Single Lab Validation of an enzymatic test for lactose in lactose-free products (K-LOLAC). AOAC First Action Method 2019

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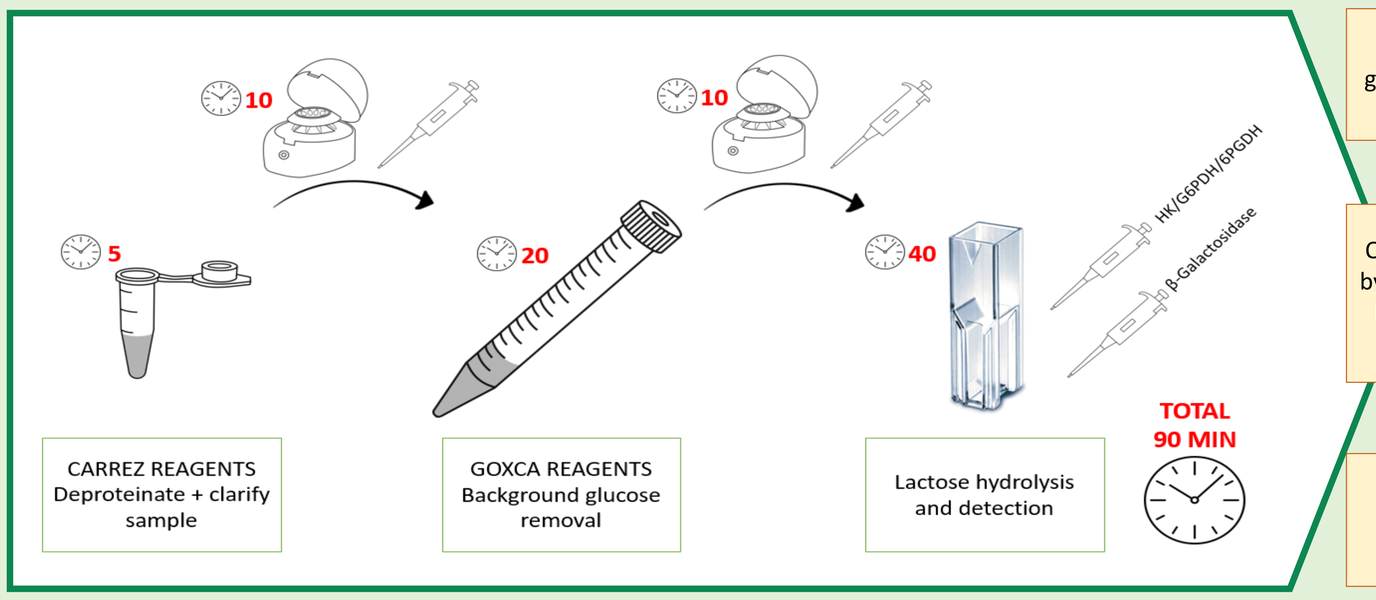
INTRODUCTION

Megazyme's Lactose Assay (K-LOLAC) is an accurate and sensitive enzymatic method for the rapid measurement of lactose in low-lactose or lactose-free products. The method includes pre-treatment steps to clarify and deproteinate samples and to remove the high levels of free D-glucose in the samples, allowing accurate measurement of lactose at very low levels. Quantification is based on the hydrolytic activity of β-galactosidase, and measurement of glucose released. The β-galactosidase employed is selective for lactose over structurally similar oligosaccharides which, when coupled with a "creep" calculation can account for overestimation that arises from the minor hydrolysis of lactose analogues.

A Single Lab Validation (SLV) is reported, analysis was performed against SMPR 2018.009 on a sample set of 36 commercial food and beverage products and a set of 10 certified reference materials. Parameters examined during the validation included Working Range and Linear Range, Selectivity, Limit of Detection (LOD), Limit of Quantification (LOQ), Trueness (*bias*),Precision (repeatability), Robustness and Stability. This method was accepted as an AOAC First Action Official Method in 2019 and is currently undergoing a multi-laboratory validation towards Final Action status.

METHODS



Sample clarification step followed by a glucose-oxidase/catalase pre-treatment to remove glucose

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Quantification of free D-glucose followed by measurement of glucose released from lactose. 45 minute assay at 340 nm and 25°C

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Calculation of results using linear extrapolation calculator with built-in 'creep' adjustment

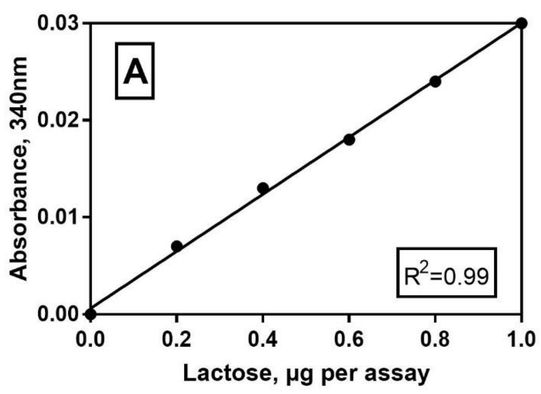
SINGLE LAB VALIDATION

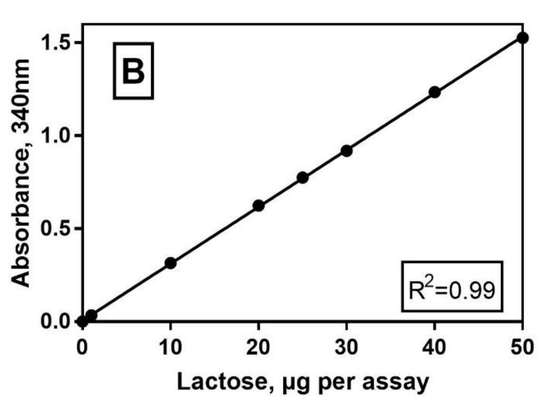
- ✓ **Linear range** – the range in which the system returns a linear response was investigated
- ✓ **LOD and LOQ** – the lowest concentration of analyte measurable using the method was determined
- ✓ **Trueness** – Results achieved for certified materials were compared to reference values
- ✓ **Precision** – samples were tested by a single analyst over a short period of time (RSD_r) and several analysts over an extended period of time (RSD_{ir})
- ✓ **Interference** – potentially interfering compounds were spiked into system (e.g. Organic acids, Salts, Amino acids and more). Recovery assessed.
- ✓ **Selectivity** – investigated potentially interfering sugars in the system
- ✓ **Robustness** - minor variations or adjustments to certain assay parameters were investigated
- ✓ **Recovery** - known amounts of lactose were added to the sample extraction and recovery was assessed.
- ✓ **Stability** – long term storage of kit components was tested under a number of different conditions

RESULTS

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.





For LIQUID samples

LOD Lactose = 0.27 mg/100 mL

LOQ Lactose = 0.89 mg/100 mL

LOD and LOQ

For SOLID samples

LOD Lactose = 0.44 mg/100g

LOQ Lactose = 1.47 mg/100g

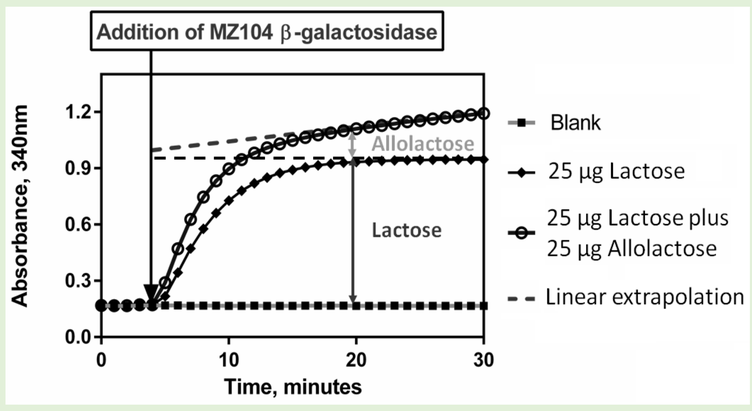
n	µg Lactose in test	STDEV	%CV
8	50	3.57	1.31
8	40	2.32	1.07
8	20	0.93	0.86
8	10	1.03	1.85
8	2	0.31	2.78
8	1	0.17	3.11
8	0.5	0.16	6.22

Assay Repeatability (RSD_r) using the 'Enzymatic Determination Reaction' procedure. A series of standards (ranging from 2.7 – 270 mg/100 mL) are the reference materials. 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).

Recovery data for 5 milk samples tested as part of recovery experiments. 36 food and beverage samples were spiked with a known amount of lactose and recovery assessed.

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/100mL	Measured Lactose (spike) mg/100 mL	Recovery (spike), %
M1; Lactose-free milk	4.44	10	10.19	101.9
M2; Lactose-free milk	26.61	10	9.74	97.4
M3; Lactose-free milk	<0.89	10	12.49	110.6
M4; Lactose-free milk	2.33	10	10.24	102.4
M5; Lactose-free milk	66.61	10	9.98	99.8



Selectivity

Reaction profile observed for 25 µg of lactose alongside a mixture of 25 µg of lactose and 25 µg of allolactose in the 'Enzymatic Determination Reaction'. 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.

DISCUSSION

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. 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. Trueness was tested using 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 10 harmonization materials tested (using values stated by the manufacturer, obtained by HPLC). 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 two lactose-free milk samples, two infant formula samples, two 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/100g or mL) varied from 94.44 – 108.28 %.

Robustness testing included the examination of incubation temperature fluctuations, time at which absorbance measurement is taken, 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. 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'). 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.

CONCLUSIONS

The Single Lab Validation shows 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. K-LOLAC is selective for lactose in low lactose and lactose-free products. K-LOLAC is a sensitive, accurate and cost effective test kit for lactose, specifically in low lactose and lactose free samples.

REFERENCES AOAC SMPR 2018.009 (2018)