Application Note

Carbohydrate Analysis

The finest HPAEC-PAD Applications for Carbohydrate Analysis

Food and Beverage
Mono- and disaccharides
Sugars in meat & fish
Carbohydrates in food according to AOAC
Carbohydrates in instant coffee

Prebiotics Food Additives
Fructans in infant formula
TGOS in food products
Profiling of FOS

Lactose Free Products
Lactose in dairy & meat
Lactose in lactose-free products

Artificial Sweeteners
Sugar alcohols

Analysis of Lactose and Isomers in 'Lactose-free' Labelled Products

- **Fast and sensitive HPAEC-PAD analysis**
- **Lactose, allolactose, epilactose and lactulose**
- **SenCell™ with Au working electrode**
- **SweetSep™ AEX200 anion-exchange column**
- **Milk, quark, and latte**

Summary

Dairy products play a vital role in a healthy and balanced diet providing essential vitamins and minerals like calcium. Lactose intolerance is a widespread condition, which prevents a large number of people from consuming dairy products as a part of their daily diet. It is estimated that an average of 65% of the global population is suffering from lactose intolerance [1]. The global market for 'lactose-free' dairy products is rapidly growing and the criteria for 'lactose-free' labelled products are becoming stricter. In the EU for instance the threshold limit for lactose has been lowered to 10 mg per 100 g product in the last years in a number of EU member states [2]. To check the lactose contents in these products there is a need for fast, sensitive, and selective analytical methods.

In this application note a method is presented for fast and sensitive analysis of lactose and its isomers using the DECADE Elite electrochemical detector and SenCell. The method is based on separation by High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). The use of a SweetSep™ AEX200 column, which is a strong anion-exchange column with highly monodisperse 5 µm particles, allowed the separation of all compounds of interest in less than 8 min with high resolution. A variety of dairy products were analyzed to demonstrate the versatility of the method.

Introduction

Lactose is the main carbohydrate found in milk and dairy products (cow milk contains approximately 5% Lactose). It is a disaccharide composed of the monosaccharides D-glucose and D-galactose, joined in a ß-1,4-glycosidic linkage. In humans lactose is metabolized into glucose and galactose in the intestines by the enzyme lactase. Lactose intolerance is a condition caused by the inability to digest lactose due to a lactase deficiency. The most common symptoms of lactase deficiency are intestinal discomforts such as cramps, diarrhea, bloating, and gas. The reduction of lactase activity in humans starts already at infancy and might develop into a lactose intolerance during adulthood [3]. It is estimated that an average of more than 65% of the global population is suffering from lactose-intolerance. Rates of lactose intolerance vary between regions, from less than 10% in Northern Europe to as high as 95% in parts of Asia and Africa.

The global demand for 'lactose-free' dairy and other food products is rapidly growing and a large amount of commercial 'lactose-free' products are available nowadays. The majority of these products are produced by enzymatic hydrolysis using lactase-containing yeast (*Kluyveromyces* strains), resulting in a reduction of lactose concentration up to < 0.01%. Currently, there is no legislation in the US and EU concerning the lactose concentration limits in 'lactose-free' labelled products, except for infant formulae [4]. However, in many EU member states a lactose threshold level of 10 mg per 100 g of product is adopted for 'lactose-free' labelled dairy [2]. To check if products meet these requirements there is a need for fast, sensitive, and selective analytical methods to quantify lactose.

Besides lactose, dairy products can also contain lactose isomers, such as allolactose, epilactose, and lactulose. These isomers can be formed enzymatically [5] or by heat treatment such as pasteurization [6]. Lactulose is a recognized laxative and food additive for digestive comfort. Furthermore, both epilactose and lactulose are considered prebiotic lactose isomers. The presence of these isomers, with their small structural differences, hampers the quantification of such low levels of lactose. Good chromatographic separation is necessary to avoid coelution and thus overestimation of the lactose contents in dairy samples. A wide range of different methods are available to measure lactose in food products based on different techniques (enzymatic assays, mid infrared, gravimetry, differential pH, polarimetry, and HPLC), and most of them are lacking sufficient selectivity and sensitivity [7].

High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) is the method of choice. It combines superior selectivity with sensitive detection. The hydroxyl groups on carbohydrates can be oxidized, therefore, carbohydrates can be detected without derivatization using pulsed amperometric detection with pico- and femtomol sensitivity [8-10]. Several HPAEC-PAD methods for quantification of lactose are reported in the literatures based on conventional Anion-exchange columns with 6.5 - 10 µm particle sizes [11,12].

In this application note, a method is presented based on a new type of HPAEC column, SweetSep™ AEX200 with highly monodisperse 5 µm polymeric particles for the fast, selective, and sensitive analysis of lactose and its isomers.

Method

The HPAEC-PAD conditions are listed in Table 1. The analysis was performed using HPLC with a quaternary low-pressure gradient pump, autosampler, and thermostatted column compartment. For detection, a DECADE Elite electrochemical detector with a SenCell flow cell (see Figure 1) was used. The DECADE Elite was controlled via a PC using the DataApex™ Clarity™ Chromatography Data System (CDS) software version 8.8.0.78.

Figure 1: Left: SenCell with Au working electrode and Pd/H_2 (HyREF) reference electrode. Right: DECADE Elite electrochemical detector.

Separation

Under alkaline conditions (pH > 12) carbohydrates can be separated using HPAEC. Carbohydrates are weak acids with pKa values ranging between 12 and 14. At high pH, they will be either completely or partially ionized depending on their pKa value. Only polymeric anion-exchange columns are suitable for

Table 1

HPAEC-PAD conditions

LC system	Quaternary HPLC system equipped with column oven
Detector	DECADE Elite electrochemical detector
Columns	SweetSep™ AEX200, 4 × 200 mm column, 5 µm SweetSep™ BIT, 4 x 50 mm borate ion trap All columns: Antec Scientific
Mobile phase (MP)	MP A: 12 mM NaOH MP B: 12 mM NaOH + 10 mM NaOAc MP C: 100 mM NaOH + 100 mM NaOAc Eluents blanketed with Nitrogen 5.0
Flow rate	0.7 mL/min
System Backpressure	About 200 bar (during isocratic elution)
Injection	10 µL
Temperature	30 °C for separation, 35 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF, AST 2
Potential waveform (4-step)	E1, E2, E3, E4: +0.1, -2.0, +0.6, -0.1 V ts, t1, t2, t3, t4: 0.2, 0.4, 0.02, 0.01, 0.07 s
I-cell	about 0.2— 0.4 µA
ADF	0.1 Hz
Range	5 µA/V

Table 2

Step-gradient program

Time (min)	*Mobile phase*	*Description*
0 - 8	12 mM NaOH + 3 mM NaOAc	Isocratic elution and detection
8 - 13	100 mM NaOH + 100 mM NaOAc	Column clean-up and regeneration
13 - 30	12 mM NaOH + 3 mM NaOAc	Equilibration, starting conditions

carbohydrate separation on this alkaline condition. The retention time of carbohydrates is inversely correlated with the pKa value and increases significantly with molecular weight. A strong anion-exchange column SweetSep™ AEX200 was chosen for the separation of lactose and isomers (Table 1). This column is based on a monodisperse 5 µm resin coated with quaternary amine functionalized nanoparticles. The high uniformity and monodispersity of the resin allow for fast and high-resolution separation of carbohydrates. A trap column was installed in the solvent line between the pump and autosampler to suppress the tailing of the fructose and lactulose peaks due to the complexation of these particular sugars with borate. The use of a pre-column filter is advised when using samples which might contain particulate matter.

The temperature for separation was set at 30 °C. The analysis is based on a step-gradient with a sodium acetate modifier, see Table 2. The use of sodium acetate in addition to sodium hydroxide reduces the retention time of all carbohydrates, resulting in a faster run time compared to without an acetate modifier. At a concentration of 12 mM NaOH, carbonate ions (CO_3^{2-}) present in the mobile phase will bind strongly to the active sites of the stationary phase resulting in a loss of retention and column efficiency. A column clean-up / regeneration step after isocratic elution with 100 mM NaOH and 100 mM NaOAc is therefore necessary to remove the bound carbonate ions and late eluting compounds like oligo-saccharides present in dairy samples. This regeneration step assures reproducible retention behavior for each run. The total cycle time for each run is 30 minutes.

For some samples, the use of sodium acetate is not always needed. In addition, it might be necessary to increase the time of the washing (and/or equilibration) step to avoid late eluting interference in subsequent runs. Note that all samples shown as examples in this application note are analyzed with a wash step of 5 min (Table 2). Under these conditions, all tested samples did not show any coeluting interferences nor late eluting interferences.

To minimize the introduction of carbonate ions in the mobile phase the eluents were carefully prepared manually using a carbonate-free 50% w/w NaOH solution (commercially available). The diluent was DI water (resistivity >18 MΩ.cm) which was sonicated and sparged with Nitrogen 5.0 prior to use. The mobile phase should be prepared in plastic bottles instead of glass to avoid release of silicate and borate ions. The appropriate amount of 50% w/w NaOH solution was carefully pipetted into the diluent under gentle stirring and Nitrogen sparging to prepare the required mobile phase solutions. The

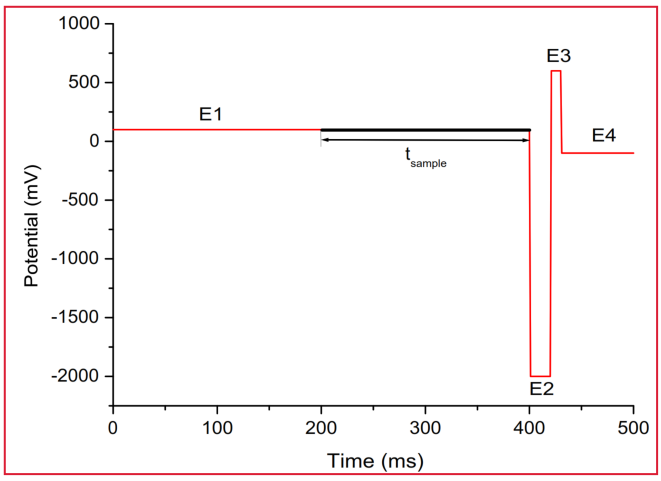

Figure 2: 4-step PAD potential waveform for the detection of monosaccharides and other carbohydrates.

bottles with mobile phase and column clean-up solution were blanketed with Nitrogen (0.5 bar overpressure) during the analysis to minimize the build-up of carbonate ions in the mobile phase and to assure a reproducible analysis.

Detection

For the pulsed amperometric detection of lactose and isomers, the Antec SenCell electrochemical flow cell is used. This novel flow cell [14] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd/ H_2) reference electrode (RE), and stainless steel auxiliary electrode (AE). A 4-step potential waveform was applied as shown in figure 2. The temperature for detection was set to 35°C. The cell current was typically about 0.2—0.4 µA with these PAD settings under the specified conditions. This particular 4-step waveform has the following benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [15], resulting in less flow cell maintenance and system downtime.

Preparation of standards, reagents and samples

Standards: 10 mM stock standards of the individual sugars were prepared in 95/5 (v/v%) water/acetonitrile. To prevent fast degradation 5% Acetonitrile was added to suppress bacterial and fungal growth. Stock standards under these conditions are stable for more than a month in the fridge at 4° C. Working standards in the concentration range of 100 nM—100 µM were prepared by dilution of the stock standards with DI water.

Carrez reagents: a Carrez clarification procedure is used for the deproteination of the dairy samples. By removing all proteins also the enzymatic activity will be quenched, eliminating any unwanted conversion of the sugars inside the sample during the analysis process. The Carrez I solution was prepared by dissolving 15.0 g potassium hexacyanoferrate(II) trihydrate in 100 mL of DI water in a volumetric flask. The Carrez II solution was prepared by dissolving 30.0 g zinc sulfate heptahydrate in 100 mL of water in a volumetric flask. Both Carrez solutions were filtered over a 0.2 µm syringe filter prior to use.

Sample preparation: The following lactose-free products were prepared and analyzed using the method described below:

- Semi-skimmed milk
- Quark
- Latte

Procedure:

1. 0.5 grams of dairy sample was weighed in a 50 mL volumetric flask and 10 mL DI water was added.
2. Subsequently, 100 µL Carrez I and 100 µL Carrez II reagent was added (the solution was shortly vortexed after each addition of reagent), followed by the addition of DI water up to a total volume of 50 mL.
3. The solution was allowed to stand for 30 minutes and vortexed again to obtain a homogeneously turbid solution.
4. A few milliliters of the turbid sample solution was centrifuged for 15 min in 2 mL Eppendorf vials at 6000 RPM.
5. The supernatant was collected in a plastic 5 mL syringe and filtered over a 0.22 µm PES (Polyethersulfone) syringe filter.
6. 10 µL of the filtered supernatant was injected into the LC system and analyzed.

Results

A chromatogram of 10 µL injection of a 10 µM standard mix is shown in Figure 3. Allolactose, lactulose, and epilactose are some lactose isomers which may occur during the heat / enzymatic treatment of dairy products [4]. Raffinose might be found in whole grain and cacao products. A concentration of 10 µM corresponds to 3.4 mg/L (ppm) of lactose and lactose isomers. All compounds of interest eluted within 8 minutes, and the total run cycle time is 30 minutes due to the wash and equilibration step. The analysis time is at least two times shorter than reported with other HPAEC columns [11,12], resulting in a significant improvement in sample throughput. The retention time of lactose was approximately 6.3 minutes.

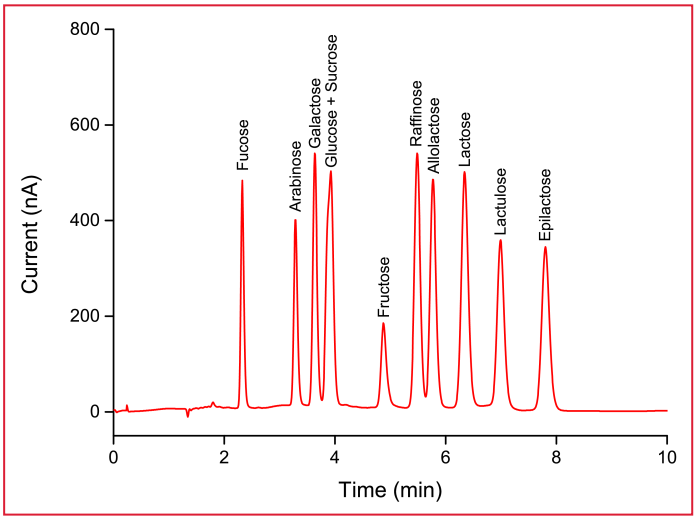

Figure 3: Chromatogram of a 10 µL injection 10 µM sugar standard mix containing fucose, arabinose, galactose, glucose, sucrose, fructose, raffinose, allolactose, lactose, lactulose, and epilactose.

Not all sugars are completely baseline separated, but under these conditions lactose and its isomers were baseline separated (resolution > 1.5). Therefore, quantification of lactose can be more reliable compared to the findings presented in reference [13] in which baseline separation of lactose and its isomers was not achieved.

The peak efficiencies found for the sugars ranged from 36,000 to 72,000 theoretical plates/meter (fucose and epilactose, respectively). The introduction of a borate trap between the pump and the injector successfully suppressed the tailing of fructose and lactulose. Only fructose showed a slight tailing (tailing factor 1.3) while the rest of the sugars does not show any significant tailing (tailing factor between 1.0—1.2).

Linearity

The linearity was investigated in the concentration range of 100 nM - 100 µM for lactose and its isomers. This corresponds with a concentration range of 34 µg/L - 34 mg/L for lactose and its isomers. In the concentration range of 100 nM—40 µM, the linearity is excellent and correlation coefficients for peak area were better than 0.999 for all 4 sugars. Above 40 µM the correlation coefficients for peak area were lower for all 4 sugars, therefore, the concentration range of 100 nM—40 µM is used for further quantification. The calibration curves for lactose and the lactose isomers, used for the actual quantification of samples, are shown in Figure 4.

Repeatability

The repeatability of the method and system was evaluated by repetitive injections with a 0.1 µM, 1 µM, and 10 µM sugar standard (mix of 11 sugars), corresponding with a concentration of 0.034 mg/L, 0.34 mg/L, and 3.4 mg/L for lactose and the isomers, respectively.

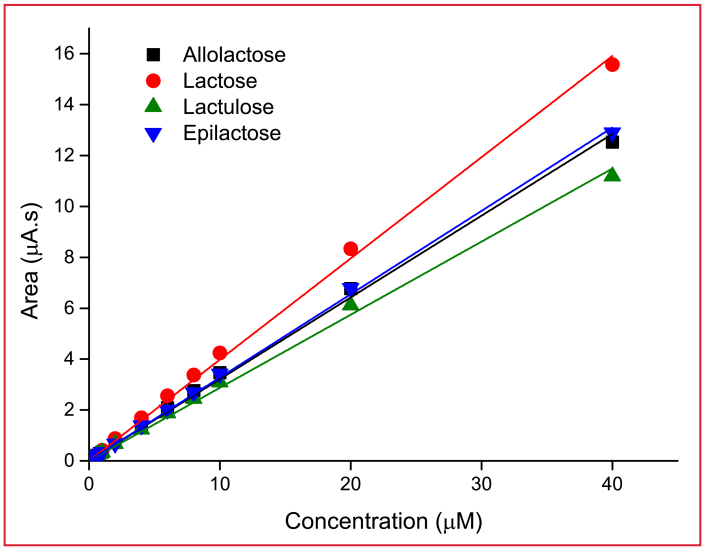

Figure 4: Calibration curve of allolactose, lactose, lactulose and epilactose in the concentration range of 0.25 - 50 mg/L.

Table 3

Repeatability of 10 µL injections of a 10, 1 and 0.1 µM sugar standard mix in water (n=10)

	RSDs (%) 10 µmol/L		RSDs (%) 1 µmol/L		RSDs (%)* 0.1 µmol/L	
Compound	t_R	*Area*	t_R	*Area*	t_R	*Area*
Fucose	0.30	0.57	0.25	1.79	0.29	2.01
Arabinose	0.21	0.43	0.19	1.15	0.21	2.23
Galactose	0.19	0.30	0.17	0.55	0.18	2.09
Fructose	0.14	0.78	0.15	1.62	0.17	1.75
Raffinose	0.15	0.56	0.19	1.08	0.21	1.59
Allolactose	0.13	0.48	0.16	0.44	0.18	1.24
Lactose	0.12	0.35	0.14	1.03	0.17	2.40
Lactulose	0.11	0.77	0.13	1.89	0.16	2.22
Epilactose	0.11	1.31	0.13	1.46	0.16	0.81

*) n=5 for the repeatability test with the 0.1 µmol/L standard. Glucose and sucrose were omitted due to coelution.

The relative standard deviations (RSD) for retention time and peak area for the different concentrations of sugar standards are listed in Table 3. The good repeatability of the method is evident from the low RSD values obtained. RSDs for retention time were < 0.3%. For peak area, the RSDs were < 2% for all sugars in the 10 µmol/L standard and about 2% for most of the sugars in the 100 nmol/L standard. It is important to keep in mind that although linear responses were obtained from 100 nmol/L up to 40 µmol/L, the determination of lactose contents at a concentration of around 100 nmol/L may not be as reproducible as at a concentration of around 1 µmol/L or 10 µmol/L. For samples with high lactose concentrations, sample dilution is recommended. Nevertheless, these data demonstrate that with this method reproducible analysis of lactose and related sugars can be achieved.

LODs and LOQs

The Limit of Detections (LODs) for all sugars are shown in Table 4 in µg/L (ppb) and nanomolar concentrations. The LODs were calculated as the analyte response corresponding to 3× the ASTM noise (average peak-to-peak baseline noise of 10 segments of 0.5 min). The noise was calculated based on a 5-minute section of the baseline. The average responses of 5 replicate injections obtained with a 100 nmol/L standard mix in the 5 µA/V range were used to calculate the LODs for all sugars.

Table 4

Limit of Detection (LOD) and Limit of Quantification (LOQ)

	Limit of Detection (LOD)		*Limit of Quantification (LOQ)*
Compound	*µg/L (ppb)*	*nmol/L*	*µg/L (ppb)*
Fucose	0.7	4.3	2.3
Arabinose	0.8	5.6	2.7
Galactose	0.7	3.9	2.3
Fructose	2.2	12.5	7.3
Raffinose	1.9	3.8	6.3
Allolactose	1.5	4.4	5.0
Lactose	1.5	4.3	5.0
Lactulose	1.2	3.5	4.0
Epilactose	2.2	6.6	7.3

* Glucose and sucrose were omitted due to the coelution.

The excellent sensitivity of the method is evident in Table 4. Detection limits around 10 nmol/L are attainable for most of the sugars. The calculated LOQ (10× S/N) for lactose is approximately 5.0 µg/L, which is a factor 200 below the upper limit of the lactose concentration expected in samples of 'lactose-free' labelled products.

Sample analysis

In total 3 commercially available 'Lactose-free' labelled products were purchased from supermarkets in the Netherlands and analyzed using the presented method, see Table 5.

Table 5

'Lactose-free' labelled products

Product	*Lactose content on the product label*
Semi-skimmed milk	Lactose < 10 mg / 100 mL
Quark	Lactose < 10 mg / 100 g
Latte	Lactose < 10 mg / 100 g

The lactose contents specified on the product labels are all < 10 mg Lactose per 100 g or mL of product. All samples were prepared following the sample preparation procedure described in the method section. The contents of lactose and lactose isomers in the samples were determined in two different ways, using:

- Calibration curve based on standards (0.25 - 50 mg/L)
- Standard addition method

The quantification with the standard addition method was based on a single point calibration by spiking the sample in the first dilution step during sample preparation with a known

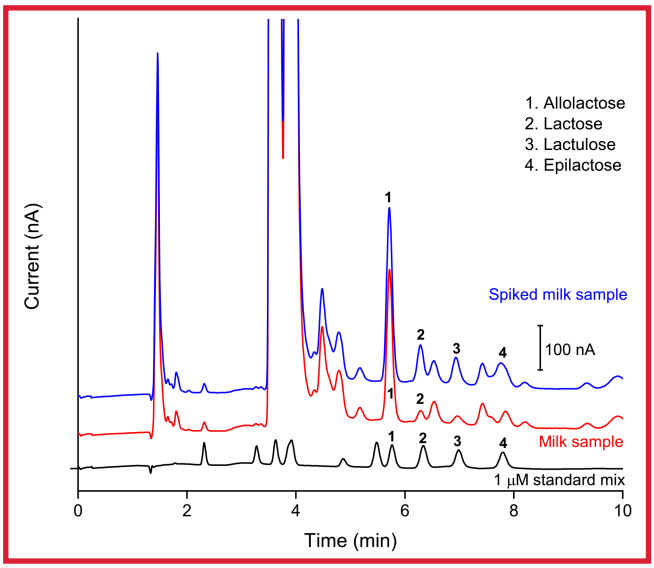

Figure 5: Chromatogram of the lactose-free milk sample (red), spiked milk sample (blue) and 1 µM standard mix (black).

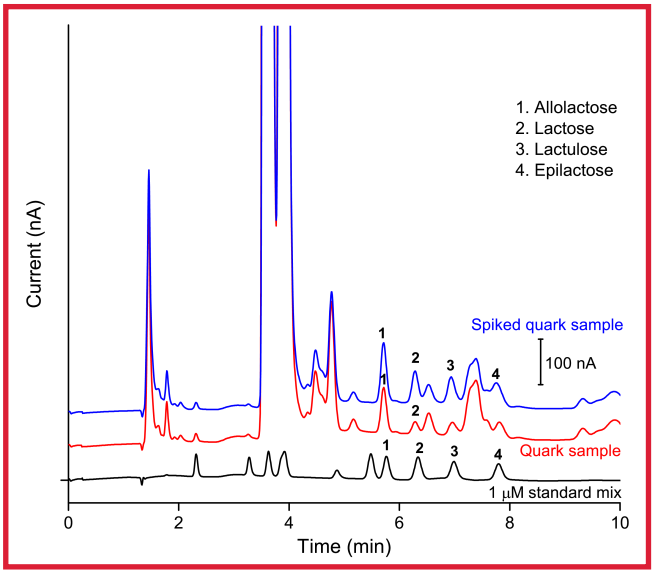

Figure 6: Chromatogram of the lactose-free quark sample (red), spiked quark sample (blue) and 1 µM standard mix (black).

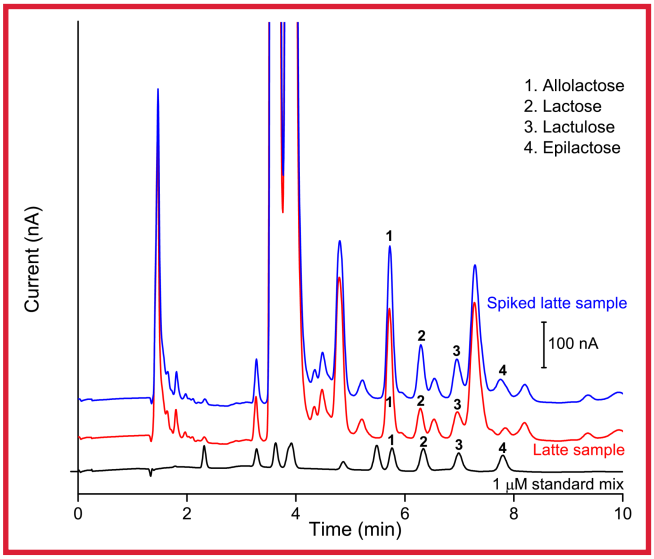

Figure 7: Chromatogram of the lactose-free latte sample (red), spiked latte sample (blue) and 1 µM standard mix (black).

amount of a standard containing allolactose, lactose, lactulose, and epilactose. The spike concentration was 1 µM (0.34 mg/L) for all 4 components in the final sample.

By using standard addition the method accuracy could be assessed, by calculating the sample recovery based on the responses of the analytes in the sample, spiked sample, and 1 µM standard.

$$\text{Recovery (\%)} = 100\% * \frac{\text{Area}_{\text{spiked sample}} - \text{Area}_{\text{sample}}}{\text{Area}_{\text{standard}}}$$

The chromatograms of the samples are shown in Figure 5 - 7. Every figure contains an overlay of chromatograms of the sample (red), spiked sample (blue), and the 1 µM standard (black). The lactose and lactose isomers-related compounds present in the specific samples are marked with a number above the peaks, corresponding with the legend in the top right corner of the plot.

Comparisons between the chromatograms of samples and spiked samples show unidentified peaks that coelute with lactulose and epilactose. Based on these comparisons, epilactose was not found in any of the analyzed samples and lactulose was present only in the latte sample (Figure 7). The concentration of lactulose in the sample was calculated based on standard addition and corresponds to 38 mg lactulose per 100 gram product (recovery 86.5%). The milk sample and quark sample show an unidentified interference that coelutes closely to lactulose. For lactose and allolactose, which are present in the majority of the 'lactose-free' products, the amounts found in the analyzed samples are listed in Table 6.

Table 6

Lactose & allolactose, contents and sample recovery

Product	*Lactose*		*Allolactose*	
	mg per 100 g product	*Recovery (%)*	*mg per 100 g product*	*Recovery (%)*
Semi-skimmed milk UHT	1.2	99.5	27.3	109.5
Quark	0.5	80.5	7.2	93.8
Latte	3.8	97.1	24.4	102.5

The lactose and allolactose contents in Table 6 were calculated using the standard addition method (single point calibration). There was a good correlation between the values in Table 6 and the concentrations calculated based on the calibration curve. The sample recovery found for lactose ranged between 80% - 100%. The lactose contents in all the 'lactose-free' products analyzed with the presented HPAEC-PAD method, fall within the specified limit of < 10 mg/100 g listed on the product labels.

Conclusion

The presented HPAEC-PAD method allows fast separation (within 8 min) of lactose and its isomers with high resolution in commercial lactose-free products. The new SweetSep™ AEX200 HPAEC column demonstrates efficient separation and excellent performance for the quantification of lactose and its isomers.

Lactose contents in three different commercial lactose-free products fall within the specified limit of lactose (< 10 mg / 100 g product) listed on the product labels. Overall, the presented method is reliable and robust for lactose analysis in lactose-free products.

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Figure 8: Recommended instrument configuration for this application: the ALEXYS Carbohydrate Analyzer
The system consists of a P6.1L pump with integrated Solvent Switch Valve (SSV) capable of running step gradients, an AS6.1L autosampler, an ET 210 Eluent tray for nitrogen sparging and blanketing, a CT 2.1 column oven with broad temperature range, and the DECADE Elite electrochemical detector. The ALEXYS Carbohydrate Analyzer can be operated under DataApex™ Clarity™ CDS (version 8.3 and up) or Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software (version 7.2 SR 5 and up).

Ordering information

Detector only	
176.0035B	DECADE Elite SCC electrochemical detector
116.4321	SenCell 2 mm Au HyREF
Recommended ALEXYS analyzer	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
Column	
260.0010	SweetSep™ AEX200, 4 x 200 mm column, 5 µm
260.0030	SweetSep™ BIT, 4 x 50 mm borate ion trap
260.0100#	Pre-column filter PEEK, 0.5 µm
Software*	
195.0035	Clarity CDS single instr. incl LC, AS module

#) In case samples might contain particulate matter it is advised to use a pre-column filter.

*) The ALEXYS Carbohydrates Analyzer can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. For the DECADE Elite electrochemical detector only also control drivers are available in Waters Empower™, Agilent OpenLab CDS and Agilent OpenLab CDS Chemstation Edition. Please contact Antec for more details.

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system and DECADE Elite detector. The actual performance may be affected by factors beyond Antec's control and may be adjusted accordingly. Specifications mentioned in this application note are subject to change without further notice.

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