactose*.— β-D-galactopyranosyl-(1→4)-D-glucose (CAS No. 63-42-3).
ii. *Allolactose*.— β-D-galactopyranosyl-(1→6)-D-glucose (CAS No. 28447-39-4).
iii. *Lactose analogues*.— compounds with a molecular structure similar to that of lactose. Generally used to refer to galactosyl-glucose compounds containing any linkage other than β-1,4.
iv. *Galactooligosaccharides (GOS)*.— Also known as oligogalactosyllactose, oligogalactose, or transgalactooligosaccharides (TOS) produced by transgalactosylation of lactose, which may or may not contain a terminal glucose.

v. *Infant formula.*— Breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding (Codex Standard 72-1981) Infant Formula and Formulas for Special Medical Purposes – 0-12 month of age; Follow-Up Formula – from 6–12 months and for young children; Young Children – 12-36 months of age; Foods for Special Medical Purposes Nutritionally complete specially formulated food for adults, consumed in liquid form, which may constitute the sole source of nourishment (AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals; 2010). Made from any combination of milk, soy, rice, whey, hydrolysed protein, starch and amino acids, with and without intact protein.

vi. *Milk and milk products.*— Milk is defined as the normal mammary secretion of a milk animal, intended for consumption as liquid milk or for further processing. Milk product is defined as a product obtained by any processing of milk. Although a milk product shall be made from milk, the definition does not hinder the milk from being subjected to various processing steps before it becomes an end product. Composite milk product is a product of a milk product and other food(s) where the milk constituents are an essential part in terms of quantity of the final product. [Bulletin of IDF 397 (2005) The Codex General Standard for the Use of Dairy Terms, Its Nature, Intent and Implications]

4. General Information

This method is based on the Lactose Assay Kit (Megazyme cat. no. K-LOLAC) which is available for purchase from Megazyme directly at www.megazyme.com.

Technical support can be provided to the user via email (contact scientist@megazyme.com).

5. Method

5.1 Scope of Method

i. *Target Analytes* — Glucose; CAS No. 50-99-7

Lactose; CAS No. 62-42-3

ii. *Matrices* — For this validation, the Lactose Assay Kit was tested with the samples outlined below. Note that where commercial samples were used, brand names and identifiers have been removed.

a. Commercial low-lactose or lactose-free milk samples

M1; Lactose-free milk

M2; Lactose-free milk

M3; Lactose-free milk

M4; Lactose-free milk

b. Commercial lactose-free food samples

F1; Lactose-free Chocolate Melting Pudding

F2; Lactose-free Cookies

F3; Lactose-free Nachos, cheese flavoured

F4; Lactose-free Jam Tart

F5; Lactose-free Cheese

F6; Lactose-free Gravy Granules

F7; Lactose-free Whey Isolate

c. Commercial low-lactose yoghurt samples

Y1; Low-lactose yoghurt

Y2; Low-lactose yoghurt

Y3; Low-lactose yoghurt

d. Commercial cheese samples

C1; Parmigiano Reggiano

C2; French Brie

C3; Mature Cheddar Cheese

C4; Mature Brie

C5; Parmigiano Reggiano

C6; Ricotta cheese

C7; Processed cheese

e. Commercial low-lactose and lactose-free infant formula samples

IF1; Lactose-free Infant Formula, Ready to feed, 125 mg/mL

IF2; Lactose-free Infant Formula, Ready to feed, 125 mg/mL

IF3; Lactose-free Infant Formula, Ready to feed, 125 mg/mL

IF4; Lactose-free Infant Formula, Ready to feed, 125 mg/mL

IF5 Lactose-free Infant Formula, Ready to feed, 125 mg/mL

IF6; Lactose-free Infant Formula, Ready to feed, 125 mg/mL

IF7; Lactose-free Infant Formula, Ready to feed, 125 mg/mL

IF8; Low-lactose Infant formula, Ready to feed, 125 mg/mL

IF9; Low-lactose Infant formula, Ready to feed, 125 mg/mL

IF10; Low-lactose Infant formula, Ready to feed, 125 mg/mL

IF11; Low-lactose Infant formula, Ready to feed, 125 mg/mL

f. Adult nutritional drinks

ND1; Lactose-free Adult Nutritional Drink, Strawberry milkshake flavour

ND2; Lactose-free Adult Nutritional Drink, Banana milkshake flavour

ND3; Adult Nutritional Drink, Vanilla flavour

ND4; Lactose-free Adult Nutritional Drink, Chocolate flavour

iii. *Certified Reference Materials (CRMs)* - For this validation this procedure was tested with the following harmonization materials provided by Muva Kempten:

CRM1; MUVA-ML-2311, UHT Milk, lactose free

CRM2; MUVA-ML-2310, UHT Milk, low lactose

CRM3; MUVA-CA-0904, sodium caseinate

CRM4; MUVA-RO-0746, Raw Milk (shock frozen)

CRM5; MUVA-KI-1105, Infant Food

CRM6; MUVA-KI-1106, Infant Food

CRM7; MUVA-KI-1107, Infant Food

CRM8; MUVA-FK-1221, Cream Cheese

CRM9; MUVA-S-0816, Nut Nougat

CRM10; MUVA-S-0814, Whole Milk Chocolate

iv. *Expression of results* – Results are expressed in the form of mg/100 g or g/100 g where applicable for solid samples, and mg/100 mL or g/100 mL where applicable for liquid samples. Where infant formula samples were analysed, samples were first formulated as 'ready to feed' solutions (25 g per 200 mL, 125 mg/mL). As such, results for infant formula samples are reported as mg/mL or mg/100 mL.

5.2 Method principle

Pre-treatment:

Free D-glucose is efficiently removed from the sample by conversion to D-gluconic acid by the enzymes glucose oxidase and catalase in the presence of oxygen (**1**) and (**2**). See Fig 1 (page 28) for schematic representation of the sample pre-treatment principle.

(1) D-Glucose + O_2 + H_2O $\xrightarrow{\text{(glucose oxidase)}}$ D-gluconic acid + H_2O_2

(2) $2H_2O_2$ $\xrightarrow{\text{(catalase)}}$ O_2 + $2H_2O$

Assay (measurement of D-glucose/lactose in samples):

Prior to lactose hydrolysis, any remaining free D-glucose is phosphorylated by the enzyme hexokinase (HK), in the presence of adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (**3**).

(3) D-Glucose + ATP $\xrightarrow{\text{(HK)}}$ G-6-P + ADP

G-6-P is oxidised by the enzyme glucose-6-phosphate dehydrogenase (G6P-DH) in the presence of nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate (gluconate-6-P) with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (**4**).

(4) G-6-P + $NADP^+$ $\xrightarrow{\text{(G6P-DH)}}$ gluconate-6-P + NADPH + H^+

Gluconate-6-P is immediately converted to D-ribulose-5-phosphate (R-5-P), carbon dioxide (CO_2), and a further molecule of NADPH by the enzyme 6-phosphogluconate dehydrogenase (6-PGDH) (**5**).

(5) Gluconate-6-P + $NADP^+$ $\xrightarrow{\text{(6-PGDH)}}$ R-5-P + CO_2 + NADPH + H^+

Lactose is hydrolysed to D-galactose and D-glucose by β-galactosidase (**6**). Glucose released from lactose enters the series of reactions catalysed by HK, G6P-DH, and 6-PGDH (**3, 4 & 5**).

(6) Lactose + H_2O $\xrightarrow{\text{(β-galactosidase)}}$ D-galactose + D-glucose

The amount of NADPH formed is stoichiometric to twice the amount of lactose as two molecules of NADPH are produced for each D-glucose molecule originating from the

lactose in the sample. See Fig 2 (page 28) for schematic representation of the assay principle.

5.3 Equipment Required

a. Volumetric flasks and glass beakers (50 mL and 100 mL)
b. Disposable plastic microfuge tubes (2 mL)
c. Disposable polypropylene tubes (13 mL)
d. Disposable plastic cuvettes (1 cm light path, 1.5 mL)
e. Micro-pipettors (e.g. Gilson Pipetman® P20 and P100)
f. Analytical balance
g. Magnetic stir plate with heating capability (required max temperature 70°C)
h. Boiling water bath (required temperature 100°C)
i. Microfuge (required speed 13,000 rpm)
j. UV-VIS Spectrophotometer (required wavelength 340 nm)
k. Vortex mixer
l. Filter papers (e.g. Whatman No. 1, 9 cm)

5.4 Chemicals and Reagents

Reagents listed below are supplied in the Megazyme Lactose Assay Kit (K-LOLAC).

Solution 1: Bottle 1 containing Buffer A (8 mL, pH 8.0) plus sodium azide (0.02% w/v) as a preservative.

Use the contents of Bottle 1 as supplied. Stable for > 2 years at 4°C.

Solution 2: Bottle 2 containing Glucose oxidase (GOX) and catalase, lyophilised powder. Stable for > 2 years below -10°C.

For use dissolve the contents of Bottle 2 in 14 mL of distilled water. To avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes below -10°C. Stable for > 2 years below -10°C

Solution 3: Bottle 3 containing Buffer B (8 mL, pH 7.6) plus sodium azide (0.02% w/v) as a preservative.

Use the contents of Bottle 3 as supplied. Stable for > 2 years at 4°C.

Solution 4: Bottle 4 containing NADP+ plus ATP. Stable for > 2 years at 4°C

For use dissolve the contents of Bottle 4 in 4 mL of distilled water. To avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes. Stable for > 1 year at 4°C or stable for > 2 years below -10°C.

Suspension 5: Bottle 5 containing Hexokinase plus glucose-6-phosphate dehydrogenase plus 6-phosphogluconate dehydrogenase suspension (1.4 mL).

Use the contents of bottles 5 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the stopper. Subsequently store the bottle in an upright position. Swirl the bottle to mix contents before use.

Stable for > 2 years at 4°C.

Suspension 6: Bottle 6 containing Z104 β-galactosidase suspension (1.4 mL).

Use the contents of bottles 6 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the stopper. Subsequently store the bottle in an upright position. Swirl the bottle to mix contents before use.

Stable for > 2 years at 4°C

Solution 7: Bottle 7 containing Lactose standard solution (5 mL, 0.25 mg/mL in 0.02% w/v sodium azide).

Use the contents of bottle 7 as supplied. Stable for > 2 years; store sealed at 4°C.

Additional reagents (not supplied in Lactose Assay Kit):

1. Concentrated Carrez I solution: 200 mL

   Dissolve 30 g of potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6].3H_2O$) (Sigma cat. no. 60279) in 200 mL of distilled water. Stable for > 3 years room temperature.
2. Concentrated Carrez II solution: 200 mL

   Dissolve 60 g of zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) (Sigma cat. no. 31665) in 200 mL of distilled water. Stable for > 3 years room temperature
3. Hydrogen peroxide (~ 30% w/w; Sigma cat. no. H1009) Use as supplied. Stable for > 3 years at 4°C.

5.5 Safety Considerations

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 based on regions.

https://www.megazyme.com/lactose-assay-kit

5.6 Other Considerations

This test should not be carried out by anyone other than a trained and experienced laboratory analyst. A lactose control should be included with analysis if there is any doubt as to the performance of the reagents or the performance of the analyst. Losses in sample handling and dilution can be identified by performing recovery experiments, i.e. by adding a known amount of lactose to the sample in the initial extraction or pre-treatment steps.

5.7 Sample extraction/clarification procedure – LIQUID SAMPLES

5.7.1 Ensure that milk sample has been mixed thoroughly before sampling

5.7.2 Pipette the following reagents into a 2 mL disposable microfuge tube:

0.9 mL of distilled water (at room temperature, ~ 20-25°C)

0.5 mL of milk sample

0.05 mL of Carrez II solution

0.05 mL of Carrez I solution

Cap the tube and mix by vortex

5.7.3 Centrifuge at 13,000 rpm for 10 min

5.7.4 Take 1.0 mL of the clear filtrate for Glucose oxidase/Catalase treatment (5.9)

5.8 Sample extraction/clarification procedure – SOLID SAMPLES

5.8.1 Accurately weigh approx. 10 g of solid sample into a 50 mL glass beaker

5.8.2 Add a stir bar and approx. 30 mL of distilled water

5.8.3 Mix on a magnetic stir plate and heat until temperature reaches 50°C. Continue stirring at temperature for approx. 15 min or until sample has solubilised or homogenised

5.8.4 Quantitatively transfer to a 50 mL volumetric flask

5.8.5 Add 0.5 mL of Carrez II solution and mix

5.8.6 Add 0.5 mL of Carrez I solution and mix

5.8.7 Make to volume (50 mL) with distilled water

5.8.8 Filter an aliquot, discarding the first few mL of filtrate (~ 5 mL)

5.8.9 Take 1.0 mL of the clear filtrate for Glucose oxidase/Catalase treatment (5.9)

5.9 Glucose Oxidase/Catalase pre-treatment procedure – ALL SAMPLES

5.9.1 Pipette the following reagents to a 13 mL polypropylene tube:

0.4 mL of distilled water (at room temperature, ~ 20-25°C)

1.0 mL of clear supernatant (from 5.7 or 5.8)

0.1 mL of Solution 1

0.2 mL of Solution 2

0.1 mL of Hydrogen peroxide (H_2O_2, ~ 30% w/w)

Cap the tube and mix by vortex

5.9.2 Incubate at room temperature (20-25°C) for 15 min

5.9.3 Slowly loosen the cap to release pressure and then re-tighten

5.9.4 Incubate in a boiling water bath (100°C) for 5 min

5.9.5 Remove from water bath and allow to cool for approx. 5 min

5.9.6 Transfer approx. 1.0 mL of the solution to a 2 mL microfuge tube and centrifuge at 13,000 rpm for 10 min

5.9.7 Carefully pipette the required volume (0.1 mL) for use in the Enzymatic Determination Reaction (5.10)

5.10 Enzymatic Determination Reaction – ALL SAMPLES

This procedure is summarised in Fig 3 (page 29).

5.10.1 Set the spectrophotometer to read absorbance at 340 nm.

5.10.2 Blank the spectrophotometer against air or water.

5.10.3 Prepare a blank cuvette by addition of the following reagents to a 1.5 mL UV cuvette:

1.0 mL of distilled water

0.1 mL of Solution 3

0.05 mL of Solution 4

Cap the cuvette using a cuvette cap or parafilm and mix by gentle inversion.

5.10.4 Prepare sample cuvettes (in duplicate) by addition of the following reagents to a 1.5 mL UV cuvette:

0.9 mL of distilled water

0.1 mL of sample solution (from 5.9)*

0.1 mL of Solution 3

0.05 mL of Solution 4

Re-cap the cuvette using the matching cuvette cap or parafilm and mix by gentle inversion.

5.10.5 If required prepare a lactose standard cuvette by addition of the following reagents to a 1.5 mL UV cuvette:

0.9 mL of distilled water

0.1 mL of Solution 7

0.1 mL of Solution 3

0.05 mL of Solution 4

Re-cap the cuvette using the matching cuvette cap or parafilm and mix by gentle inversion.

5.10.6 After 3 min at 25°C read the absorbances of the blank and then samples (A_1).

5.10.7 Remove the cuvette caps or parafilm, taking care to avoid spillage of liquid.

5.10.8 Start the reaction by addition of 0.02 mL of Suspension 5. Re-cap the cuvettes after addition and mix by gentle inversion.

5.10.9 After 10 min at 25°C read the absorbances of the blank and samples (A_2).

5.10.10 Remove the cuvette caps or parafilm, taking care to avoid spillage of liquid.

5.10.11 Start the next reaction by addition of 0.02 mL of Suspension 6. Re-cap the cuvettes after addition and mix by gentle inversion.

5.10.12 After 15 min at 25°C read the absorbances of the blank and samples (A_3).

5.10.13 Ensure that the reaction has terminated by reading the absorbance of the blank and sample after a further 5 min at 25°C.**

**If the absorbance value has increased at this time, continue to read at 5 min intervals until either there is no further increase in absorbance (plateau) or the rate of increase remains constant over time (linear creep). If this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of Suspension 6. This can be simplified using the Megazyme MegaCalc tool for creep calculation (available on the Megazyme website). The creep reaction is described in more detail in section 6.2.8 (Selectivity).

*For samples exhibiting lower lactose concentrations (i.e. absorbance values below 0.05), the sample volume can be increased to 0.4 mL and a linear response is expected (Fig 4, page 30). If sample volume is increased, the amount of water added in 5.10.4 should be reduced proportionally.

5.11 Calculation of results

Determine the absorbance difference caused by hydrolysis of lactose (A_3-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{lactose}$. The value of $\Delta A_{lactose}$ should as a rule be in the range of 0.02-1.2 absorbance units to achieve sufficiently accurate results.

The concentration of lactose can be calculated as follows:

$$c_{lactose} = \frac{V \times MW}{\varepsilon \times d \times v \times 2} \times \Delta A_{lactose} \times F \quad [mg/mL]$$

where:

V = Enzymatic Determination Reaction final volume [mL]
= 1.19

MW = molecular weight of lactose [g/mol]
= 342.3

ε = extinction coefficient of NADPH at 340 nm [l x mol^{-1} x cm^{-1}]
= 6300

d = light path [cm]
= 1

v = sample volume [mL]
= 0.1

2 = 2 moles of NADPH produced for each mole of D-glucose or lactose

F = dilution factor
= for LIQUID SAMPLES

$$= \frac{V1}{v1} \times \frac{V2}{v2}$$

$$= \frac{1.5}{0.5} \times \frac{1.8}{1.0} = \mathbf{5.4}$$

= for SOLID SAMPLES

$$= \frac{V2}{v2}$$

$$= \frac{1.8}{1.0} = \mathbf{1.8}$$

where:

V1 = final volume in liquid extraction procedure [mL]

v1 = sample volume in liquid extraction procedure [mL]

V2 = final volume in glucose oxidase/catalase treatment [mL]

v2 = sample volume in glucose oxidase/catalase treatment [mL]

It follows for SOLID SAMPLES:

$$[g/100g] = \frac{c_{lactose}\ [mg/mL]}{\text{extract concentration [mg/mL]}} \times 100$$

where:

extract concentration = 200

$$= \frac{10\ g}{50\ mL}$$

100 = conversion to g/100g

6. Validation

6.1 Planning

The purpose of this report is to verify and validate the Lactose Assay Kit (K-LOLAC) for the analysis of lactose concentration in low-lactose and lactose-free milk, milk products, and products containing dairy ingredients. The assay requires the addition of two enzymes to begin the reactions. Absorbance (A_2) was taken 10 min after the addition of the hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase suspension (Suspension 5, hereafter referred to as HK/G6PDH/6PGDH). Absorbance (A_3) was taken 15 min after the addition of the β-galactosidase (Suspension 6). All absorbances were read at 340 nm and the Enzymatic Determination Reactions were carried out at 25°C unless stated otherwise. Samples that exhibited a 'creep' in the reaction curve as outlined in the Method section (5) were corrected by extrapolation calculation as shown in Fig 14-17 (page 35-36). The creep reaction is described in more detail in section 6.2.8 (Selectivity). The first reaction is complete within approximately 5 min, as shown in Fig 7 (page 31), and the second reaction is complete within approximately 12.5 min, as shown in in Fig 8 (page 32). Nonetheless it is recommended that the user measure absorbance 10 min and 15 min after enzyme addition, for A_2 and A_3 respectively.

6.2 Performance characteristics

Performance characteristics that are investigated within include; Working Range, Limit of Detection (LOD), Limit of Quantification (LOQ), Trueness (*bias*), Precision (reproducibility and repeatability), Interference, Selectivity, Robustness and Stability.

6.2.1 Working Range

The assay follows the 'Enzymatic Determination Reaction – ALL SAMPLES' procedure (5.10). For glucose (A_2), 0.1 mL of a glucose standard was used as sample across a range of concentrations (1-25 mg/100 mL) corresponding to 1-25 µg of glucose per cuvette (Fig 5, page 30). For lactose (A_3), 0.1 mL of lactose standard was used as sample across a range of concentrations (0.2-50 mg/100 mL lactose) corresponding to 0.2-50 µg of lactose per cuvette (Fig 6, page 31).

For liquid samples treated as per the 'Sample extraction/clarification procedure – LIQUID SAMPLES' (5.7) the Working Range of 1-50 µg of lactose per cuvette equates to a concentration range of 1.35-67.5 mg/100 mL in original sample when using the maximum volume of 0.4 mL in the Enzymatic Determination Reaction.

For solid or semi-solid samples treated as per the 'Sample extraction/clarification procedure – SOLID SAMPLES' (5.8) the working range of 1-50 µg of lactose per cuvette equates to a concentration range of 2.25-112.5 mg/100g in original sample when using the maximum volume of 0.4 mL in the Enzymatic Determination Reaction.

Linearity was also examined for a variety of real samples containing a range of lactose concentrations (Fig 4, page 30).

6.2.3 LOD and LOQ

The calculated limit of detection (LOD) and the calculated limit of quantification (LOQ) for the purpose of this report is based on the analysis of samples that have been taken through the procedure.

The LOD is the lowest concentration of the analyte that can be detected by the method. LOD is calculated as 3 x s0; where s0 is the standard deviation of a number of blanks A_3-A_2 readings. The LOQ is the lowest level at which the assay performance

is acceptably repeatable. LOQ is calculated as kQ x s0; where s0 is the standard deviation of a number of blanks A_3-A_2 reading. The IUPAC default value for kQ is 10.

For the results outlined below the following value was used:

s0 = 0.002

s0 LOD = 0.002 x 3 = 0.006

s0 LOQ =0.002 x 10 = 0.02

Replicate measurements for the purposes of this report = 20

For samples containing lactose, with no pre-treatment, treated as per the 'Enzymatic Determination Reaction' procedure (5.10).

LOD – For 0.4 mL of sample (maximum volume)

Lactose = 0.05 mg/100 mL

LOQ – For 0.4 mL of sample (maximum volume)

Lactose = 0.16 mg/100 mL

The above detection limits are for samples as used in the assay (i.e. after any required sample preparation such as glucose removal and deproteinisation). The dilution used in pre-treatment must be accounted for while establishing the detection limits for specific samples. It follows that for samples treated as per the sample preparation guidelines described above, the following limits apply:

For liquid or milk samples treated as per the 'Sample extraction/clarification procedure – LIQUID SAMPLES' (5.7)

LOD – For 0.4 mL of sample (maximum volume)

Lactose = 0.27 mg/100 mL

LOQ – For 0.4 mL of sample (maximum volume)

Lactose = 0.89 mg/100 mL

For solid or semi-solid samples treated as per the 'Sample extraction/clarification procedure – SOLID SAMPLES' (5.8)

LOD – For 0.4 mL of sample (maximum volume)

Lactose = 0.44 mg/100 g

LOQ – For 0.4 mL of sample (maximum volume)

Lactose = 1.47 mg/100 g

For this SLV, where sample analysis returned an absorbance value lower than s0 as determined for the LOQ calculation (i.e. an absorbance value lower than 0.03), these samples were reported as containing 'less than' the LOQ values specified (i.e. < 0.89 mg/100 mL for LIQUID SAMPLES and < 1.47 mg/100 g for SOLID SAMPLES)

6.2.4 Trueness (*Bias*)

Comparison of the mean of the results (x) achieved with the Lactose Assay Kit 'Enzymatic Determination Reaction – ALL SAMPLES' procedure with a suitable reference value (xref) (Table 1). For this report, Relative Bias is calculated in per cent as: *b*(%) = x – xref / xref x 100. The reference material for this purpose is lactose supplied with the Lactose Assay Kit (K-LOLAC) at 25 mg/100 mL.

Comparison of the mean of the results (x) achieved with the Lactose Assay Kit 'Sample extraction/clarification procedure – LIQUID SAMPLES' (5.7) with a variety of suitable reference values (xref) across the liner range of the assay (Table 2, page 39). For this report, Relative Bias is calculated in per cent as: *b*(%) = x – xref / xref x 100. The reference materials for this purpose are lactose standards formulated in-house from 2.7-270 mg/100 mL.

Trueness was also assessed using MUVA-Kempten certified reference materials. Reference values (xref) were provided by the manufacturer based on results achieved by HPLC. For this report, Relative Bias is calculated in per cent as: *b*(%) = x – xref / xref x 100. Result are reported in Table 3 (page 39). The poor recovery observed for sodium caseinate can be attributed to the insolubility of this material in water using the extraction procedure outlined within this report.

6.2.5 Precision

This report details the reproducibility of the Lactose Assay Kit (K-LOLAC), it is a measure of the variability in results when tested by a single analyst over a short period of time and several analysts over an extended period of time.

Assay Repeatability (RSD_r) using the 'Enzymatic Determination Reaction' procedure (5.10) as outlined in this report. For the purpose of this report a series of standards (formulated in-house and ranging from 2.7-270 mg/100 mL) are the reference materials (Table 4, page 40). These materials were analysed in order to provide system repeatability data (i.e. the suitability of the system for analysis of the

specified lactose concentrations, disregarding any possible matrix or sample influence on repeatability).

Sample repeatability (RSD_r) and **Sample Intermediate precision (RSD_{ir})** using the procedure for either 'Sample extraction/clarification procedure – LIQUID SAMPLES' (5.7) or 'Sample extraction/clarification procedure – SOLID SAMPLES' (5.8) as outlined in this report. Seven commercial samples (infant formulas, lactose free milks and cheeses) with lactose concentrations between 2.1- 135 mg/100 mL were analysed in order to provide sample repeatability data (i.e. the suitability of the system for analysis of the specified sample type). For RSD_r (repeatability) the experiments were carried out by one analyst over a period of three days, with two extractions per sample per day, analysed in duplicate. For RSD_{ir} (intermediate precision), these results were compared to results achieved by a second analyst using separate reagents and equipment over a period of three days, also with two extractions per sample per day, analysed in duplicate. Each 'Extraction' as described in the table refers to a separate dilution of the sample material by the analyst (Tables 6-12, pages 41-47).

6.2.6 Recovery

Recovery was first assessed by addition of a known amount of lactose to the extraction, without sample, for both the solid and liquid sample extraction and clarification procedures (i.e. the recovery of a lactose spike, disregarding any possible matrix or sample influence on recovery).

Real samples were then tested, by spiking these samples with known quantities of lactose in the initial extraction phases, prior to treatment for assay. The recoveries were assessed by comparison to sample assays (without spike) and assay of lactose spike (without sample). A wide range of samples was chosen for recovery testing as shown in 5.1 (ii) in order to obtain as much information and cover as many sample matrices as possible. Table 13 (page 47) contains recovery data, showing the percentage recovery of lactose spike for the 36 samples. Spiking of real samples (including lactose-free milks, infant formulas, adult nutritional drinks, yoghurts, food samples, cheeses) resulted in recoveries between 93% and 114%.

6.2.7 Robustness

Occasionally the user may lack the capability to control certain factors during the test (e.g. temperature at which the assay is run or time at which the absorbance is measured). Robustness experiments were carried out to show that the method does not suffer due to minor variations or adjustments to certain parameters.

6.2.7.1 Time of measurement

The time at which absorbance measurement (A_3) is taken was also evaluated for robustness. In general, the enzymatic determination reaction is complete within 15 min of addition of β-galactosidase (Suspension 6). The assay was carried out as per the procedure (at 25°C) using the Megazyme lactose standard solution (Solution 7). Fig 9 (page 32) shows that the results do not suffer when adjustments are made to the time at which the absorbance measurement is made. Results remain the same when absorbance values are taken at 10, 12.5, 15 (recommended for assay), 17.5, 22.5, 25 and 27.5 min.

6.2.7.2 Assay temperature

The temperature at which the 'Enzymatic Determination Reaction' takes place was examined as part of the Robustness experiments. The assay was carried out as per the 'Enzymatic Determination Reaction' procedure (5.10) using the lactose standard solution (Solution 7) at the top of the linear range (50 µg per test) over a range of temperatures. Fig 10 (page 32) shows that the results do not suffer when adjustments are made to the temperature at which the assay is carried out. Results remain the same after incubation at 20°C, 25°C and 37°C though there is a small variation in the time to completion of the assay. This variation in time to completion does not negatively affect the result of the assay as absorbance (A_3) should be read at 15 min.

6.2.7.3 Units of β-galactosidase added

Dilutions of the β-galactosidase suspension (Suspension 6) were made in 3.2M ammonium sulphate in order to create a range of enzyme concentrations (137.5 U/mL: 2,200 U/mL, the equivalent of 2.75-44 Units per test when using 0.02 mL). 0.02 mL of each suspension was added to the test as in the 'Enzymatic Determination Reaction' procedure (5.10). The assay was carried out using the

lactose standard solution (Solution 7) at the top of the linear range (50 µg per test) and results can be seen in Fig 11 (page 33). A reduction in the number of Units of β-galactosidase per test caused a reduction in time-to-completion of assay, however it can be seen that the β-galactosidase as supplied (2,500 U/mL, the equivalent of 50 Units per test when using 0.02 mL) is greatly in excess of the 22 Units per test required to complete the reaction within the time frame specified for measurement in the 'Enzymatic Determination Reaction' (5.10) outlined in this report.

6.2.7.4 Glucose removal

The robustness of the sample pre-treatment step was evaluated by pre-treatment of a range of glucose solutions using the 'Glucose oxidase/Catalase pre-treatment procedure' (5.8) and analysis using the 'Enzymatic Determination Reaction' procedure (5.10). The absorbance values (A_1 and A_2) were measured as per the standard procedure and an increase in absorbance between A_1 and A_2 was observed at higher glucose concentrations (the equivalent of > 6% glucose in sample), indicating that glucose was not fully removed by the glucose oxidase/catalase pre-treatment system (Fig 12, page 34).

Low-lactose and lactose-free milk products generally contain ~ 2.5% of glucose, and in such products the glucose oxidase/catalase system is capable of removing all background glucose. During the course of this SLV no issues were reported regarding poor removal of glucose from real samples. Where there is concern that background glucose in the samples to be analysed is above the threshold indicated (or there is a large observed increase between A_1 and A_2 during the analysis i.e. A_2 - A_1 value > 0.5), it is possible to increase the time of incubation during the glucose oxidase/catalase pre-treatment procedure (5.9.2) and a further reduction in background glucose would be expected over time.

6.2.8 Selectivity

It is well documented that along with its primary hydrolytic function, β-galactosidase also catalyses a transglycosylation process in which the released galactose can be transferred to lactose or pre-formed glucose, galactose or galactooligosaccharides (GOS). This reaction is employed industrially utilising high concentrations of lactose to produce GOS as a prebiotic ingredient. Transglycosylation also occurs to some extent during the hydrolysis of lactose for the production of low-lactose and lactose-

free products. In this process, trace quantities of a range of galactosyl-glucosyl oligosaccharides are formed. While the concentrations of the various transglycosylation products are very low, they can occur at levels similar to, or greater than, that of the residual lactose present, which complicates the measurement of lactose in these samples.

This enzymatic method relies on the β-galactosidase mediated hydrolysis of lactose and the subsequent measurement of the glucose released as a result. The presence of the aforementioned galactosyl-glucosyl oligosaccharides in low-lactose and lactose-free samples (all of which have the potential to interfere in the measurement by falsely inflating glucose measurements if hydrolysed) necessitates the utilisation of a β-galactosidase enzyme that is selective for lactose in the presence of these potentially interfering sugars. The oligosaccharide profile of a typical commercial low-lactose milk product (obtained using HPAEC-PAD) is shown in Fig 13 (page 34). A substantial number of the sugars represented (i.e. β-1,6-D-Galactosyl-galactose, β-1,3-D-Galactosyl-galactose and β-1,4-D-Galactosyl-galactose) do not contain glucose and therefore will not inflate measured lactose values if hydrolysed by the β-galactosidase.

β-1,6-D-Galactosyl-D-glucose (hereafter referred to as allolactose) is usually the principal component of the transglycosylation products with concentrations frequently in excess of the lactose concentration, but in certain samples it can be present in even higher quantities. Hydrolysis of allolactose in the presence of lactose was examined in a series of tests in which the lactose content was maintained at 25 µg while the allolactose content was varied from 0 to 50 µg across the linear range for lactose (Fig 16, page 36). The MZ104 β-galactosidase shows a clear preference for lactose over allolactose as the lactose hydrolysis reaction reaches completion almost immediately while the allolactose hydrolysis reaction exhibits a slow linear increase in absorbance, indicating a slow release of glucose. There is an observed linear relationship between the quantity of allolactose added to the system and the slope of the absorbance increase (hereafter referred to as ‘creep’). This linear relationship is expanded on in Fig 14-17 (pages 35-36). The slow hydrolysis of allolactose forms the basis for the linear extrapolation calculations mentioned in 5.10.

Fig 18 (page 37) shows the hydrolysis profile in the enzymatic system of β-1,4-D-galactosyl-lactose when added to the test at concentrations at the middle and the

top of the linear range for lactose (0, 25 and 50 µg per test). It is clear that the β-galactosidase employed can also slowly hydrolyse the β-1,4-D-galactosyl-lactose but again shows a significant preference for lactose. Any contribution to the A_3 absorbance measurement by this sugar is also negated by the use of the linear extrapolation calculation.

The hydrolysis profiles of other minor transglycosylation products were also examined. There was no hydrolysis of β-1,6-D-galactosyl-lactose (no change in A_3 when this sugar is present at concentrations of 0, 25 and 50 µg per test, data not shown) so there should be no inflation in results due to the presence of this sugar. There was full hydrolysis of β-1,3-D-galactosyl-glucose (data not shown), however this sugar appears in relatively low abundance in low-lactose and lactose-free products and will contribute very slightly to measured values for lactose.

6.2.9 Interference

Interference was first assessed during the initial stages of method development by analysis of a wide variety of 'real' samples and examination of the linearity profiles of these samples. For a small percentage of samples it became clear that an increase in sample volume in the 'Enzymatic Determination Reaction' did not result in a linear increase in absorbance. All samples that exhibited this non-linearity were examined in greater detail and it was shown experimentally that they contained elevated levels of D-galactose (i.e. levels greater than the ~ 2.5% galactose generally seen in low-lactose milks). Experiments were then carried out to examine the recovery of a known amount of lactose when an increasing amount of D-galactose is added to the system. Fig 19 (page 37) shows that there was good recovery of a lactose standard up to a concentration of 4 mg D-galactose per test. Using the maximum sample volume of 0.4 mL and the 'Sample extraction/clarification procedure – LIQUID SAMPLES' (5.7), this equates to > 5.4 g/100 mL of D-galactose in the original sample. In order to minimise the effect of the non-linearity caused by high levels of galactose in some samples, the sample volume allowable in the 'Enzymatic Determination Reaction' is limited to a maximum of 0.4 mL, as all samples tested exhibited linearity up to this volume (during this investigation samples were tested over the range of 0.1-1 mL in 0.1 mL increments).

The effect of a variety of common food components and chemicals related to food and beverage processing on the functionality of the system was investigated by the

addition of these chemicals during the initial extraction steps. Known amounts of lactose and glucose were extracted as per the 'Sample extraction/clarification procedure – SOLID SAMPLES' (5.8), along with 1 g of the relevant interferent. The potential interferent was added at 1 g per 50 mL extraction volume in order to simulate the addition of 10 g of sample containing 1 g of interferent (i.e. replicating a concentration of 10% w/w interferent in a real sample when using the 'Sample extraction/clarification procedure – SOLID SAMPLES'). Lactose was included at a concentration close to the top of the linear range of the assay (the equivalent of 40 µg per test) in order to ensure that the system was close to a stress point before addition of the potential interferent. Glucose was added at a concentration close to the top of the experimentally determined range for glucose removal by the system (glucose removal shown in Fig 12 (page 34) and discussed in section 6.2.7.5), again ensuring that the system was close to a stress point before testing interference. It is also worth noting that the glucose concentration included in the inhibition testing was higher than the expected levels of glucose in standard low-lactose or lactose-free dairy product (typical β-galactosidase treated milk products contain ~ 2.5% glucose). The results are reported in Tables 14 and 15 (pages 50-52). It is possible to conclude that there should be no interference from any chemicals listed in Table 14 (pages 50-51), addition of which resulted in an acceptable recovery of spiked lactose and an acceptable level of glucose removal (data for glucose removal not shown). Where interference was observed, either by a reduced A_3 value (poor recovery of lactose) or an inflated A_2 value (poor removal of glucose), the test was repeated over a range of inhibitor concentrations until both a full recovery of lactose and full removal of glucose were achieved. These results are shown in Table 15 (page 52). Elevated levels of acetaldehyde, arginine and copper(I) sulphate do cause inhibition in the Enzymatic Determination Reaction (reduced A_3 value resulting in a poor recovery of lactose standard) and not in the Glucose Oxidase/Catalase system (glucose was removed successfully), suggesting that these chemicals have an inhibitory effect on either the glucose determination enzymes (HK/G6PDH/6PGDH) or the β-galactosidase enzyme utilised in the system. In practise, it is highly unlikely that acetaldehyde (or other aldehydes), arginine or copper(I) sulphate will be present in food or beverage samples at levels anywhere near the experimentally determined levels shown to cause interference.

The possibility that there may be some interference due to the presence of residual β-galactosidase (remaining after the industrial removal or reduction of lactose) was also taken into consideration. In this case the concern exists that any residual β-galactosidase present in the sample could theoretically further hydrolyse any lactose present in the sample upon extraction (resulting in an under-reporting of lactose in the sample). The treatment of samples using the 'Sample extraction/clarification procedure – LIQUID SAMPLES' removes the potential for interference as the immediate treatment with Carrez reagents fully denatures and deactivates any protein present. In samples such as cheeses and other solid samples where solubilisation is required before analysis, in theory it is possible that β-galactosidase could be active. In practise, however, no such interference was observed during the numerous recovery experiments using real solid samples (Table 13, pages 48-49).

It is also possible to further use the 'real' sample recovery data as an indicator of potential inhibition or interference. During recovery experiments a wide range of samples were tested for lactose (outlined in section 6.2.6, results shown in Table 13, pages 48-49). Where a good recovery of lactose was achieved it is possible to say that no sample components inhibited the measurement system, further proving that the method does not suffer from any major drawbacks in terms of chemical inhibition.

Where there is concern that there may be interfering substances present in the sample for analysis, an internal standard can be included by the analyst. 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 to the sample in the initial extraction steps).

6.2.10 Stability

The Lactose Assay Kit is formulated by Megazyme with a two-year stability guarantee when the components are stored as described on the individual kit component label. Kit components may be provided with longer stability guarantees, the user can find this information on the product label (specific expiry date is stated on each component). Regular quality control testing is performed in the Megazyme QC laboratory. The functionality of one set of kit components over a period of 3 years can be seen in Fig 20 (page 38). As the Lactose Assay Kit has been commercially available for a number of years, the historical data available from the

Quality Control laboratory shows that the stability of each component does not vary from batch to batch (data not shown).

The nature of the enzyme kit components (Suspension 5, HK/G6PDH/6PGDH and Suspension 6, β-galactosidase) is such that they are the most likely to display poor stability. The HK/G6PDH/6PGDH has been used for decades in this formulation in several other Megazyme test kits and has not exhibited any stability issues over time (data not shown). A detailed stability study was carried out on the β-galactosidase suspension, measuring enzyme activity. Enzyme storage stability at 4°C (recommended storage for β-galactosidase) is shown in Fig 21 (page 38) (U/mL values are converted to % activity for the purposes of this report). Note that a significant allowance has been made when formulating the enzyme components for any potential loss of activity that may occur (due to incorrect storage conditions or similar) as both enzyme suspensions are supplied greatly in excess of the U/mL concentration required for full functionality in the test.

7. Discussion

The Single Lab Validation as outlined in this report proves that the Lactose Assay Kit (K-LOLAC) is fit for purpose and applicable for the determination of lactose in low-lactose or lactose-free products, including infant formula and adult nutritional drinks, conventional dairy samples and a variety of food samples.

The test is user-friendly and the assay, excluding pre-treatment, is complete within 30 min.

This SLV included investigation into a variety of performance characteristics including Working Range, Limit of Detection (LOD), Limit of Quantification (LOQ), Trueness (*bias*), Precision (repeatability and intermediate precision), Selectivity, Interference, Robustness and Stability.

The assay was shown to be linear over a range of 1-25 µg per test for glucose and 1-50 µg per test for lactose. For samples treated as per the method outlined for liquid samples in this report the Working Range of 1-50 µg of lactose per cuvette equates to a concentration range of 1.35-67.5 mg/100 mL of lactose in the original sample when using the maximum assay volume of 0.4 mL. For samples treated as per the method outlined for liquid samples in this report the Working Range of 1-50 µg of lactose per cuvette equates to a concentration range of 2.25-112.5 mg/100g of lactose in the original sample when using the maximum assay volume of 0.4 mL.

The Limit of Detection and Limit of Quantification were determined for the Enzymatic Determination Reaction and subsequently for both liquid and solid samples, when sample are analysed using the relevant sample preparation example as outlined in the methods section (5). Trueness was tested using in-house lactose reference materials and the results were excellent across a range of concentrations. Experiments showed good correlation between results achieved using this procedure and expected results for 9 out of 10 harmonisation materials tested (using values stated by the manufacturer, obtained by HPLC). Good correlation was not achieved with the sodium caseinate material as this sample did not solubilise fully using the procedure outlined in this SLV.

A set of 7 commercial samples were analysed to provide repeatability and intermediate precision data. Samples were extracted and analysed by two analysts over a three-day period. Each sample was extracted twice per day by each analyst and analysed in duplicate. For these samples (including 2 lactose-free milk samples, 2 infant formula samples, 2 cheese samples and an adult nutritional drink), the highest RSD_r value was 8.48, while the highest RSD_{ir} value was 9.98.

A set of 36 samples (covering a wide range of matrix types) were analysed and the recovery of a spiked lactose standard was measured. For Infant formula samples, samples were spiked with a lactose standard at 5 mg/100 mL (for samples referred to as 'lactose-free' by the manufacturer) and 10 mg/100 mL (for samples referred to as 'low-lactose' by the manufacturer). Recoveries across the 11 samples varied from 93.2-109.34%. For all other samples tested within the lower range (10-100 mg/100 g), recoveries varied from 93.21-114.10%. A number of the Muva Kempten reference materials contained lactose at concentrations higher than the 1000 mg/100 g level that could be considered 'low-lactose'. These samples were included in the SLV in order to demonstrate that traditional dairy samples can be analysed using this method also. Recoveries obtained for samples in the higher range (i.e. > 100 mg/100 g or mL) varied from 94.44-108.28%.

Robustness testing included the examination of incubation temperature in the 'Enzymatic Determination Reaction' (20°C, 25°C, and 37°C), time at which absorbance measurement is taken (10, 12.5, 15, 17.5, 22.5, 25 and 27.5 min), Units of β-galactosidase per test (2.75-44 U per test) and glucose removal in the 'glucose oxidase/catalase pre-treatment' step. No parameter investigated during robustness testing was found to influence the result in any way when the test is performed as described within the Methods section of this report and for sample types outlined within this report.

The method can be considered to be selective for lactose in the matrices specified, under the assumption that the user utilises the linear extrapolation calculation effectively where interfering sugars are present (as indicated by the presence of a 'creep', i.e. a gradual increase in absorbance values over time after the expected assay completion time). Minor overestimation of lactose is observed where samples contain β-1,3-galactosyl-glucose, however this oligosaccharide is present in relatively low concentrations in low-lactose and lactose-free products.

All assay components were shown to have at least 3 years stability when stored as recommended and both enzyme components (HK/G6PDH/6PGDH and β-galactosidase) exhibit excellent stability for at least 3 years when stored as recommended (4°C).

It is possible to conclude that the Low Lactose Assay method, as outlined within this document, is fit for purpose

8. Figures and Tables

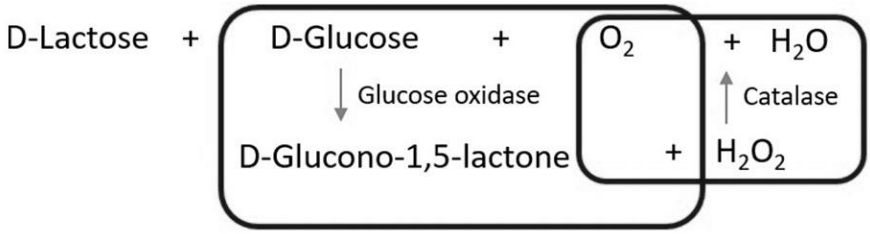

Fig 1: Schematic representation of the Glucose oxidase/Catalase sample pre-treatment.

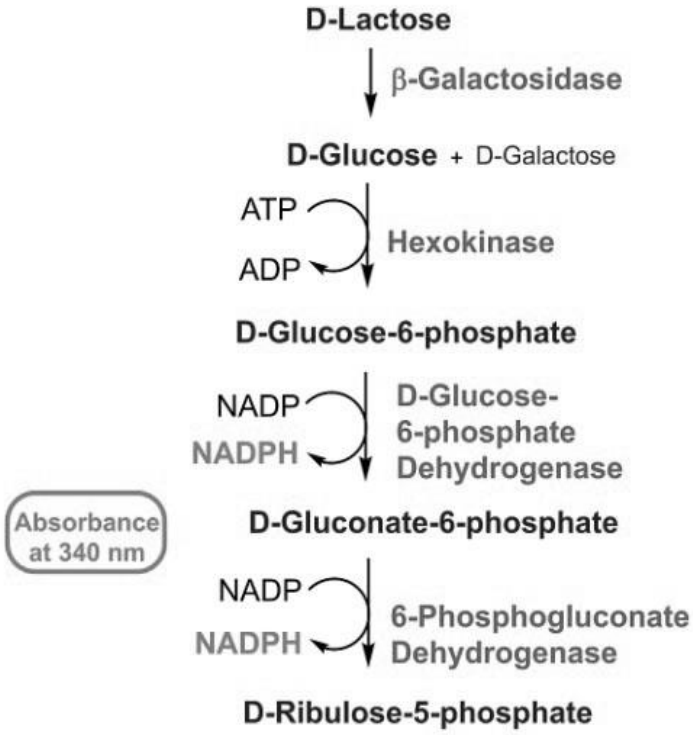

Fig 2: Schematic representation of the assay principle.

Wavelength	340 nm	
Cuvette	1.5 mL, 1 cm light path (glass or plastic)	
Temperature	25°C	
Final volume	1.19 mL	
Sample solution	0-50 µg of lactose per test in 0.1-0.4 mL sample volume	
Blank the spectrophotometer against air (without a cuvette in the light path) or against water.		
Pipette into cuvettes	Blank	Sample
Distilled water (~ 25°C) Sample Solution 3 Solution 4	1.0 mL - 0.10 mL 0.05 mL	0.9 mL 0.10 mL 0.10 mL 0.05 mL
Cap cuvettes, mix by gentle inversion and read the absorbances of the solutions (A_1) after approx. 2 min. Start the reaction by addition of:		
Suspension 5	0.02 mL	0.02 mL
Mix by inversion and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 10 min). Start the next reaction by addition of:		
Suspension 6	0.02 mL	0.02 mL
Mix by inversion and read the absorbances of the solutions (A_3) at the end of the reaction (approx. 15 min). If the reaction has not stopped after 15 min, continue to read the absorbances at 5 min intervals until the absorbances remains constant over 15 min.		

Fig 3: Summary of enzymatic determination reaction procedure as outlined in section 5 (Methods).

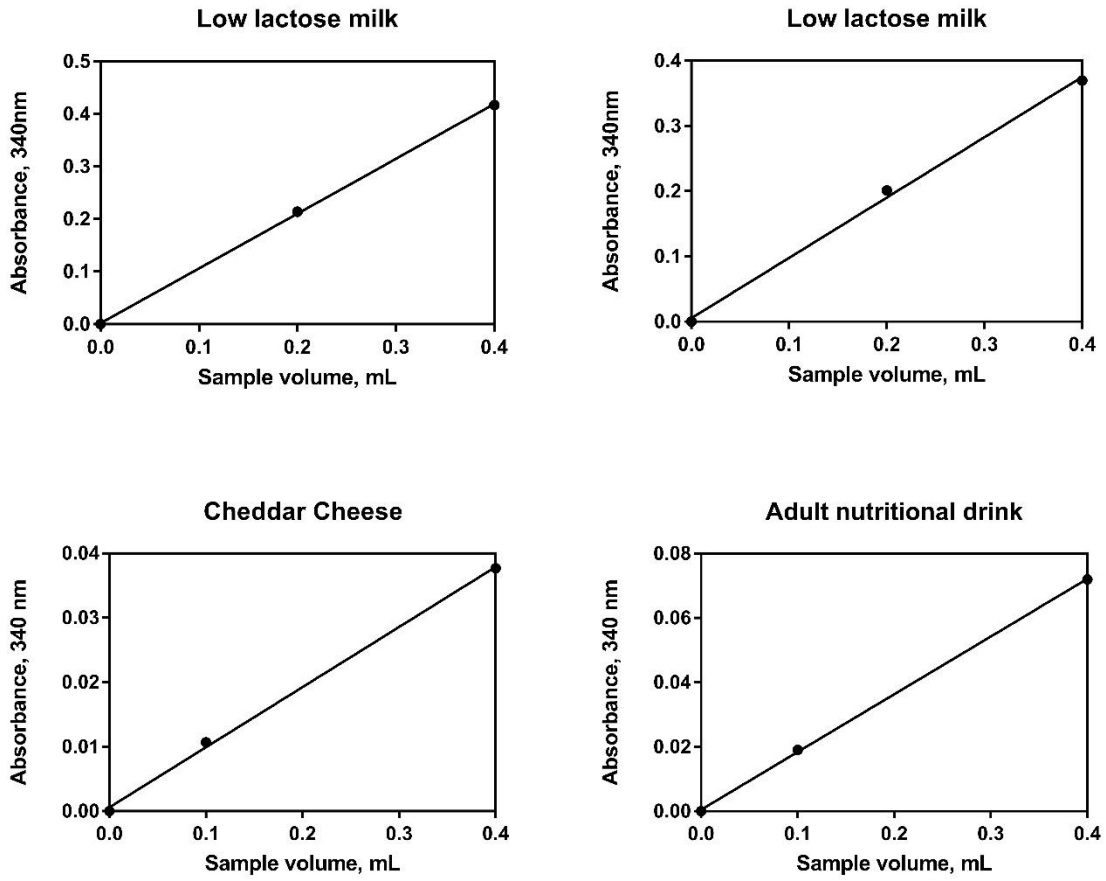

Fig 4: Examination of the linearity of the assay when increasing sample size across a range of real samples.

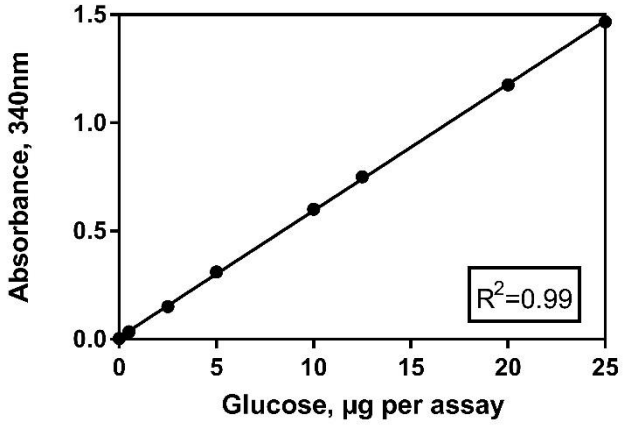

Fig 5: Examination of the linearity of the assay over a range of glucose concentrations (0-25 µg glucose per test).

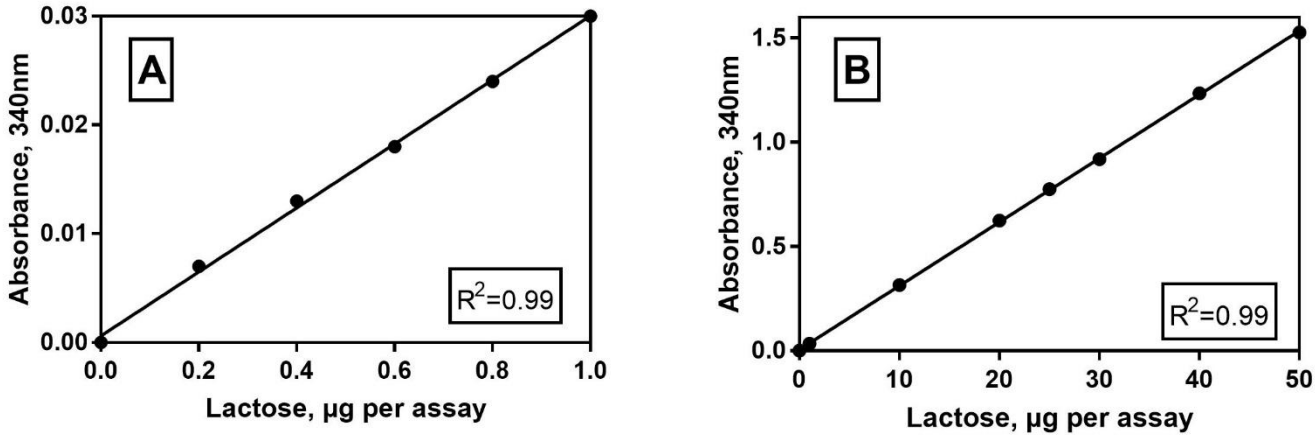

Fig 6: Examination of the linearity of the assay over a range of lactose concentrations A) 0-1 µg lactose per test and B) 0-50 µg lactose per test.

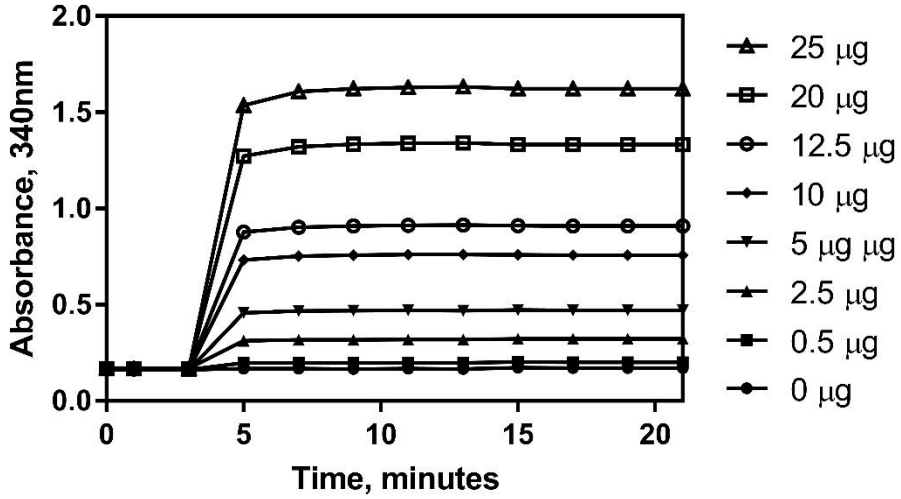

Fig 7: Reaction kinetic graph showing the time of reaction completion for glucose after addition of HK/G6PDH/6PGDH using a range of glucose concentrations across the linear range of the assay (0-25 µg glucose per test).

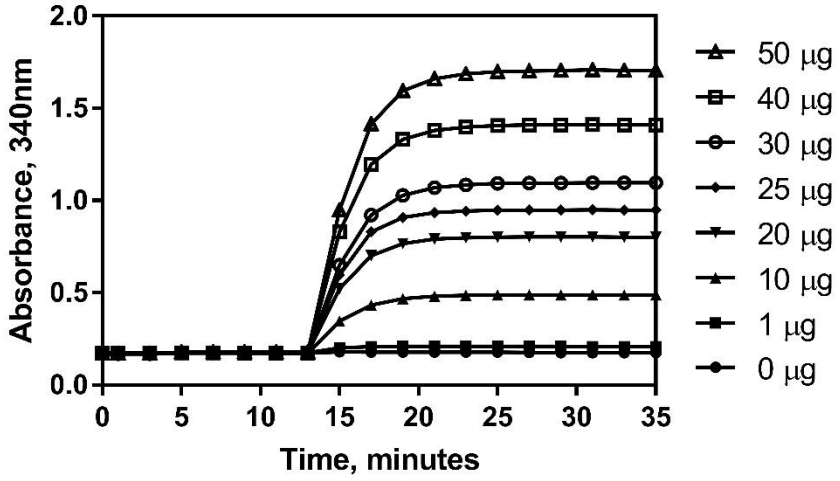

Fig 8: Reaction kinetic graph showing the time of reaction completion for lactose after addition of β-galactosidase using a range of lactose concentrations across the linear range of the assay (0-50 µg lactose per test). HK/G6PDH/6PGDH suspension was added after 3 min and β-galactosidase suspension was added after 13 min.

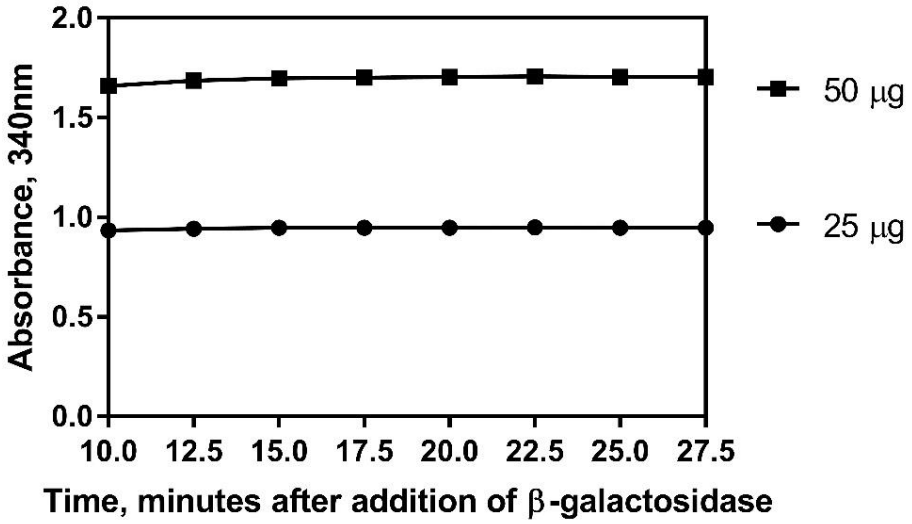

Fig 9: Examination of the robustness of the assay by adjusting the time at which the absorbance measurement for lactose is taken using two concentrations of lactose at the middle and the top of the linear range (25 and 50 µg). Absorbance at 340 nm was measured at 10, 12.5, 15 (recommended for assay), 17.5, 22.5, 25 and 27.5 min after addition of β-galactosidase.

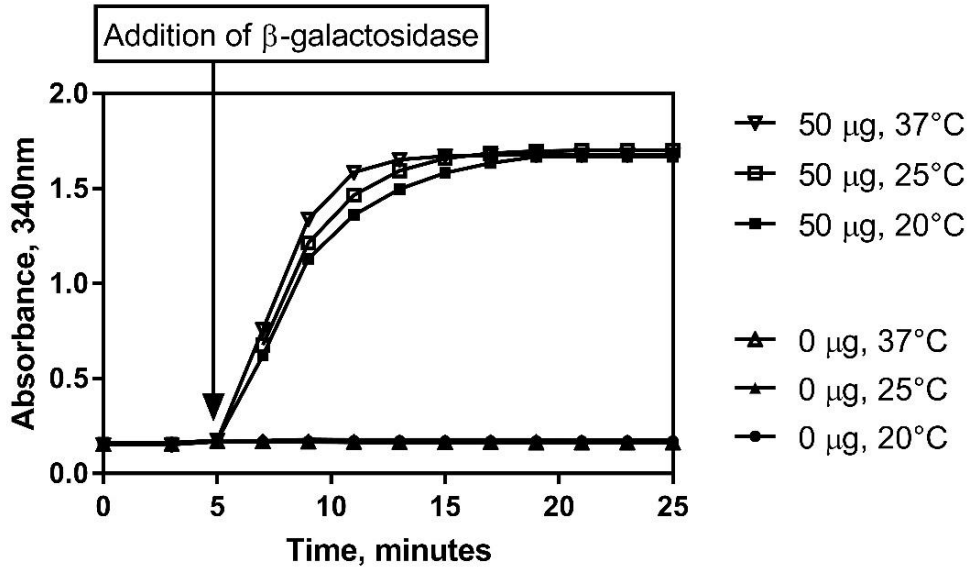

Fig 10: Examination of the robustness of the assay by adjusting the temperature at which the enzymatic determination reaction takes place. Assay carried out at 20°C, 25°C and 37°C with 0 and 50 µg of lactose per test. The β-galactosidase suspension was added after 5 min.

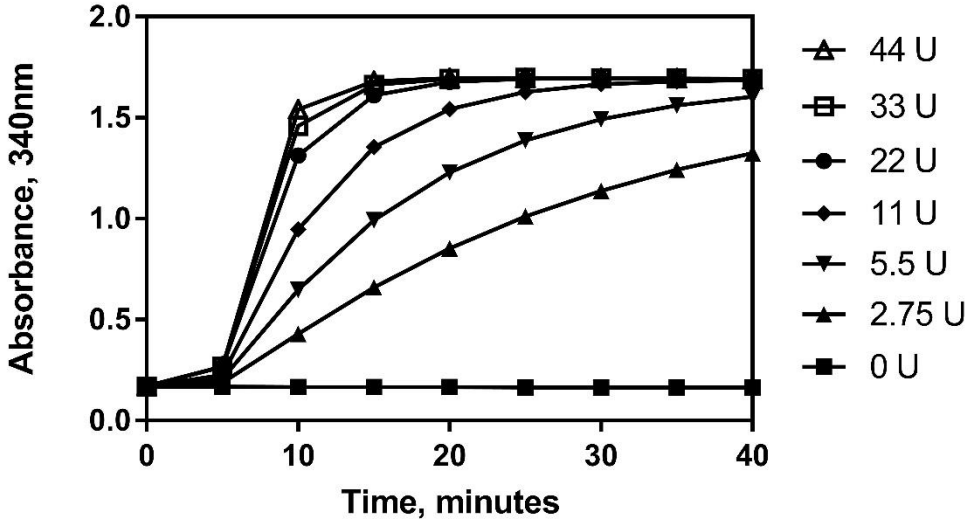

Fig 11: Examination of the robustness of the assay by adjusting the concentration of β-galactosidase enzyme in the 'Enzymatic Determination Reaction' (5.10, page 11). Assay carried out with 50 µg of lactose per test. β-galactosidase suspension (0.02 mL, 137.5 U/mL to 2,200 U/mL) was added after 5 min.

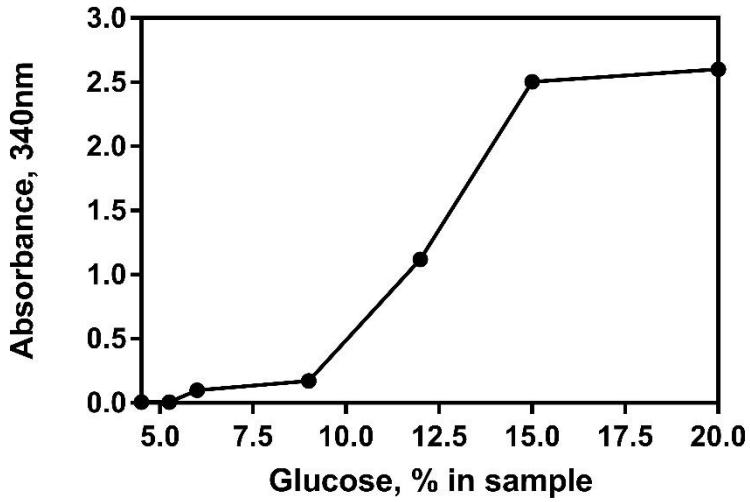

Fig 12: Examination of the robustness of the sample pre-treatment step by extraction of a range of glucose solutions as per the 'Glucose oxidase/Catalase pre-treatment procedure' (5.X) and analysis using the 'Enzymatic Determination Reaction' procedure (5.10, page 11). Measured absorbance for glucose (A_2) is shown on the y axis.

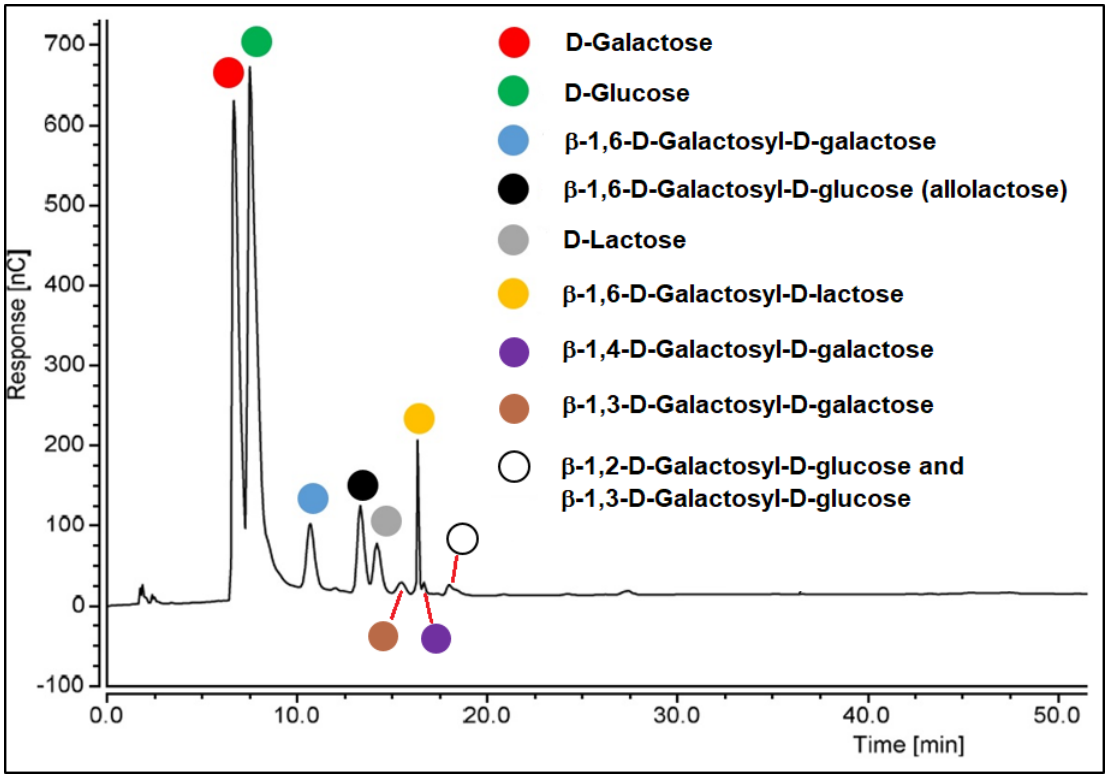

Fig 13: Oligosaccharide profile of a lactose-free milk product, graph obtained by HPAEC-PAD analysis.

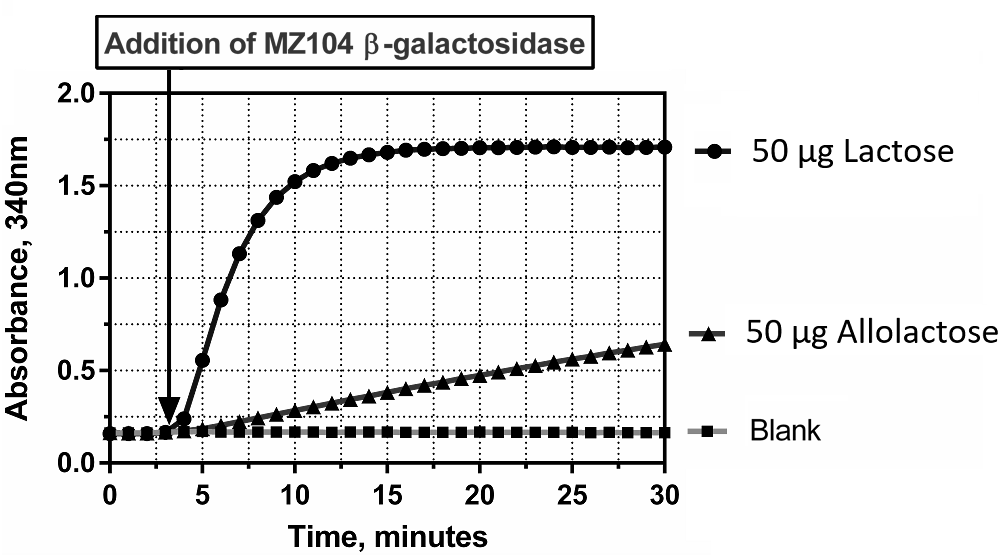

Fig 14: Reaction profile observed for 50 µg of lactose and 50 µg of allolactose in the 'Enzymatic Determination Reaction' (5.10, page 11). Note the slow, linear hydrolysis of allolactose.

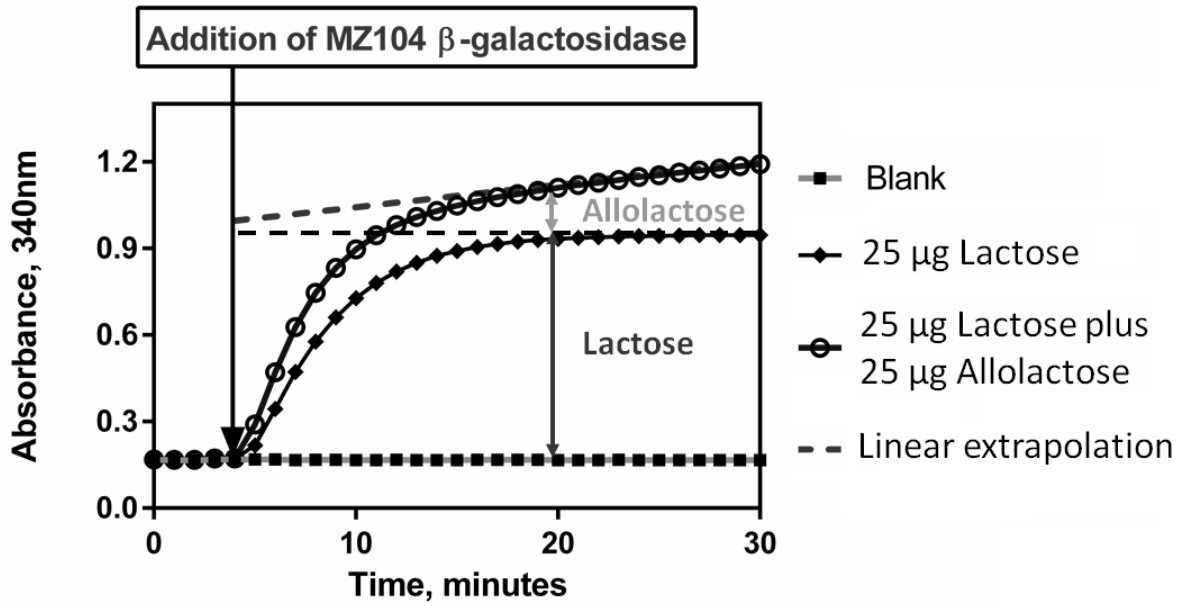

Fig 15: Reaction profile observed for 25 µg of lactose alongside a mixture of 25 µg of lactose and 25µg of allolactose in the 'Enzymatic Determination Reaction' (5.10, page 11). Note that the slow, linear hydrolysis of allolactose in the mixture can be extrapolated back to the point of addition of β-galactosidase, which provides the absorbance value corresponding to lactose hydrolysis only.

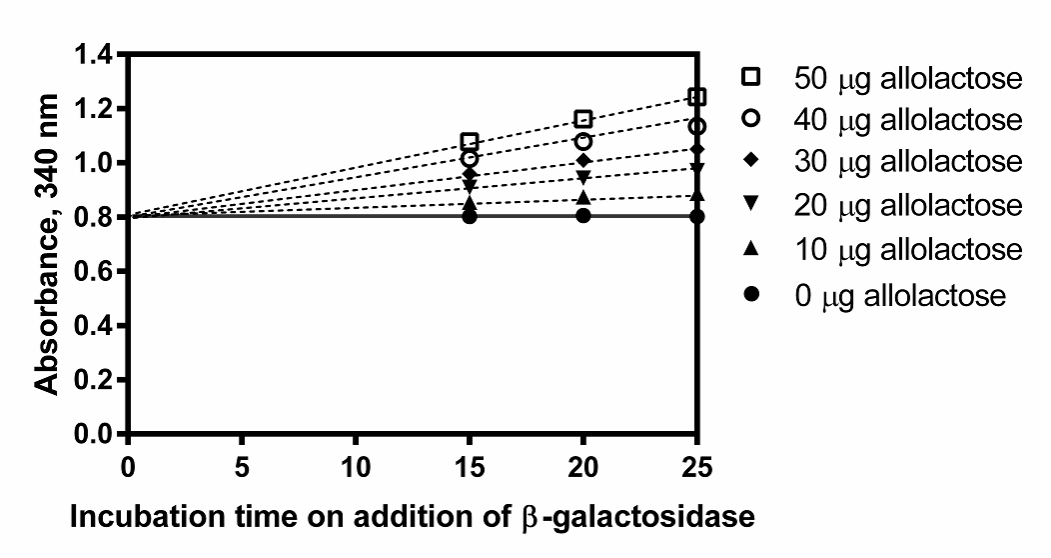

Fig 16: Demonstration of the effect of 0-50 μg of allolactose in the presence of 25 μg of lactose in the 'Enzymatic Determination Reaction' (5.10, page 11). In each mixture, linear extrapolation back to the point of addition of β-galactosidase provides the absorbance value corresponding to lactose hydrolysis only.

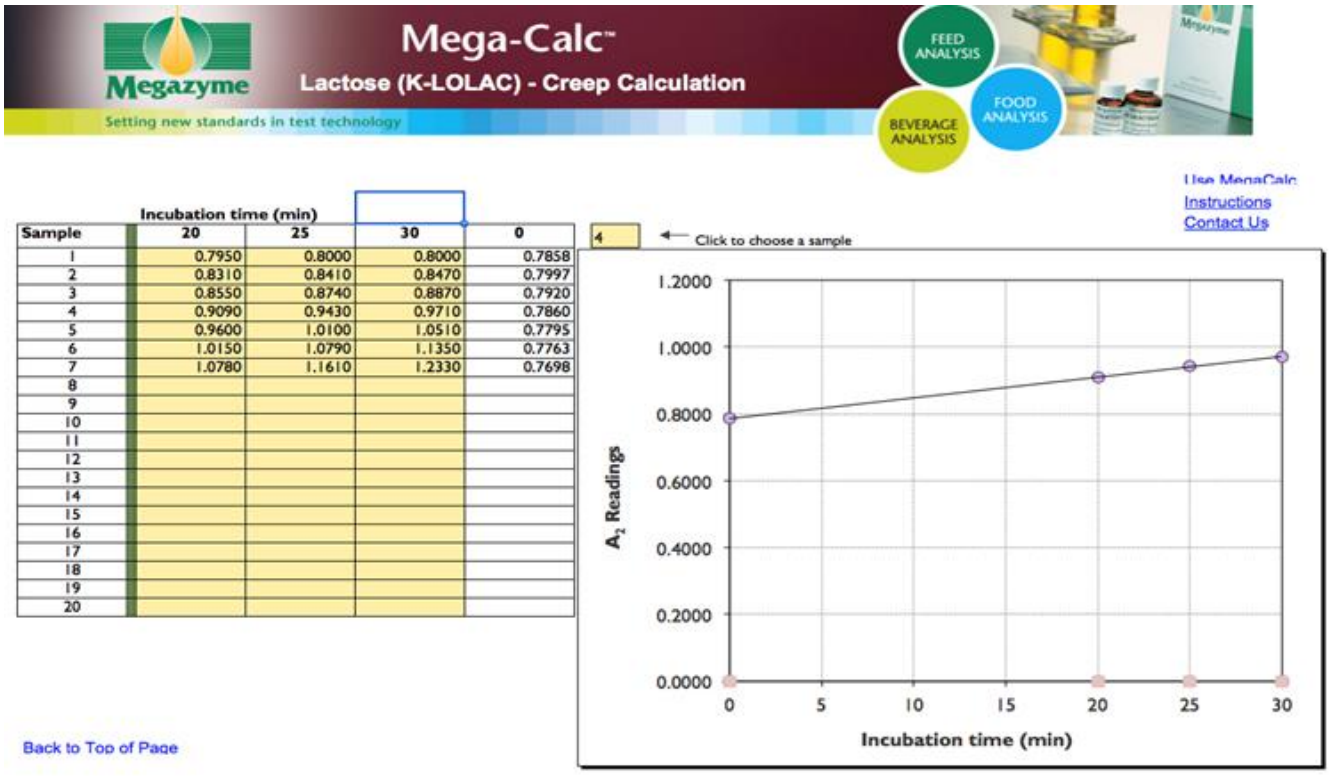

Fig 17: Demonstration of the application of the Megazyme creep calculator to obtain the absorbance corresponding to lactose hydrolysis in a mixture of lactose and allolactose. By entering the absorbance readings obtained at 5 min intervals after completion of the recommended 20 min β-galactosidase incubation, the absorbance value corresponding to lactose hydrolysis only is automatically generated.

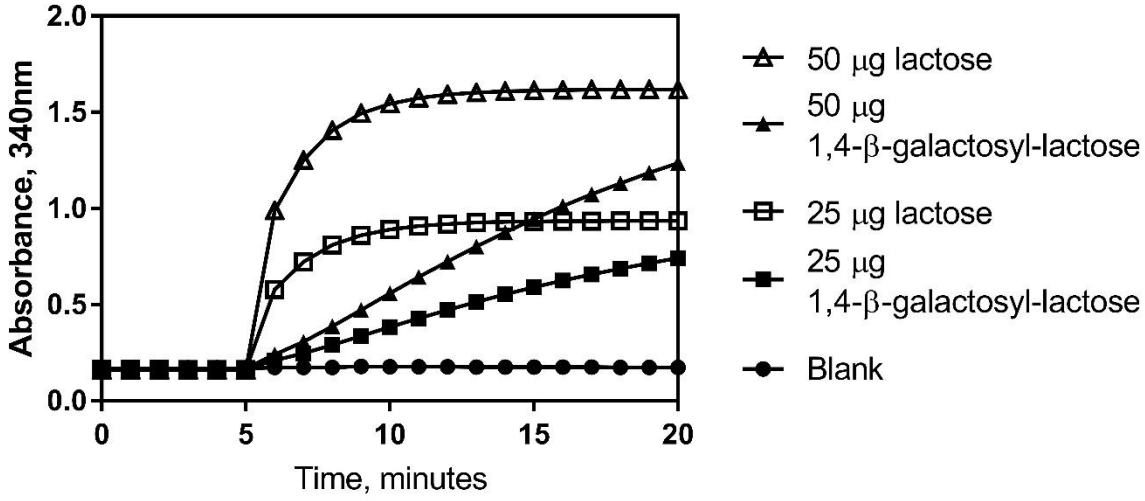

Fig 18: Reaction profile observed for both 25 µg and 50µg of lactose and 1,4-β-D-galactosyl-lactose in the 'Enzymatic Determination Reaction' (5.10, page 11).

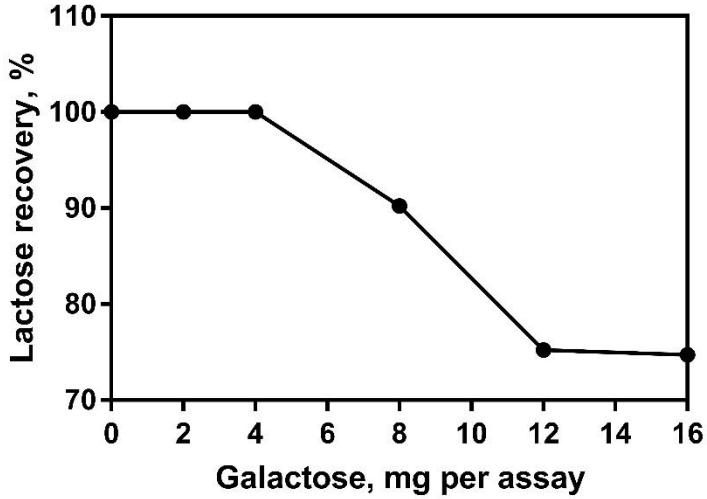

Fig 19: Recovery of a lactose standard when varying amounts of D-galactose are added to the 'Enzymatic Determination Reaction' (5.10, page 11).

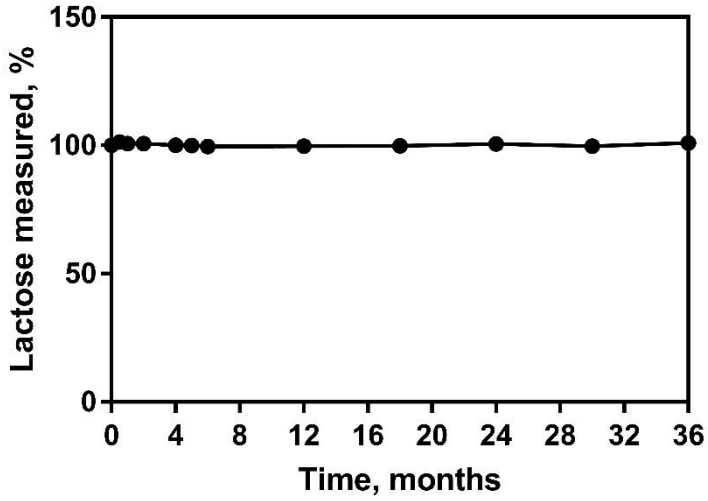

Fig 20: Stability of one set of kit components over a period of 36 months. The lactose standard was assayed at the highest concentration (50 µg per cuvette) in order to thoroughly test the system for stability. Note that glucose removal was also tested at these times and no issues were reported (data not shown).

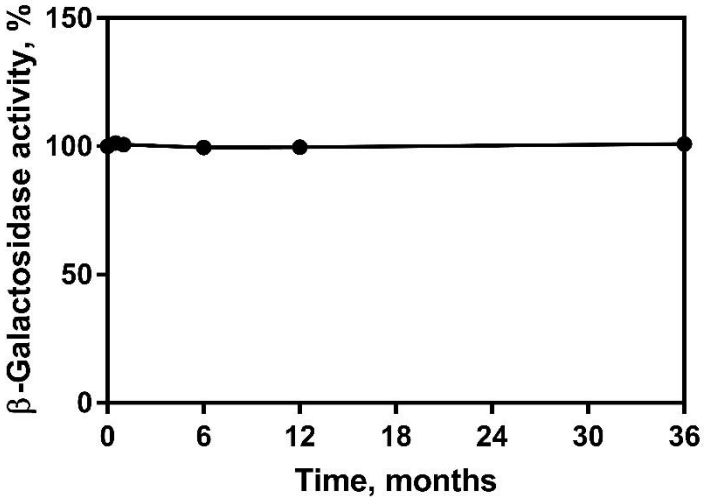

Fig 21: Stability of β-galactosidase (Suspension 6) over a period of 36 months. The enzyme was stored at 4°C (recommended storage) and assayed for activity (U/mL) which was then plotted as percentage activity remaining.

Table 1: Relative Bias *b*(%) using a single lactose standard, the 'Enzymatic Determination Reaction' (5.10, page 11), using 0.1 mL of standard per test

	n	Ref Material (mg/100mL)	MEAN (mg/100mL)	*b*(%)
Lactose	30	25	25.06	0.24

Table 2: Relative Bias *b*(%) using a variety of internal lactose standards in the 'Enzymatic Determination Reaction' after sample treatment as per 'Sample extraction/clarification procedure – LIQUID SAMPLES' (5.7, page 10) and the 'Glucose Oxidase/Catalase pre-treatment procedure' (5.9, page 10), using 0.1 mL of sample in the 'Enzymatic Determination Reaction'

	n	µg Lactose in cuvette	Ref Material (mg/100 mL)	MEAN (mg/100 mL)	*b*(%)
Lactose	8	50	270.0	272.5	0.94
	8	40	216.0	216.6	0.29
	8	20	108.0	109.0	1.01
	8	10	54.0	55.4	2.67
	8	2	10.8	10.9	1.19
	8	1	5.4	5.5	2.10
	8	0.5	2.7	2.6	-2.69

Table 3: Relative Bias *b*(%) using a number of Certified Reference Materials, after sample extraction and pre-treatment as appropriate for each sample matrix and analysis in the 'Enzymatic Determination Reaction' (5.10, page 11)

Sample	Expected lactose, g/100g (xref)	Measured Lactose, g/100g	Recovery, %	*b*(%)
CRM1	0.007	0.006	90.09	-11.00
CRM2	0.212	0.205	96.74	-3.37
CRM3	0.023	0.017	71.98	-38.93
CRM4	4.917	4.995	101.59	0.14
CRM5	24.277	23.954	98.67	-1.35
CRM6	22.100	21.397	96.82	-3.28
CRM7	22.670	22.872	100.89	0.88
CRM8	2.810	2.638	93.89	-6.50
CRM9	4.060	3.794	93.46	-7.00
CRM10	9.440	8.737	92.56	-8.04

Table 4: Repeatability (RSD_r) using a lactose standard (Solution 7) in the 'Enzymatic Determination Reaction' (5.10), with 0.1 mL used as sample

	n	Ref Material (mg/100 mL)	MEAN (mg/100 mL)	STDEV	%CV
Lactose	30	25	26.05	0.24	0.96

Table 5: Repeatability (RSD_r) using a range of internal lactose standards in the 'Enzymatic Determination Reaction' (5.10, page 11) after sample treatment as per 'Sample extraction/clarification procedure – LIQUID SAMPLES' (5.7, page 10) and the 'Glucose Oxidase/Catalase pre-treatment procedure' (5.9, page 10).

	n	µg Lactose in cuvette	Ref Material (mg/100 mL)	MEAN (mg/100 mL)	STDEV	%CV
Lactose	8	50	270.0	272.5	3.57	1.31
	8	40	216.0	216.6	2.32	1.07
	8	20	108.0	109.0	0.93	0.86
	8	10	54.0	55.4	1.03	1.85
	8	2	10.8	10.9	0.31	2.78
	8	1	5.4	5.5	0.17	3.11
	8	0.5	2.7	2.6	0.16	6.22

Tables 6-12: Repeatability (RSD_r) and intermediate precision (RSD_{ir}) for 7 commercial food and beverage samples analysed by two analysts over a three-day period. Each 'Extract' is a separate extraction of the sample using the relevant sample preparation example as outlined in the Methods section (5).

		Extraction	mg/100 mL	MEAN	STDEV	RSD_r	RSD_{ir}
Sample M4	Analyst 1	Day 1, Extract 1	2.536	2.467	0.19	7.68	9.98
		Day 1, Extract 1	2.566				
		Day 1, Extract 2	2.731				
		Day 1, Extract 2	2.611				
		Day 2, Extract 1	2.293				
		Day 2, Extract 1	2.051				
		Day 2, Extract 2	2.393				
		Day 2, Extract 2	2.565				
		Day 3, Extract 1	2.459				
		Day 3, Extract 1	2.462				
	Analyst 2	Day 1, Extract 1	2.255	2.196	0.19	8.84	
		Day 1, Extract 1	2.361				
		Day 1, Extract 2	2.387				
		Day 1, Extract 2	2.421				
		Day 2, Extract 1	1.974				
		Day 2, Extract 1	2.064				
		Day 2, Extract 2	1.954				
		Day 2, Extract 2	1.930				
		Day 3, Extract 1	2.331				
		Day 3, Extract 1	2.284				

		Extraction	mg/100 mL	MEAN	STDEV	RSD_r	RSD_{ir}
Sample M5	Analyst 1	Day 1, Extract 1	67.624	65.269	3.13	4.80	4.17
		Day 1, Extract 1	68.625				
		Day 1, Extract 2	63.149				
		Day 1, Extract 2	60.746				
		Day 2, Extract 1	59.332				
		Day 2, Extract 1	66.064				
		Day 2, Extract 2	66.262				
		Day 2, Extract 2	67.554				
		Day 3, Extract 1	66.157				
		Day 3, Extract 1	67.182				
	Analyst 2	Day 1, Extract 1	66.268	67.941	1.56	2.30	
		Day 1, Extract 1	69.148				
		Day 1, Extract 2	69.137				
		Day 1, Extract 2	67.688				
		Day 2, Extract 1	66.117				
		Day 2, Extract 1	66.262				
		Day 2, Extract 2	66.245				
		Day 2, Extract 2	69.643				
		Day 3, Extract 1	69.404				
		Day 3, Extract 1	69.501				

		Extraction	mg/100 mL	MEAN	STDEV	RSD_r	RSD_{ir}
Sample ND4	Analyst 1	Day 1, Extract 1	2.491	2.620	0.20	7.60	7.59
		Day 1, Extract 1	2.492				
		Day 1, Extract 2	2.962				
		Day 1, Extract 2	2.998				
		Day 2, Extract 1	2.531				
		Day 2, Extract 1	2.493				
		Day 2, Extract 2	2.559				
		Day 2, Extract 2	2.518				
		Day 3, Extract 1	2.473				
		Day 3, Extract 1	2.684				
	Analyst 2	Day 1, Extract 1	2.654	2.544	0.18	7.25	
		Day 1, Extract 1	2.741				
		Day 1, Extract 2	2.531				
		Day 1, Extract 2	2.425				
		Day 2, Extract 1	2.827				
		Day 2, Extract 1	2.771				
		Day 2, Extract 2	2.418				
		Day 2, Extract 2	2.486				
		Day 3, Extract 1	2.293				
		Day 3, Extract 1	2.290				

		Extraction	mg/100 mL	MEAN	STDEV	RSD_r	RSD_{ir}
Sample IF1	Analyst 1	Day 1, Extract 1	2.784	2.821	0.11	3.73	2.97
		Day 1, Extract 1	2.953				
		Day 1, Extract 2	2.907				
		Day 1, Extract 2	2.821				
		Day 2, Extract 1	2.722				
		Day 2, Extract 1	2.779				
		Day 2, Extract 2	2.859				
		Day 2, Extract 2	2.928				
		Day 3, Extract 1	2.859				
		Day 3, Extract 1	2.603				
	Analyst 2	Day 1, Extract 1	2.885	2.803	0.06	2.23	
		Day 1, Extract 1	2.752				
		Day 1, Extract 2	2.806				
		Day 1, Extract 2	2.811				
		Day 2, Extract 1	2.843				
		Day 2, Extract 1	2.668				
		Day 2, Extract 2	2.838				
		Day 2, Extract 2	2.784				
		Day 3, Extract 1	2.827				
		Day 3, Extract 1	2.812				

		Extraction	mg/100 mL	MEAN	STDEV	RSD_r	RSD_{ir}
Sample IF3	Analyst 1	Day 1, Extract 1	2.255	2.218	0.15	6.64	6.01
		Day 1, Extract 1	2.221				
		Day 1, Extract 2	2.360				
		Day 1, Extract 2	2.476				
		Day 2, Extract 1	2.079				
		Day 2, Extract 1	2.165				
		Day 2, Extract 2	2.130				
		Day 2, Extract 2	2.015				
		Day 3, Extract 1	2.373				
		Day 3, Extract 1	2.108				
	Analyst 2	Day 1, Extract 1	2.203	2.127	0.10	4.92	
		Day 1, Extract 1	1.994				
		Day 1, Extract 2	2.025				
		Day 1, Extract 2	2.137				
		Day 2, Extract 1	2.217				
		Day 2, Extract 1	2.104				
		Day 2, Extract 2	2.319				
		Day 2, Extract 2	2.040				
		Day 3, Extract 1	2.102				
		Day 3, Extract 1	2.130				

		Extraction	g/100 g	MEAN	STDEV	RSD_r	RSD_{ir}
Sample C6	Analyst 1	Day 1, Extract 1	0.150	0.143	0.00	2.95	6.84
		Day 1, Extract 1	0.147				
		Day 1, Extract 2	0.142				
		Day 1, Extract 2	0.141				
		Day 2, Extract 1	0.137				
		Day 2, Extract 1	0.138				
		Day 2, Extract 2	0.141				
		Day 2, Extract 2	0.138				
		Day 3, Extract 1	0.141				
		Day 3, Extract 1	0.146				
		Day 3, Extract 2	0.146				
		Day 3, Extract 2	0.147				
	Analyst 2	Day 1, Extract 1	0.123	0.127	0.01	4.06	
		Day 1, Extract 1	0.121				
		Day 1, Extract 2	0.124				
		Day 1, Extract 2	0.124				
		Day 2, Extract 1	0.121				
		Day 2, Extract 1	0.125				
		Day 2, Extract 2	0.129				
		Day 2, Extract 2	0.127				
		Day 3, Extract 1	0.128				
		Day 3, Extract 1	0.134				
		Day 3, Extract 2	0.135				
		Day 3, Extract 2	0.135				

		Extraction	mg/100 g	MEAN	STDEV	RSD_r	RSD_{ir}
Sample C7	Analyst 1	Day 1, Extract 1	32.651	31.198	0.78	2.49	2.31
		Day 1, Extract 1	32.767				
		Day 1, Extract 2	30.929				
		Day 1, Extract 2	31.010				
		Day 2, Extract 1	31.301				
		Day 2, Extract 1	31.219				
		Day 2, Extract 2	31.027				
		Day 2, Extract 2	30.107				
		Day 3, Extract 1	30.457				
		Day 3, Extract 1	30.859				
		Day 3, Extract 2	31.062				
		Day 3, Extract 2	30.981				
	Analyst 2	Day 1, Extract 1	31.388	31.905	0.48	1.52	
		Day 1, Extract 1	31.394				
		Day 1, Extract 2	31.458				
		Day 1, Extract 2	31.446				
		Day 2, Extract 1	32.261				
		Day 2, Extract 1	32.570				
		Day 2, Extract 2	31.749				
		Day 2, Extract 2	32.820				
		Day 3, Extract 1	32.238				
		Day 3, Extract 1	32.081				
		Day 3, Extract 2	31.807				
		Day 3, Extract 2	31.644				

Table 13: Recovery data for 36 food and beverage samples spiked with a known amount of lactose. Measured lactose (sample) = Concentration of lactose measured in sample without spiking, Expected lactose (spike) = Concentration of spike added to test, Measured lactose (spike) = Concentration of spike measured (taking away separately determined value for sample).

Sample identifier	Measured Lactose (sample), mg/100 mL	Expected Lactose (spike) mg/100 mL	Measured Lactose (spike) mg/100 mL	Recovery (spike), %
M1	4.44	10	10.19	101.90
M2	26.61	10	9.74	97.40
M3	<0.89	10	12.49	110.60
M4	2.33	10	10.24	102.40
M5	66.61	10	9.98	99.80

Sample identifier	Measured Lactose (sample), mg/100 g	Expected Lactose (spike) mg/100 g	Measured Lactose (spike) mg/100 g	Recovery (spike), %
Y1	27.5	10	10.14	101.4
Y2	15.1	10	9.85	98.5
Y3	38.8	10	10.15	101.5

Sample identifier	Measured Lactose (sample), mg/100 g	Expected Lactose (spike), mg/100 g	Measured Lactose (spike), mg/100 g	Recovery (spike), %
F1	2.54	7	7.82	111.65
F2	<1.48	7	7.10	101.47
F3	<1.48	7	7.92	113.21
F4	2.65	7	7.27	103.91
F5	<1.48	7	7.39	105.58
F6	28.14	10	10.04	100.41
F7	<1.48	10	10.12	101.24

Sample identifier	Measured Lactose (sample), mg/100 g	Expected Lactose (spike) mg/100 g	Measured Lactose (spike) mg/100 g	Recovery (spike), %
C1	<1.48	10	10.66	106.60
C2	2.35	10	9.99	99.90
C3	1.58	10	11.41	114.10
C4	<1.48	10	11.15	111.50
C5	<1.48	10	9.77	97.71
C6	135	10	10.11	101.11
C7	31.54	10	11.01	110.14

Sample identifier	Measured Lactose (sample), mg/100 mL	Expected Lactose (spike) mg/100 mL	Measured Lactose (spike) mg/100 mL	Recovery (spike), %
IF 1	2.81	5	5.12	102.40
IF 2	4.41	5	5.15	103.13
IF 3	2.17	5	5.34	106.26
IF 4	<0.89	5	5.18	103.14
IF 5	<0.89	5	4.99	99.16
IF 6	<0.89	5	4.66	93.21
IF 7	1.28	5	5.03	100.61
IF 8	2.42	10	10.85	108.50
IF 9	3.23	10	9.86	98.64
IF 10	<0.89	10	10.93	109.34
IF 11	<0.89	10	10.30	103.02

Sample identifier	Measured Lactose (sample), mg/100 mL	Expected Lactose (spike) mg/100 mL	Measured Lactose (spike) mg/100 mL	Recovery (spike), %
ND1	<0.89	10	11.28	112.82
ND2	<0.89	10	10.68	106.84
ND3	73.51	10	10.75	107.51
ND4	2.58	10	10.21	102.11

Sample identifier	Measured Lactose (sample), mg/100 g	Expected Lactose (spike) mg/100 g	Measured Lactose (spike) mg/100 g	Recovery (spike), %
CRM1	9.89	10	10.16	101.61
CRM2	222.70	100	100.88	100.88
CRM3	16.56	10	10.78	107.85
CRM4	4924.13	1000	944.47	94.44
CRM5	23953.68	5000	5027.06	100.54
CRM6	21398.12	5000	5351.96	107.04
CRM7	22872.30	5000	5414.21	108.28
CRM8	2638.40	1000	945.52	94.52
CRM9	3794.54	1000	989.04	98.90
CRM10	8737.54	5000	4748.97	94.97

Table 14: Table showing percentage recovery of a lactose standard when extracted as per the 'Sample extraction/clarification procedure – SOLID SAMPLES' (5.8, page 10) with 1 g of the specified potential interferent in the extraction (the equivalent of 1 g per 10 g of sample, or 10% *w/w* interferent). Samples were treated as per the 'Glucose Oxidase/Catalase pre-treatment procedure' (5.9, page 10) and then analysed in the 'Enzymatic Determination Reaction' using the maximum sample volume of 0.4 mL per test.

Chemical	CAS number	Interferent, % (equivalent g per 100 g of sample weight)	Lactose recovery, %
Salts			
Calcium chloride	10043-52-4	10	98.51
Magnesium chloride	7786-30-3	10	99.53
Manganese chloride	7773-01-5	10	97.52
Potassium chloride	7447-40-7	10	100.41
Sodium chloride	7647-14-5	10	100.49
Zinc chloride	7646-85-7	10	99.246
Sugar alcohols			
Erythritol	149-32-6	10	98.88
Glycerol	56-81-5	10	99.53
Lactitol	585-86-4	10	97.84
Maltitol	585-88-6	10	98.31
Mannitol	87-78-5	10	99.87
myo-Inositol	87-89-8	10	100.91
Sorbitol	50-70-4	10	99.38
Xylitol	87-99-0	10	97.49
Organic acids			
Acetic acid	64-19-7	10	99.64
Ascorbic acid	50-81-7	10	93.61
Aspartic acid	617-45-8	10	98.59
Citric acid	77-92-9	10	99.21
Galacturonic acid	685-73-4	10	101.95
Gluconic acid	133-42-6	10	98.66
Glucuronic acid	6556-12-3	10	99.22
Glutamic acid	617-65-2	10	100.70
Lactic acid	50-21-5	10	99.81
Malic acid	6915-15-7	10	98.04
Succinic acid	110-15-6	10	97.97
Tartaric acid	526-83-0	10	100.92

Chemical	CAS number	Interferent, % (equivalent g per 100 g of sample weight)	Lactose recovery, %
Amino acids			
Glycine	56-40-6	10	104.41
Histidine	71-00-1	10	104.63
Isoleucine	73-32-5	10	99.34
Lysine	56-87-1	10	97.27
Tryptophan	54-12-6	10	100.81
Tyrosine	60-18-4	10	97.80
Valine	516-06-3	10	97.40
Other			
Aspartame	22839-47-0	10	99.80
Casein	9000-71-9	10	101.12
Cellobiose	528-50-7	10	104.649
Cellotriose	33404-34-1	10	102.05
Fructan	9013-95-0	10	99.93
Galactomannan	900-30-0	10	101.94
Glycerol triacetate	102-76-1	10	99.84
Isomalt	64519-82-0	10	98.33
Lactulose	4618-18-2	10	101.14
Maltose	69-79-4	10	98.567
Maltotriose	1109-28-0	10	101.37
Sodium alginate	9005-38-3	10	97.73
Starch	9005-25-8	10	102.89
Sucralose	56038-13-2	10	97.35
Sucrose	57-50-1	10	100.5
β-glucan	9012-72-0	10	101.14

Table 15: Table showing percentage recovery of a lactose standard when extracted as per the 'Sample extraction/clarification procedure – SOLID SAMPLES' (5.8, page 10) with varying amounts of the specified interferent in the extraction (the equivalent of 0.2-0.8 g per 10 g of sample, or 2-8 % *w/w* interferent). Extracted samples were treated as per the 'Glucose Oxidase/Catalase pre-treatment procedure' (5.9, page 10) and then analysed in the 'Enzymatic Determination Reaction' using the maximum sample volume of 0.4 mL per test.

Chemical	**CAS number**	**Interferent, % (equivalent g per 100 g of sample weight)**	**Lactose recovery, %**
Acetaldehyde	75-07-0	2	100.01
		4	98.123
		6	95.496
		8	93.830
Arginine	7200-25-1	2	98.864
		4	98.093
		6	94.956
		8	92.411
Copper (I) Sulphate	17599-81-4	2	99.543
		4	95.581
		6	96.721
		8	93.656

7. Supplementary information – Additional sample preparation examples

7.1 Cheese solubilisation

The melting point of cheese can vary based on parameters such as moisture content and age. A solubility assessment should be carried out by visual inspection. Although the solution may not be free of turbidity after solubilisation, there should be no lumps present or noticeable lack of homogeneity. A sample preparation example is outlined below:

7.1.1 Using a standard kitchen hand grater, grate cheese through a fine sieve

7.1.2 Accurately weigh approx. 10 g of grated sample into a 50 mL glass beaker

7.1.3 Add a stir bar and approx. 30 mL of distilled water

7.1.4 Mix on a magnetic stir plate and heat until temperature reaches 50°C. Continue stirring at temperature for approx. 15 min and assess the level of solubilisation*

7.1.5 If the cheese has not solubilised at 50°C slowly increase the temperature by 5°C and further assess the level of solubilisation

7.1.6 Continue to increase the temperature in increments of 5°C up to a temperature of 70°C if solubilisation or homogenisation has not occurred at lower temperatures

7.1.7 Quantitatively transfer to a 50 mL volumetric flask

7.1.8 Add 0.5 mL of Carrez II solution and mix

7.1.9 Add 0.5 mL of Carrez I solution and mix

7.1.10 Make to volume (50 mL) with distilled water

7.1.11 Filter an aliquot, discarding the first few mL of filtrate (~ 5 mL)

7.1.12 Take 1.0 mL of the clear filtrate for Glucose oxidase/Catalase treatment (5.9, page 10)

7.2 Samples containing carbon dioxide

Samples containing a significant amount of carbon dioxide should be degassed by increasing the pH to approx. 7.6 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

7.3 Strongly coloured samples

If used undiluted, strongly coloured samples should be treated by the addition of Polyvinylpolypyrrolidone. 0.2 g of PVPP should be added to 10 mL of sample. Shake the tube vigorously over a 5 min period and then filter through Whatman No. 1 filter paper.

7.4 Homogenisation of solid food samples

Homogenise solid samples if necessary, using a blender such as a Nutribullet or similar.

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