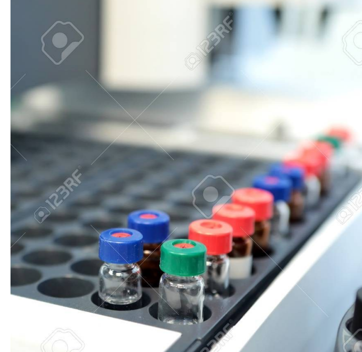
Antec® Scientific

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
Carbohydrates in Dutch candy
Carbohydrates in honey
Oligo– and Polysaccharides in honey
Sugars in beer

Prebiotics Food Additives
Analysis of Maltodextrin in Syrups
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
Sucralose

Glycoproteins
N-glycans
Monosaccharides
Sialic acids

High-throughput lactose analysis

- **ALEXYS™ High-throughput Carbohydrate Analyzer**
- **SweetSep™ AEX200 anion-exchange column**
- **Fast, sensitive, and accurate analysis**
- **Lactose and isomers in commercial lactose-free products**

Summary

High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) is a well-established technique for sensitive carbohydrate analysis, routinely used in testing and quality control laboratories. In this application note, analysis of lactose and its isomers in 'lactose-free' labelled products is demonstrated using a high-throughput HPAEC-PAD method that utilizes a dual-column configuration with a switching valve to alternate between 'online' analysis and 'offline' regeneration step. A fast cycle time of only 15 minutes per sample was achieved, effectively doubling sample throughput compared to conventional single-column methods described in the application note 220_009 [1].

The method was evaluated using the ALEXYS™ High-throughput Carbohydrate Analyzer and the 2.1 mm ID SweetSep™ AEX200 column, which is optimized for the high-resolution separation of lactose and its isomers, including allolactose, lactulose, and epilactose. Several commercially available 'lactose-free' labeled products were analyzed to demonstrate the method's suitability for routine analysis of lactose in complex matrices. The results highlight the method's applicability in delivering a high throughput, selective, and sensitive analysis of lactose and its isomers.

Introduction

Routine analysis in many laboratories, including contract research organizations (CROs) and quality control laboratories, demands high throughput to efficiently process the large sample quantities. These laboratories must consistently deliver reliable and reproducible results within a very short turnaround time to meet both the customer demands and regulatory requirements. Several strategies have been developed to improve sample throughput, including optimizing sample preparation and reducing the analysis time. This application note demonstrates the use of the method described in the technical note 220_043, where one effective approach involves using two columns in alternating manner to reduce the analysis time [2].

While throughput is important, maintaining high sensitivity and accuracy is equally critical. High-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) is one of the most sensitive analytical technique suitable for carbohydrate analysis. Typically carbohydrates can be detected using HPAEC-PAD up to pico– and femtomole sensitivity [3]. The sensitivity of the PAD method is vital for trace-level analysis of carbohydrates such as lactose in lactose-free labelled products. In the European Union (EU), the threshold limit for lactose in these products is set to 10 mg per 100 g product [4—5]. Therefore, HPAEC-PAD is the method of choice for sensitive analytical methods for quantification of lactose.

Dairy products may also contain lactose isomers such as allolactose, lactulose, and epilactose. These isomers can be formed enzymatically [6] or by heat treatment such as pasteurization [7]. Due to their small structural differences, these isomers are challenging to separate and can potentially interfere with lactose quantification. Therefore, a highly selective chromatographic separation is necessary. In this application, the Antec Scientific SweetSep™ AEX200 200 × 2.1 mm ID analytical column is employed to achieve high-resolution separation of lactose and its isomers [8]. The microbore column format minimizes both mobile phase and sample consumption, aligning with the green analytical chemistry principle. In this application, a fast, selective, and sensitive method for analyzing lactose and its isomers in various lactose-free labelled products is demonstrated using the ALEXYS High-throughput Carbohydrate Analyzer (Figure 1).

Figure 1. ALEXYS High-throughput Carbohydrate Analyzer. The CT2.1 column thermostat is an optional part.

Method

Instrumentation

The analysis of lactose and its isomers was performed using the ALEXYS™ High-throughput Carbohydrate Analyzer as shown in Figure 1. This dedicated HPAEC-PAD system consists of the ET210 eluent tray (for storage and N_2 blanketing of mobile phases during the analysis), two P6.1L quaternary LPG pumps, AS6.1L autosampler, an optional CT2.1 column thermostat, and the DECADE Elite electrochemical detector. The SenCell™ with Au working electrode and HyREF (Pd/H_2) reference electrode was selected for detection of the carbohydrates. A detailed description of the high-throughput method can be found in Technical Note 220_043 [2].

Separation

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. Therefore, under alkaline conditions (pH > 12) carbohydrates can be separated by means of HPAEC [9]. Due to the extreme alkaline conditions, only polymeric anion-exchange columns are suitable for separation of the carbohydrates. The separation of the lactose isomers and several other sugars were carried out using the SweetSep™

Table 1

HPAEC-ECD Conditions

HPLC system	ALEXYS™ High-Throughput Carbohydrate Analyzer, SCC (Antec Scientific)
Columns	2 x SweetSep™ AEX200, 2.1 x 200 mm analytical column, 5 µm 2 × Pre-column filter PEEK, 0.5 µm 2 × Borate ion trap, 2.1 x 50 mm column, 10 µm (all columns from Antec Scientific)
Mobile phase (MP)	A: DI Water B: 100 mM NaOH C: 200 mM NaOH + 500 mM NaOAc D: 200 mM NaOH Eluents blanketed with Nitrogen 5.0
Flow rate	0.18 mL/min
Back pressure	about 130—150 bar
Injection	3 µL
Temperature	40 °C for separation, 35 °C for detection*
Flow cell	SenCell with Au WE, stainless steel AE and HyREF Palladium RE, AST 2
Potential waveform	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 0.1— 0.3 µA
ADF	0.1 Hz
Range	2 µA/V

*In the case the system is not equipped with an optional column thermostat, the columns can be installed in the detector oven compartment set to 40°C for separation and detection.

Table 2

Gradient program ('online' column, elution)

Time (min)	*Mobile phase*	*%A*	*%B*	*%C*	*%D*	*Description*
0—15	12 mM NaOH	88	12	0	0	Elution & detection
14.98	Valve position change					

Table 3

Gradient program ('offline' column, wash & equilibration)

Time (min)	*Mobile phase*	*%A*	*%B*	*%C*	*%D*	*Description*
0	12 mM NaOH	88	12	0	0	Start
0.02—3	200 mM NaOH + 100 mM NaOAc	0	0	20	80	Column clean-up and regeneration
3.02—15	12 mM NaOH	88	12	0	0	Equilibration to starting conditions
14.98	Valve position change					

AEX200 columns (2.1 × 200 mm analytical column), which are anion-exchange columns containing highly monodisperse 5 µm ethyl vinylbenzene-divinylbenzene copolymer (80% crosslinked) coated with quaternary amine functionalized nanoparticles [8]. In addition, borate ion trap columns (2.1 × 50 mm) were installed in the solvent line between the pump and autosampler/switching valve as a precaution to eliminate borate ions from the mobile phase.

The separation of lactose and its isomers was performed following the isocratic elution program described in Table 2. A column clean-up/regeneration step (Table 3) is employed after the elution step. This regeneration step is kept for 3 min to elute any strongly retained components and to remove carbonate ions (CO_3^{2-}) buildup on the column. After the clean-up step the column is equilibrated for 12 minutes to the starting conditions, resulting in a cycle time of only 15 minutes per sample. The optimal separation temperature was found to be 40°C at this condition.

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 and electrochemical grade sodium acetate salt (all commercially available). The diluent was DI water (resistivity 18.2 MΩ.cm, TOC <5 ppb), which was sparged with Nitrogen 5.0 (purity 99.999%). During analysis the mobile phase headspace is also blanketed with Nitrogen gas (0.2—0.4 bar N_2 gauge pressure) using the ET210 eluent tray. The inert gas atmosphere will prevent the introduction of CO_2 (from the air) into the mobile phase and the subsequent formation of CO_3^{2-} ions, ensuring reproducible analysis.

Detection

For the pulsed amperometric detection of the analytes, the Antec SenCell™ electrochemical flow cell was used. This flow cell [10] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd) reference electrode (RE) and stainless steel auxiliary electrode (AE). A 4-step potential waveform was applied as described in Table 1. The temperature for detection was set to 35°C. The cell current was typically about 0.1—0.3 µA using these PAD settings under the specified conditions. This particular 4-step waveform with a pulse duration of 500 ms has several benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [11], resulting in less flow cell maintenance and system down time.

Preparation of standards, reagents and samples

Standards: stock standards of the 10 individual sugars were prepared by dissolving a known amount of the individual compounds in deionized (DI) water to a final concentration of 10 mM. Stock standards under these conditions are stable for more than a month in the freezer at -30°C. A combined stock standard solution with a concentration of 1 mM for each carbohydrate was prepared by mixing 100 µL of each individual standard. Working standards were prepared by serial dilution of the combined stock standards with DI water. The working standards were prepared in the concentration range of 0.1 - 80 µM.

Samples: the three lactose-free products analyzed in the application note 220_009 were re-analyzed using the high-throughput method. The details of the sample preparation is outlined in the application 220_009 [1]. The three products were semi-skimmed milk, quark, and coffee latte. Each sample was also spiked with a known amount of standard to ensure correct peak identification and to assess the method accuracy based on the sample recovery. The final concentration of standards spiked into each sample after dilution was 1 µM.

Results

An example chromatogram for each column obtained with the 3 µL injection of the 10 µM standard mix is shown in Figure 2. The method presented in this application note resulted in elution of all sugars in the standard mix within 10 minutes. The overall cycle time is reduced to 15 minutes per sample, which is twice as fast as the method presented in the application note 220_009 [1]. Most of the sugars, including lactose and its isomers were baseline separated (Rs > 1.5). Only galactose and sucrose coeluted under this condition. Both columns showed nearly identical retention times for all analytes, confirming the good batch-to-batch reproducibility of the SweetSep™ AEX200 columns. Raffinose, a trisaccharide frequently found in food

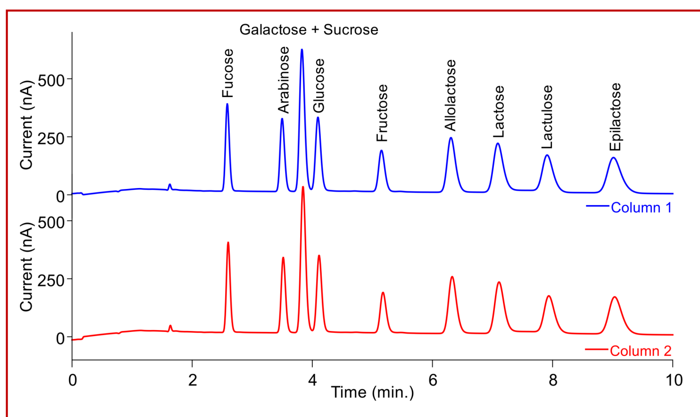


Figure 2. The chromatograms obtained from a 3 µL injection of a 10 µM standard mix in DI water on column 1 (blue curve) and on column 2 (red curve).

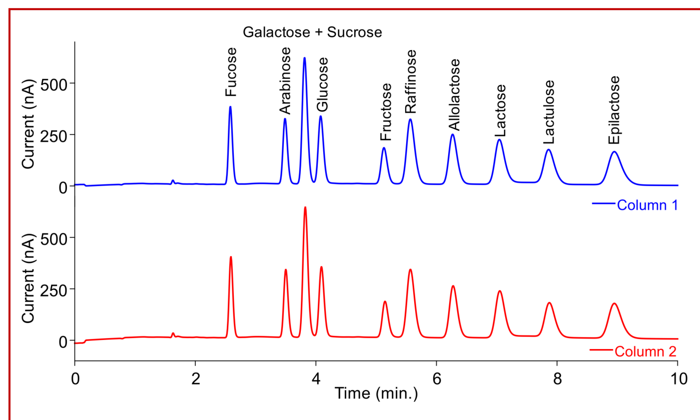


Figure 3. The chromatograms obtained from a 3 µL injection of a 10 µM standard mix including raffinose in DI water on column 1 (blue curve) and on column 2 (red curve).

matrices containing lactose, may interfere with the lactose analysis. However, under the conditions described in Tables 1–3, raffinose is baseline separated from other sugars and lactose isomers (Figure 3), ensuring lactose analysis without interference from raffinose.

Repeatability

The repeatability of the method was evaluated based on the relative standard deviation (RSD) of the retention times and peak areas. The RSD values were determined by 10 repetitive injections of the 0.1 µM, 1 µM, and 10 µM standard mix (excluding raffinose) in DI water. At 0.1 µM, the retention times for both columns were stable as shown in Table 4. The retention time RSD were ≤ 0.25% for all compounds at all concentrations, indicating excellent repeatability.

The RSD values for peak areas of all compounds at 0.1 µM were < 1.2%. The RSD of peak areas for all compounds decreased with increasing concentration; at 10 µM, the RSD values for all compounds were below 0.5% (data not shown). Both the RSD

Table 4

Repeatability of 10 µL injections of 0.1 µM standard mix in DI water (n = 10)

	RSD (%) Column 1		*RSD (%)* Column 2	
Compound	t_R	*Area*	t_R	*Area*
Fucose	0.1	0.86	0.14	0.95
Arabinose	0.11	0.67	0.12	1.23
Glucose	0.12	1.14	0.18	1.00
Fructose	0.16	1.04	0.14	1.17
Allolactose	0.19	0.74	0.21	0.89
Lactose	0.19	0.87	0.19	0.89
Lactulose	0.12	0.87	0.16	0.71
Epilactose	0.18	0.78	0.15	0.95

values for retention time and peak area are comparable to the single-column approach [1, 2]. These data demonstrate a remarkable repeatability, not only for the analysis of lactose and its isomers, but also for the other sugars (except for galactose and sucrose) using the presented method.

Linearity

The linearity was evaluated for all sugars except for galactose and sucrose (due to coelution) in the concentration range of 0.1—80 µM. For lactose and its isomers, this corresponds to a wide dynamic range equivalent to 34 µg/L—27 mg/L. Two sets of measurements were done (one for each column), and therefore two sets of calibration curves were evaluated. All calibration curves were fitted quadratically (ignoring the origin) with a weighting factor of 1/concentration^2. For fucose, arabinose, and glucose, signal saturation occurred above 60 µM (at the 2 µA/V range); therefore, these compounds were fitted only up to this concentration. Across both columns, the relative standard error of the calibration curves ranged between 1.3—2.7% for all compounds (Table 5). The relative residual error for all compounds in all calibration levels for both columns are within ± 4%, indicating excellent accuracy. At a larger current range setting of 20 µA/V, all compounds were linear up to 80 µM. However, at this range setting, the noise level is higher compared to at 2 µA/V by a factor of 2 - 2.5× (data not shown).

Limit of detection (LOD) and limit of quantification (LOQ)

The Limit of Detection (LOD) and Limit of Quantification (LOQ) for all compounds are shown in Table 6. 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 from t = 10 minutes to t = 15 minutes. The average response obtained with the 10 injections of the 0.1 µM standard mix was used to calculate the LOD and LOQ. The LOQs were calculated in a similar way as the LODs, based on the 10× S/N ratio. The calculated LODs for all sugars range between 0.9 to 4.7 ng/mL, and the calculated LOQs range between 3.0 to 18.2 ng/mL, demonstrating excellent detection sensitivity. Specifically for lactose, the calculated LOQs are 11.2 ng/mL and 11.5 ng/mL on column 1 and column 2, respectively. These values are approximately 85-fold below the upper limit of the lactose concentration expected in the lactose-free labelled products.

Table 5

Relative standard error of the calibration curves

Compound	*RSE (%)*	
	Column 1	*Column 2*
Fucose*	1.7	1.8
Arabinose*	1.6	1.6
Glucose*	1.7	2.1
Fructose	1.6	1.5
Allolactose	1.7	1.6
Lactose	1.3	1.8
Lactulose	2.2	2.7
Epilactose	1.8	1.7

*Fitted from 0.1—60 µM

Table 6

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

Compound	*Limit of detection*				*Limit of quantification*	
	Column 1		*Column 2*		*Column 1*	*Column 2*
	ng/mL	*nM*	*ng/mL*	*nM*	*ng/mL*	*ng/mL*
Fucose	0.9	5.6	0.9	5.6	3.0	3.0
Arabinose	1.0	6.6	1.0	6.7	3.3	3.3
Glucose	1.2	6.5	1.2	6.5	3.8	3.9
Fructose	2.1	11.6	2.4	13.5	7.0	8.1
Allolactose	2.9	8.6	3.2	9.2	9.8	10.4
Lactose	3.3	9.7	3.5	10.2	11.2	11.5
Lactulose	4.7	13.6	5.4	15.9	15.6	18.2
Epilactose	4.5	13.0	4.7	13.6	14.8	15.3

Sample analysis

Chromatograms of the lactose-free labelled products are shown in Figure 4, 5 and 6. Due to similarity in chromatograms

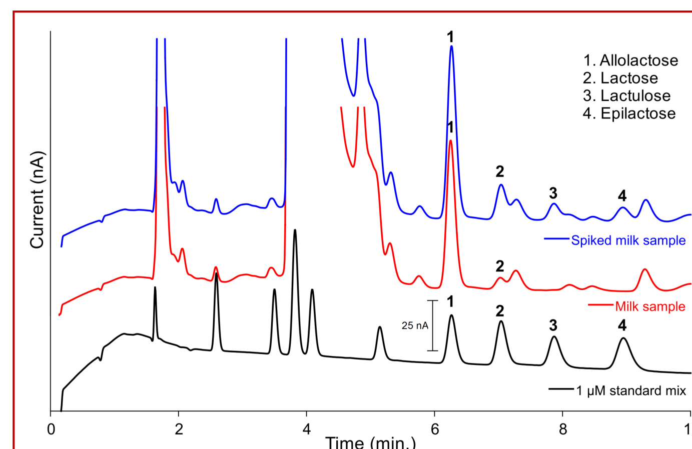


Figure 4. Chromatogram of the 1 µM standard mix (black), lactose-free semi skimmed milk sample (red), and spiked milk sample (blue). The final spike concentration is 1 µM.

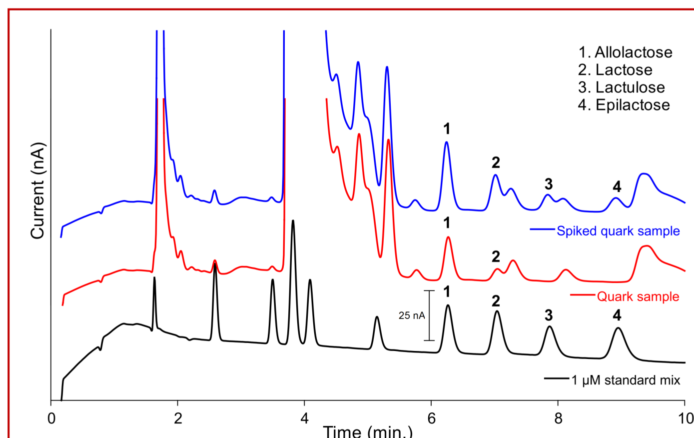


Figure 5. Chromatogram of the 1 µM standard mix (black), lactose-free quark sample (red), and spiked quark sample (blue). The final spike concentration is 1 µM.

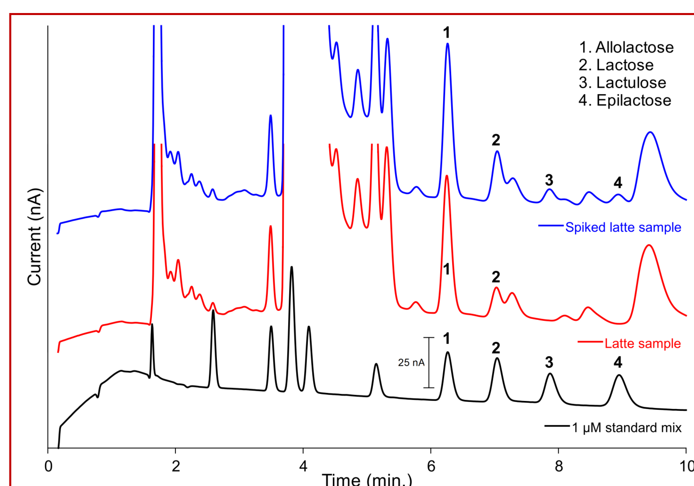


Figure 6. Chromatogram of the 1 µM standard mix (black), lactose-free latte sample (red), and spiked latte sample (blue). The final spike concentration is 1 µM.

from both columns, only one set of representative chromatograms was shown. To ensure correct peak identification, the samples were spiked with a known amount of standard containing the sugars of interest. The final spike concentration in the spiked sample was 1 µM for all sugars. Taking into account the dilution factor, this concentration corresponds to 3.4 mg / 100 g product, which is 3× below the upper limit of the regulatory threshold. Lactose and its isomer allolactose were detected in all samples. Quantification of lactose and allolactose was performed using the calibration curve based on the standards (0.1—80 µM). The amounts found in the analyzed samples were listed in Table 7. In all samples, lactose levels were below the regulatory threshold of 10 mg / 100 g product, consistent with the product labels.

During production of lactose-free products, lactose is often enzymatically processed to form galactose and glucose. However, allolactose may also form as a side product during this process, and is therefore present in all tested samples. Allolactose concentrations were quite consistent between the two columns. In contrast, slight differences in lactose concentrations were observed between the two columns. These differences may be attributed to a partially coeluting interference next to lactose, complicating the peak integration and quantification.

The sample recovery was evaluated to assess method accuracy, based on the amount of the analytes in the sample, spiked sample, and the amount of standard mix added to the spiked sample.

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

The sample recoveries are also listed in the Table 7. The sample recoveries found for all samples ranged between 80% - 105%, indicating excellent accuracy for all compounds analyzed in the sample using the presented method [12].

Quantification of lactose and allolactose was also performed with different calibration approaches to investigate the effect of having a separate calibration for each column. The different calibration approaches are the single calibration averaging the responses of both columns, and shared calibration across both columns (column 2 uses calibration of column 1 or vice versa). For lactose, the amounts determined using either calibration

Table 7

Lactose & allolactose, contents and sample recovery

Product	*Lactose*				*Allolactose*			
	Column 1		*Column 2*		*Column 1*		*Column 2*	
	mg/100 g product	*Recovery (%)*	*mg/100 g product*	*Recovery (%)*	*mg/100 g product*	*Recovery (%)*	*mg/100 g product*	*Recovery (%)*
Semi-skimmed milk UHT	2.2	105	1.5	100	20.4	102	20.7	102
Quark	1.9	103	1.5	100	5.8	101	6.0	100
Latte	6.2	94	4.0	88	19.0	99	18.9	80

model differ by only 0.1 mg/L compared to the separate calibration model shown in Table 7. The separate calibration model gave recovery values closest to 100%, while shared or averaged calibrations has ±3% recovery values differences (See example on Table 8) across both columns, indicating that the separate calibration model is the most accurate amongst the tested calibration approaches.

Overall, these results demonstrated that selective and sensitive high-throughput analysis of lactose and its isomers in lactose-free products was successfully achieved using the presented method with the ALEXYS carbohydrate analyzer.

Table 8

Lactose & allolactose in lactose-free quark sample recovery based on different calibration approaches

Compounds	Amount recovery on column 1 (%)			Amount recovery on column 2 (%)		
	Cal1	Cal2	Avg. cal	Cal1	Cal2	Avg. cal
Allolactose	101%	102%	102%	98%	100%	99%
Lactose	103%	106%	105%	98%	100%	100%

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Conclusion

A high-throughput HPAEC-PAD method for sensitive and selective analysis of lactose and its isomers in lactose-free labelled product was established using The ALEXYS™ Carbohydrate Analyzer in combination with the SweetSep™ AEX200 HPAEC column. The total cycle time was reduced to just 15 minutes per run, doubling sample throughput compared to conventional single-column approach. The results showed excellent repeatability, linearity, and sensitivity, with LOQs for lactose approximately 85-fold lower than the upper limit of the regulatory threshold (10 mg/100g). Successful analyses of several lactose-free labelled products indicated that the presented method is highly suitable for routine quality control and compliance testing in food laboratories.

Ordering information

ALEXYS analyzer	
180.0059WA	ALEXYS High-throughput Carbohydrate Analyzer, SCC
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
Columns	
260.0011	SweetSep™ AEX200, 2.1 x 200 mm column, 5 µm
260.0031	Borate ion trap, 2.1 x 50 mm column, 10 µm
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.

#) Antec ECD drivers are available for Chromeleon CDS, OpenLAB CDS and Empower CDS. The ALEXYS Carbohydrates Analyzer (full system) can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

Reagents, standards and sample prep accessories

NaOH 50%, carbonate –free	Fisher Scientific, pn SS254-500
Sodium acetate (NaOAc), 100%	Sigma Aldrich, pn 79714
DI water 18.2 MΩ.cm, TOC < 5 ppb	YoungIn Chromass Aquapuri Essence+ 393
Fucose	Sigma Aldrich, pn F2252-5G
Arabinose	Sigma Aldrich, pn A3131
Galactose	Sigma Aldrich, pn G0750
Sucrose	Sigma Aldrich, pn S9378
Glucose	Sigma Aldrich, pn G8270
Fructose	Sigma Aldrich, pn F0127
Allolactose	Carbosynth, pn OG09259
Lactose	Carbosynth, pn OL04771
Lactulose	Sigma Aldrich, pn 61360-5G
Epilactose	Carbosynth, pn OG04727
Potassium hexacyanoferrate(II) trihydrate	Fluka, pn 60280
Zinc sulfate heptahydrate	Sigma Aldrich, pn 31665-500g-M

For research purpose only not for use in diagnostic procedures. 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. Specifications mentioned in this application note are subject to change without further notice.

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