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Fast determination of lactose in dairy products

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Keywords

Lactose-free, Dionex CarboPac PA20-Fast-4µm, HPAEC-PAD, time-savings

Goal

To develop a fast, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method for the determination of trace lactose in “lactose-free" and "reduced-lactose" labeled products using new column technology.

Introduction

Lactose is the primary disaccharide found in milk products, with a mass fraction ranging from 2.8% to 5.7%. In skimmed milk powder, the mass fraction is as high as 51%.[1,2] Lactose accounts for approximately 30% of the caloric value of whole milk. To be absorbed and used by the body, lactose must be catabolized by lactase to glucose and galactose. In most adult humans the activity of lactase decreases after infancy, potentially leading to lactose intolerance. While for many Western European adults lactase activity remains constant, approximately 95% of the Asian adult population are lactase deficient.[3] Because of the uncomfortable symptoms associated with lactose-intolerance, there is a significant demand for lactose-free products.

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Commercially available lactose-free products are manufactured by enzymatic treatment of the traditional dairy product, resulting in varying levels of residual lactose. Today a widely accepted specification for lactose-free products is a lactose mass fraction of below 0.1% to 0.01% *(vide infra).* Consequently, there is a need for simple, reliable, and accurate analytical methods to quantify low amounts lactose in food products.

Over the last few decades, a number of different methods based on infrared spectroscopy, polarimetry, enzymatic assays, colorimetry, gravimetry, and chromatography have been developed to quantify lactose in milk and milk products. Some of these methods have been recognized as standards by the International Dairy Federation (IDF) and ISO or AOAC. More recently, high-performance liquid chromatography (HPLC) and differential pH methods, based on an enzymatic reaction, have been proposed as IDF and ISO standards. Every one of these alternatives exhibits advantages and disadvantages regarding the costs, analytical complexity, performance, limit of quantification (LOQ), and the degree to which these are affected by other components or ingredients present in the dairy products.[4] For example, in 2007 ISO published an HPLC method based on ligand exchange chromatography and refractive index (RI) detection for the determination of lactose in milk and milk products. This method was tested for milk, cream, and milk powder with mass fractions between 1.5% and 50%. The method, however, is not applicable for fermented milk and milk products with added oligosaccharides[5] and is also not suited for the evaluation of lactose-free products due to its chromatographic selectivity and detection sensitivity.[3]

To date, EU authorities have not defined universal limit values for the lactose content of low lactose and lactose-free food products. For a “lactose-free declaration”, national limits range from 100 mg lactose per 100 g in Germany, Slovenia, and Hungary,[6] to 10 mg lactose per 100 g in Scandinavia, to “no lactose present” in Ireland. In contrast, the definition “low lactose” has a more broadly accepted value of 1 g lactose per 100 g.[7] Given these numbers and considering that most of the approaches involve sample preparation (e.g., deproteinization by Carrez precipitation and dilution), methods of lactose determination must be capable of achieving sensitive, selective, and accurate analysis at low mg/L to µg/L concentrations. Furthermore, with laboratories specializing in lactose determinations facing increased demand for services, sample throughput and analysis times must also be optimized.

HPAEC-PAD is widely used for the determination of mono-, di-, oligo- and smaller polysaccharides, as well as sialic acids and other sugar acids. This technique combines high chromatographic selectivity and efficiency with the high detection sensitivity and specificity of amperometric detection. In this application note, the Thermo Scientific™ Dionex™ CarboPac™ PA20-Fast-4µm column is used for the fast and high-resolution separation of lactose in various conventional and lactose-free labeled dairy products. This column contains a hydrophobic, polymeric, microporous anion exchange resin stable over the entire range of pH 0–14,[8] and uses smaller resin particles compared to the legacy Dionex CarboPac PA20 column,[9] resulting in more efficient separation, improved peak integration accuracy, and more reliable results.

Experimental

Equipment

Thermo Scientific™ Dionex™ ICS-3000 IC system* consisting of:

- Dionex DP Gradient Pump Quaternary with Degasser (P/N 061712)
- Dionex AS-AP Autosampler (P/N 074925)
- Dionex DC Module (P/N 063772)
- Dionex ICS-3000 ED Electrochemical Detector (P/N 061718)
- Dionex ED Detector Cell (P/N 061756)
- Dionex ED Detector Reference Electrode (Ag/AgCl) (P/N 061719)
- Dionex Gold ED Electrode (P/N 061749)
- Dionex EO Eluent Organizer Trays with four 2 L bottles (P/N 062628)
- Thermo Scientific™ Chromeleon™ Chromatography Data System 7.2

*A Thermo Scientific™ Dionex™ ICS-5000+ system or Thermo Scientific™ Dionex™ ICS-6000 system can also be used.

Reagents, standards, and materials

Reagents

- Deionized water (DI), Type I reagent grade, 18 MΩ·cm resistivity or better, treated with 20 mg/L NaN_3 to avoid mold and bacterial growth and filtered through a 0.2 µm filter immediately before use.
- Sodium hydroxide solution (Honeywell Fluka™, P/N 71686)
- Sodium acetate p.A. (Sigma®, P/N 71182)
- Sodium azide (Sigma, P/N 71289)
- Nitrogen 5.0 (Air Liquide®)
- Potassium hexacyanoferrate (II) trihydrate (Honeywell Riedel de Haen™, P/N 31254)
  - Carrez I solution: w($K_4Fe(CN)_6 \cdot 3H_2O$) = 15% in water
- Zinc sulfate heptahydrate (Honeywell Riedel de Haen™, P/N 31665)
  - Carrez II solution: w($ZnSO_4 \cdot 7H_2O$) = 30% in water

Standards

- Fructose (Honeywell Fluka, P/N 47739)
- Sucrose (Honeywell Fluka, P/N 84097)
- Lactulose (Honeywell Fluka, P/N 2250277)
- Lactose monohydrate (Honeywell Fluka, P/N 2005592)
- Galactose (Honeywell Fluka, P/N 48259)

Materials

- Qualitative filter paper (Macherey-Nagel, P/N 531018)
- Syringe Filter (Nylon, 0.45 µm) (ROTH®, P/N KY64.1)

Chromatographic conditions

Columns:	Dionex CarboPac PA20-Fast-4µm Guard Column, 2 × 30 mm (P/N 302750) Dionex CarboPac PA20-Fast-4µm Analytical Column, 2 × 100 mm (P/N 302749)		
Injection Volume:	10 µL		
Eluents:	A: Water B: 0.2 M NaOH C: 0.1 M NaOAc D: 1 M NaOAc in 0.2 M NaOH		
Flow:	0.2 mL/min		
Temperature:	30 °C		
Curve:	5		
Working Electrode:	Au (1 mm)		
Gasket:	1.0 mil (25.4 µm)		
Reference Electrode:	Ag/AgCl		
Amperometric detection:	Carbohydrate waveform (four potential)		
Waveform:	*Time (s)*	*Potential (V)*	*Integration*
	0.00	+0.1	
	0.20	+0.1	Begin
	0.40	+0.1	End
	0.41	–2.0	
	0.42	–2.0	
	0.43	+0.6	
	0.44	–0.1	
	0.50	–0.1	
Background:	15 nC		
Noise:	5–20 pC		
System Backpressure:	2700 psi (18.5 MPa)		
Analytical Gradient:	Table 1		
Rinsing Gradient:	Table 2		

Table 1. Analytical gradient

Time (min)	Event	Eluent	(%)
0.0	Inject	B	6
		A	94
	Autozero		
10.0		B	6
		A	94
11.0		B	10
		A	90
14.0		B	10
		C	2.5
		A	87.5
18.0		B	10
		C	2.5
		A	87.5
18.1		B	6
		A	94
20.5	Stop Run	B	6
		A	94

Table 2. Rinsing gradient (no data acquisition)

Time (min)	Event	Eluent	(%)
0.0	Run	B	6
		A	94
0.1		B	25
		D	75
5.0		B	25
		D	75
5.1		B	100
10.0		B	100
10.1		B	6
		A	94
20.5	Stop Run	B	6
		A	94

The analytical gradient and rinsing conditions are listed separately, as the rinsing protocol could be performed either directly as an integrated part of the elution protocol, or separately, for instrument configurations incorporating a second pump and column switching capabilities. Optimal performance was achieved by performing the rinsing protocol directly following the analytical gradient.

Preparation of reagents and solutions

Samples

For method development, commercially available milk and dairy products, as well as quality control materials (QSE GmbH, Germany, P/N 6017), were used.

Sample preparation (Carrez precipitation)

To begin, 1.5 g of the homogeneous sample was weighed into a 100 mL volumetric flask, and 70 mL of DI water were added. Then, 1 mL of Carrez I solution and 1 mL Carrez II solution were added, shaking after each addition, and the solution was brought to volume with DI water. The solution was filtered using filter paper to remove precipitates, such as proteins and other high molecular weight compounds, and the resulting solution filtered again through a syringe filter (0.45 µm) and diluted appropriately.

Eluent solutions

The general procedure for eluent preparation is described in Technical Note 71.[10] Plastic laboratory equipment, including pipettes and bottles, was used for the preparation and handling of all eluents.

Eluent 1 (0.2 M NaOH)

To prepare 1 L of the 0.2 M sodium hydroxide solution, 10.4 mL of sodium hydroxide (w(NaOH) = 50%) was transferred by pipette into 1 L of degassed DI water. This solution was immediately transferred to the plastic eluent bottle on the HPAEC-PAD system and blanketed with nitrogen at 14 kPa (2 psi).

Eluent 2 (0.1 M NaOAc)*

To prepare 1 L of the 0.1 M sodium acetate solution, 8.20 g of high-purity anhydrous sodium acetate was dissolved in approximately 800 mL of DI water. This solution was filtered under vacuum through a 0.2 µm nylon filter to remove particles introduced from the sodium acetate. After filtration, the solution was brought to volume and immediately transferred to the plastic eluent bottle on the HPAEC-PAD system and blanketed with nitrogen at 14 kPa (2 psi).

**Note: It is usually not advisable to use NaOAc-eluents without NaOH added as this could potentially lead to biological contamination of the instrument.[10] In this approach, however, the water used to prepare the solution contained a small amount of NaN_3, preventing the growth of mold and bacteria (see Reagents). The presence of NaN_3 in eluents can cause a small increase in background signal and noise.*

Eluent 3 (0.2 M NaOH in 1 M NaOAc)

To prepare 1 L of this eluent solution, 82.04 g of sodium acetate was dissolved in approximately 800 mL of DI water. This solution was filtered under vacuum through a 0.2 µm nylon filter to remove particles introduced from the sodium acetate. After filtration, the solution was transferred to a plastic 1 L volumetric flask, 10.4 mL of NaOH (w(NaOH) = 50%) added, and the solution brought to volume with DI water. This solution was immediately transferred to a plastic eluent bottle on the HPAEC-PAD system and blanketed with nitrogen at 14 kPa (2 psi).

Working standard solutions

To prepare the initial stock solutions (1 g/L), the appropriate amount of carbohydrate was dissolved in DI water. The stock solutions were mixed and diluted in DI water to obtain the working standards (0.1–20 mg/L, six levels). The ready-to-use stock solutions and working standard solutions were stored at 4 °C and remained stable for several weeks.

Results and discussion

External calibration was carried out using aqueous standards in the concentration range between 0.1 and 20 mg/L. Table 3 summarizes representative calibration data, showing a high correlation between the experimental data and the chosen calibration model ($r^2 > 0.999$). All components of interest eluted off the column within 15 min after injection (Figure 1). Throughout the studies reported in this application note the gradient was maintained for 21 min before initiating the rinsing step (Table 1). Further reduction of cycle time may potentially be achieved by performing the column rinsing protocol at 15 min.

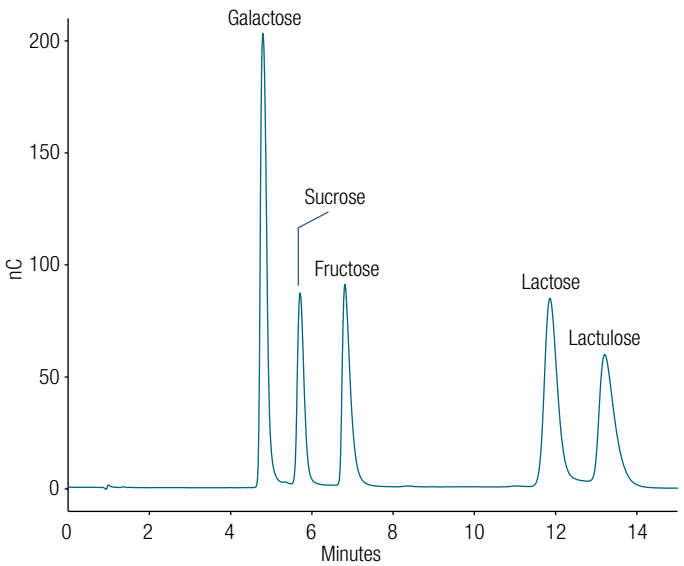

Figure 1. Standard solution containing mono- and disaccharides at a concentration of 10 mg/L each

Table 3. Representative calibration data. Calibration range is 0.1–20 mg/L for all carbohydrates.

Peak Name	Cal. Type	Eval. Type	Number of Levels	Relative Standard Deviation	Coefficient of Determination (r^2)
Galactose	Quad, AddZero, Avg	Area	7	3.5	0.9994
Sucrose	Quad, AddZero, Avg	Area	7	0.97	0.9999
Fructose	Quad, AddZero, Avg	Area	7	1.58	0.9999
Lactose	Quad, AddZero, Avg	Area	7	1.99	0.9998
Lactulose	Quad, AddZero, Avg	Area	7	0.45	1.0

The applicability of the fast method was tested by analyzing qualified control standards (Figure 2) with a specified lactose content of 0.04%. Recovery compared to this value was 98%.

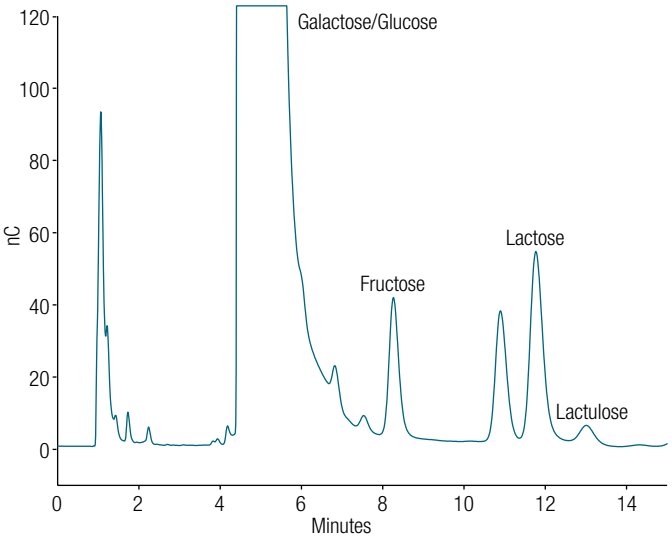

Figure 2. Fast determination of lactose in a control sample with a specified lactose content, w(lactose monohydrate) = 0.04%, recovery = 98%

Retention time precision, tested with relevant commerical samples (Figure 3), showed RSDs below 0.4%. The robustness of the chromatographic separation was evaluated by comparing the retention times of lactose for samples on two different column sets (guard and separator columns). The deviation of the retention time from the average value was approximately 3%. Due to the high reproducibility of the retention times and negligible impact on the peak characteristics, quantification was possible with just one calibration. Repeat analysis of the sample shown in Figure 2 on two different columns (n = 10) revealed an analytical precision of ±4–5% for the lactose content (w(lactose monohydrate) = 0.04 ± 0.002%). The respective evaluation for one column shows lower RSDs at a level of approximately 1.5%.

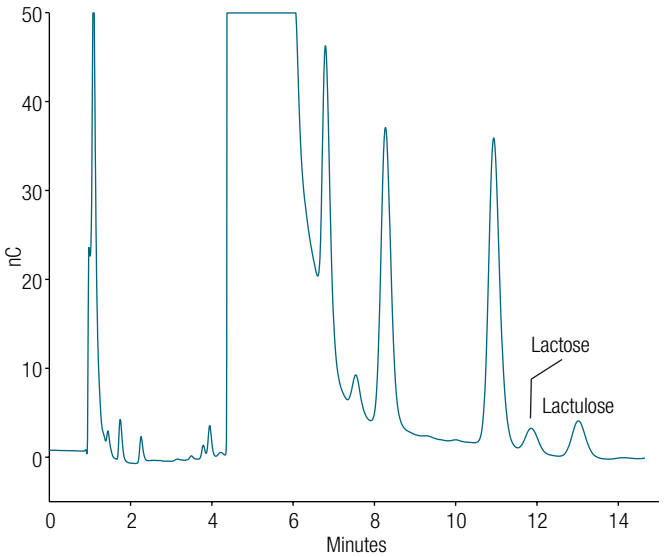

Figure 3. Lactose determination in lactose-free milk. w(lactose monohydrate) = 0.002% (RSD = 1.4 %), w(lactulose) = 0.004% (RSD = 1.7%); n = 5

The impact of different columns on the calibration stability was also evaluated. In this experiment, the same calibration experiments were repeated using both column sets, keeping the detection cell, working electrode, and reference electrode the same. The calibration model remained unchanged, and the slopes of the calibration functions, chosen for comparison, for all the carbohydrates shown in Figure 1 on both columns were within ±1% of the average value.

LOD and LOQ were calculated as $3 \times \sigma$ and $10 \times \sigma$, respectively, with σ being the standard deviation of 10 repetitive injections, using the summarized data from both column experiments. We chose to perform this evaluation using the data obtained from the injection of samples (Figure 3), rather than standard solutions. Based on the level of w(lactose monohydrate) = 0.002%, we estimated the LOD to be 0.0003% and the LOQ to be 0.0008%. The respective data for lactulose using the same sample are identical.

Figure 4 shows the determination of lactose and lactulose in cream cheese and lactose-free milk. For the milk samples, the values were comparable to the data shown in Figure 3, while the lactose monohydrate and lactulose content in the cream cheese were both approximately 0.004%.

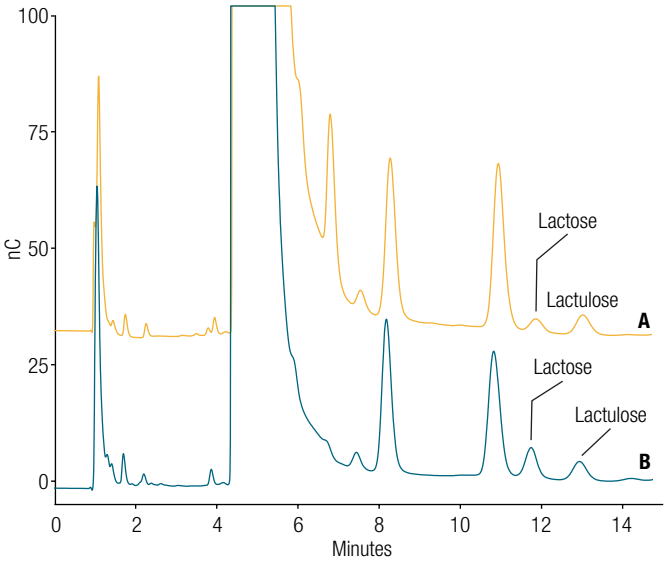

Figure 4. Determination of lactose and lactulose in lactose-free milk (A) and lactose-free cream cheese (B)

This method is also applicable for the determination of higher concentrations, as demonstrated in Figure 5 for the determination of lactose in whole milk. After purification of the sample and dilution, lactose was determined as the primary carbohydrate.

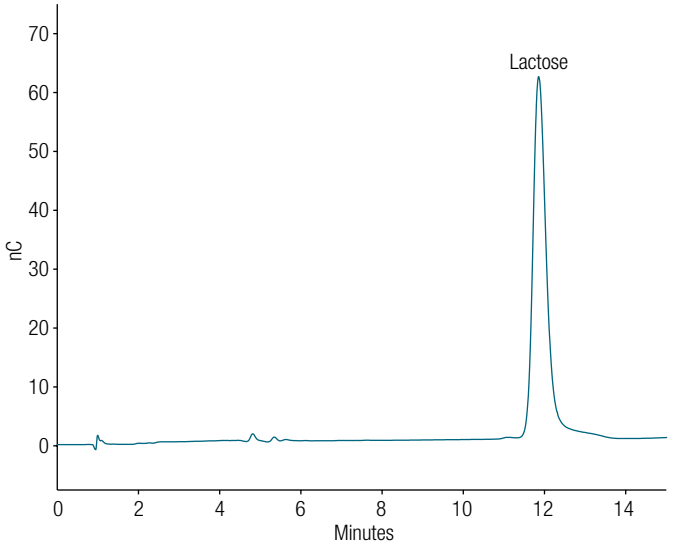

Figure 5. Determination of lactose in whole milk, w(lactose monohydrate) = 4.8%, RSD = 1.7%, n = 10

The method performs well for the determination of lactose in unripened or fresh cheese. The determination of lactose in matured cheese (e.g., hard cheese like Gouda, Emmental) should follow the method described in AN248[9] using a Dionex CarboPac PA20 (8 µm, 3 × 150 mm) column with higher column capacity to avoid an overestimation of lactose.

Conclusion

The method described in this application note allows the fast, sensitive, and accurate determination of lactose and lactulose in fresh dairy products and unripened cheese, including milk and cream cheese. The high reproducibility and sensitivity of the method allow the trace determination of both disaccharides with LODs and LOQs of less than 0.001% using the conditions described. Lower values are possible using larger injection volumes or lower dilutions of the sample. The sample analysis time, compared to previous approaches, was significantly reduced. For the analysis of lactose in matured products such as hard cheese, the method described in application note AN248 should be followed.

Acknowledgement

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