Application Note

Food, Beverage,

Life Sciences

The finest LC-EC Applications for Food & Beverage analysis

Phenols
Bisphenol A
Catechins
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Other polyphenols

Carbohydrates
Monosaccharides
Lactose
Mono- and Disaccharides
Other oligo- and Polysaccharides

Vitamins, minerals etc.
A, C, D, E, and K
Iodide
Q10, ubiquinols

Analysis of lactose and isomers in dairy and meat products

- **ALEXYS Carbohydrates Analyzer**
- **Fast and sensitive HPAEC-PAD analysis**
- **Lactose, allolactose, epilactose and lactulose**
- **Dairy, meat, and meat analogues**
- **SenCell™ with Au working electrode**

Summary

Dairy products play a vital role in a healthy and balanced diet providing essential vitamins and minerals like calcium. Lactose-intolerance is a wide-spread condition, which prevents a large number of people of consuming dairy products as a part of their daily diet [1]. The global demand for low-lactose food products is rapidly growing and a large number of commercial 'lactose-free' product are available nowadays. While commonly found in dairy products, lactose can also be present in processed food products such as deli meats and meat analogues [2]. High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) is a fast, sensitive and selective analytical method to quantify the lactose contents in such products [3].

This application note is complementary to the previously published application note 220_009 - Analysis of Lactose and Isomers in 'Lactose-free' Labelled Products [3]. In application note 220_009 a fast, reliable, and sensitive method is presented for the analysis of lactose and isomers using the DECADE Elite electrochemical detector and SenCell in combination with a narrow-bore (2mm ID) anion-exchange column with 4 µm particle size. In this publication the same method is evaluated on the new Antec ALEXYS Carbohydrates Analyzer in combination with a standard-bore (4mm ID) anion-exchange column. The ALEXYS Carbohydrates Analyzer is a dedicated LC system solution for the quantification of lactose in 'lactose-free' products. A variety of commercial food samples including dairy, meat and meat analogues were analysis to demonstrate the performance and versatility of the system.

Introduction

Lactose is a disaccharide composed of the monosaccharides D-glucose and D-galactose, joined in a ß-1,4-glycosidic linkage. It is the main carbohydrate found in milk and most dairy products. The concentration of this disaccharide depends on the origin of the milk, and ranges from 7.0 mg/100mL in human breast milk to 4.2 mg/100 ml in goat milk [4]. To digest and absorb lactose from the digestive system it is hydrolyzed into glucose and galactose in the intestines by the enzyme lactase.

While lactose is the most important energy source during the first year of human life, most humans cease to produce the enzyme lactase after the weaning phase and as a result become lactose intolerant [4]. As the lactose is not digested due to a deficiency of lactase, it is fermented by intestinal bacterial flora, resulting in the production of gas leading to diarrhea, flatulence, and/or abdominal pain. Most people with lactose intolerance can still tolerate some amount of lactose, and the digestive symptoms can be reduced by limiting the intake of lactose to a level that can be tolerated.

Lactose is not only present in dairy products but can also be found as a food additive in processed food products such as meat or meat analogues. For example, lactose can be used to add texture or bind water in processed meats, such as sausages and cold cuts [2]. In order to know if these products can be considered 'lactose free' (< 0.01% w/w), and safe to consume for lactose intolerant people [5], there is a need for fast and accurate analytical method to quantify lactose.

In this application note a LC solution is presented for fast and sensitive analysis of lactose, lactose isomers and lactulose using the new ALEXYS Carbohydrates analyzer based on the DECADE Elite electrochemical detector and SenCell. A variety of commercial food samples including dairy, meat and meat analogues were analysis using a standard-bore anion-exchange column to demonstrate the performance and versatility of the ALEXYS HPAEC-PAD system.

Method

The analysis was performed using the ALEXYS Carbohydrates Analyzer as shown in figure 1. The ALEXYS Carbohydrates Analyzer consists of a P6.1L pump with integrated Solvent Switch Valve (SSV) capable of running step gradients, AS6.1L autosampler, ET 210 eluent tray for Helium blanketing, CT 2.1 column oven and the DECADE Elite electrochemical detector. The SenCell with Au working electrode and HyREF reference electrode was selected for sensitive detection of the sugars. The system was operated under the Thermo Scientific™

Figure 1: ALEXYS Carbohydrates analyzer consisting of the ET 210 eluent tray, P6.1L analytical pump, AS6.1L autosampler, CT2.1 column thermostat and DECADE Elite electrochemical detector.

Chromeleon™ Chromatography Data System (CDS) software version 7.2.10.

Separation

Under alkaline conditions (pH > 12) carbohydrates can be separated by means of 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. Due to the extreme alkaline conditions only polymeric anion-exchange columns are suitable for carbohydrate separation. The retention time of carbohydrates is inversely correlated with pKa value and increases significantly with molecular weight. An anion-exchange column was chosen for the separation of lactose and isomers (Table 1).

This column type with small particle size (4 µm) results in high-resolution separation, allowing short analysis time. No trap column was required as no tailing of the fructose and lactulose peaks was present. The temperature for separation was set at 30 °C using the CT 2.1 column thermostat. The analysis is based on a step-gradient. Both, potassium hydroxide (KOH) or sodium hydroxide (NaOH) can be used to prepare the mobile phase for separation and the column clean-up step.

During isocratic elution, 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 with a strong eluent as rinsing solution is therefore necessary to remove the

Table 1

Conditions

HPLC system	ALEXYS Carbohydrate Analyzer
Detector	Antec DECADE Elite electrochemical detector
Columns	Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast -4µm guard column, 30 x 4.0 mm ID, 4 µm Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast -4µm analytical column, 150 x 4.0 mm ID, 4 µm
Mobile phase (MP)	A: see gradient program B: see gradient program Eluents prepared & blanketed with Helium 5.0
Flow rate	0.8 mL/min
Back pressure	about 300 bar
Injection	10 µL
Temperature	30 °C for separation, 35 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF RE, 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	0.05 Hz
Range	10 µA/V

Table 2

Step-gradient program dairy samples

Time (min)	*Mobile phase*	*Description*
0 - 9	14 mM KOH*	Isocratic elution and detection
9 - 14	100 mM KOH	Column clean-up and regeneration
14 - 30	14 mM KOH	Equilibration, starting conditions

*) Both KOH or NaOH can be used as eluent.

bound carbonate ions and late eluting compounds, see table 2. This regeneration step assures reproducible retention behaviour for each run. The total run time is 30 minutes.

To minimize the introduction of carbonate ions in the mobile phase the eluents were carefully prepared manually using a carbonate-free 50% w/w KOH or NaOH solution and electrochemical grade sodium acetate salt (all commercially available). The diluent was DI water (resistivity >18 MΩ.cm), which was sparged with Helium 5.0 using the sparging function of the ET 210 eluent tray (figure 2). The eluent tray was also used to pressurize the head space above the mobile phase with Helium gas (0.2—0.4 bar He overpressure) during the analysis. Blanketing of mobile phase with an inert gas minimizes the introduction of CO_2 in the mobile phase and the subsequent formation of CO_3^{2-} ions, ensuring reproducible analysis.

Detection

For the pulsed amperometric detection of lactose and isomers

Figure 2: ET 210 eluent tray with PPCO bottles. The eluent tray enables blanketing & sparging of LC mobile phases with an inert gas atmosphere in an user-friendly and easy way.

the Antec SenCell electrochemical flow cell is used. This flow cell [6] 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 3. The temperature for detection was set to 35°C. The cell current was typical about 0.2—0.4 µA with these PAD settings under the specified conditions. This particular 4-step waveform with a pulse duration of 500 ms has been claimed to have as benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [7], resulting in less flow cell maintenance and system down time.

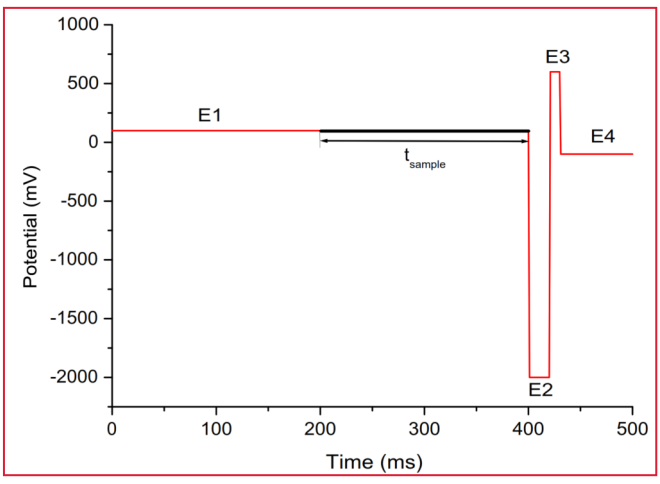

Figure 3: 4-step PAD potential waveform for the detection of carbohydrates.

Preparation of standards, reagents and samples

Standards: 50 mM stock standards of the individual sugars were prepared in 95/5 (v/v%) water/acetonitrile to increase the storage life. Stock standards under these conditions are stable for approximately 1 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 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 qualitative filter paper prior to use.

Sample preparation dairy products:

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

- Semi-skimmed milk
- Whipping cream
- Cream cheese
- Yoghurt

Procedure:

1. 0.5 gram of dairy sample was weighted in a 50 mL volumetric flask and 10 mL DI water 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 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. The turbid sample solution was centrifuged 15 min in 50 mL conical tubes at 2500 xG. For the lactose-free whipping cream, no clear supernatant could be obtained by centrifugation. Therefore the solution was allowed to stand for another 30 min, and clear supernatant was aspirated from the bottom of the tube.
5. The supernatant was collected in a plastic 5 mL syringe and filtered over a 0.20 µm PES (Polyethersulfone) syringe filter.
6. 10 µL of the filtered supernatant was injected into the LC system and analyzed.

Results

In figure 4 a typical chromatogram of a 10-µL injection of the 10 µM standard mix is shown. Not all carbohydrates are completely baseline separated (resolution > 1.5). For example raffinose elutes closely to lactose and lactulose. Raffinose might be found in whole grain and cacao products. The peak efficiencies found for the sugars ranged from 27.000 to 65.000 theoretical plates per meter with peak tailing factors < 1.5.

Separation

The elution order of the 11 carbohydrates, as shown in figure 4, is the same as previously shown in the application note [3]. Elution with a mobile phase of 14 mM hydroxide results in the best separation for this set of carbohydrates. However, if component from the sample matrix interferes with the analysis, the concentration of OH^- can be adjusted to prevent coelution and improve the separation. The retention times of the carbohydrates in the standard mix as function of the concentration hydroxide ranging from 13 to 20 mM are shown in figure 5.

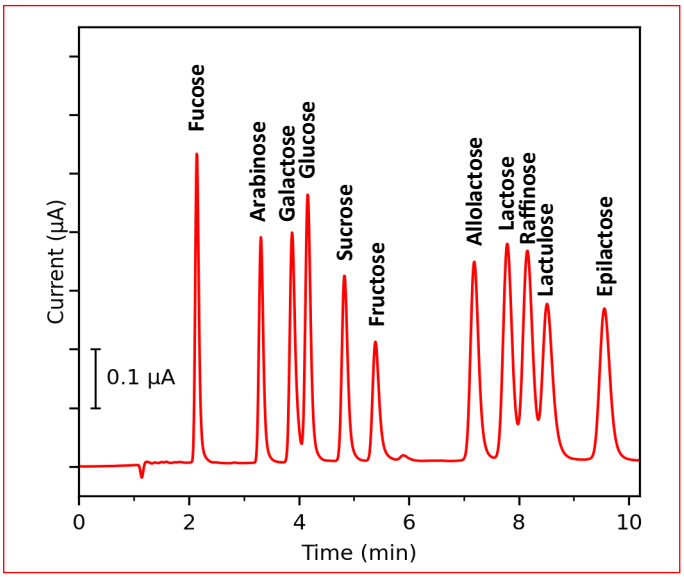

Figure 4: Chromatogram of a 10 µL injection of a 10 µM standard mix in water (eluent 14 mM KOH). The mix is composed of the following sugars: fucose, arabinose, galactose, glucose, sucrose, fructose, allolactose, lactose, raffinose, lactulose and epilactose.

As shown in figure 5, the retention times of the different carbohydrates are not affected equally by a change in hydroxide concentration (pH) which can result in a swap in elution order. For example, the retention time of the

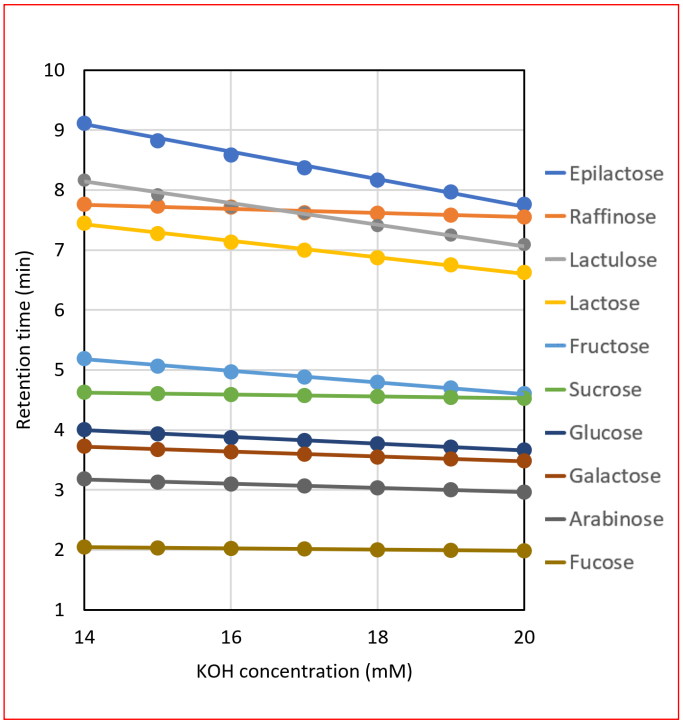

Figure 5: Retention times of all carbohydrates (except allolactose) in the standard mix as function of the hydroxide concentration.

disaccharide sucrose is less affected by an increasing concentration of hydroxide than the closely eluting monosaccharide fructose. The same holds for the trisaccharide raffinose and the closely eluting disaccharides lactose and epi-lactose. By selecting the right concentration of hydroxide, coelution of the sugars of interest and interferences can be prevented and the separation optimized.

Two chromatograms of 500 nM standard mix eluted with a mobile phase containing 14 mM NaOH (black) or 14 mM KOH (red) are shown in figure 6. It is evident that the counter ion of the hydroxide has no influence on the separation.

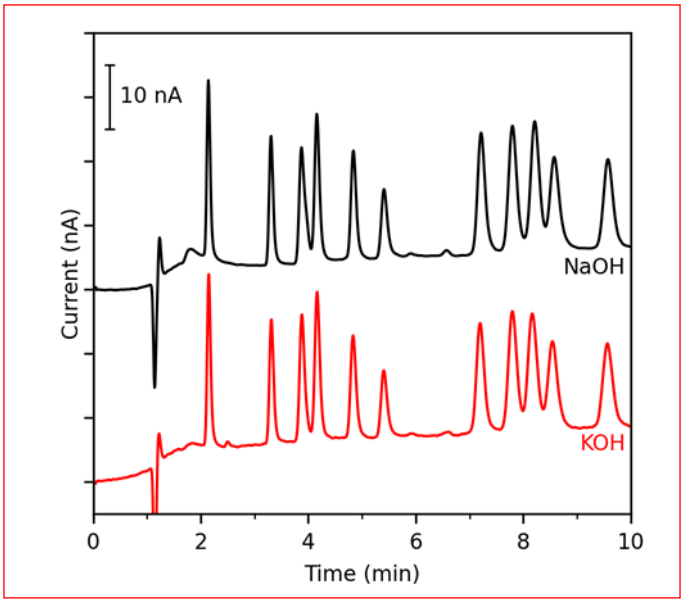

Figure 6: Chromatograms of a 10 µL injections of the 500 nM standard mix eluted with 14 mM KOH (red) and 14 mM NaOH (black).

Repeatability

The relative standard deviation (RSD) of the retention time and peak area were determined for 10 repetitive injections with a 0.1, 1 and 10 µM standard, corresponding with 0.034, 0.34 and 3.4 mg/L for lactose and the isomers, respectively. Retention times were stable, with RSD values below 0.20% for all 11 carbohydrates independent of concentration. The RSD values for peak area are shown in table 5. The RSD's for peak area were < 3% for the 0.1 µM standard and typically < 0.5% for the 1 and 10 µM standard. The only exception is raffinose, which has slightly higher RSD since this component is not baseline separated under these LC conditions. The data demonstrate that with this method reproducible analysis of all the components can be achieved.

Table 5

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

Compound	*RSD area (%) 0.1 µM*	*RSD area (%) 1 µM*	*RSD area (%) 10 µM*
Fucose	1.07	0.22	0.08
Arabinose	1.28	0.31	0.09
Galactose	1.11	0.40	0.16
Glucose	1.31	0.20	0.15
Sucrose	2.37	0.25	0.13
Fructose	2.97	0.50	0.30
Allolactose	2.15	0.50	0.10
Lactose	2.07	0.49	0.43
Raffinose	3.28	0.43	0.67
Lactulose	2.91	0.53	0.42
Epilactose	2.59	0.40	0.36

LOD/LOQ and linearity

The Limit of Detection (LOD) and limit of quantification (LOQ) for all sugars are shown in table 6. The LOD's and LOQ's were calculated as the analyte response corresponding to 3x and 10x the ASTM noise (average peak-to-peak baseline noise of 10 segments of 0.5 min), respectively. The noise was calculated based on a 5-minute section of the baseline close to the peaks of interest. The average responses of 5 replicate injections obtained with the 0.1 µM standard mix were used to calculate the LOD's for all sugars.

Table 6

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

	Limit of detection		*Limit of Quantification*	
Compound	*nM*	*µg/L*	*nM*	*µg/L*
Fucose	7.7	1.3	25.8	4.2
Arabinose	10.5	1.6	34.9	5.2
Galactose	9.6	1.7	32.0	5.8
Glucose	8.9	1.6	29.7	5.3
Sucrose	12.7	4.4	42.4	14.5
Fructose	19.8	3.6	65.9	11.9
Allolactose	11.9	4.1	39.7	13.6
Lactose	11.1	3.8	37.0	12.7
Raffinose	11.2	5.7	37.4	18.9
Lactulose	14.6	5.0	48.6	16.6
Epilactose	15.6	5.3	52.1	17.8

The LOQ of lactose of 12.7 µg/L demonstrates the excellent sensitivity of the method. During sample preparation the dairy product is 100 x diluted. Therefore, a concentration of 10 mg lactose in 100 gram product corresponds to a concentration of 1000 µg/L (ppm) lactose in the sample vials. Therefore. the reported LOQ is factor 70 below the upper limit of the lactose concentration expected in 'lactose-free' products.
The linearity was investigated for the concentration range of 0.1 to 100 µmol/L. The calibration curves for lactose and the lactose isomers, used for the actual quantification of samples,

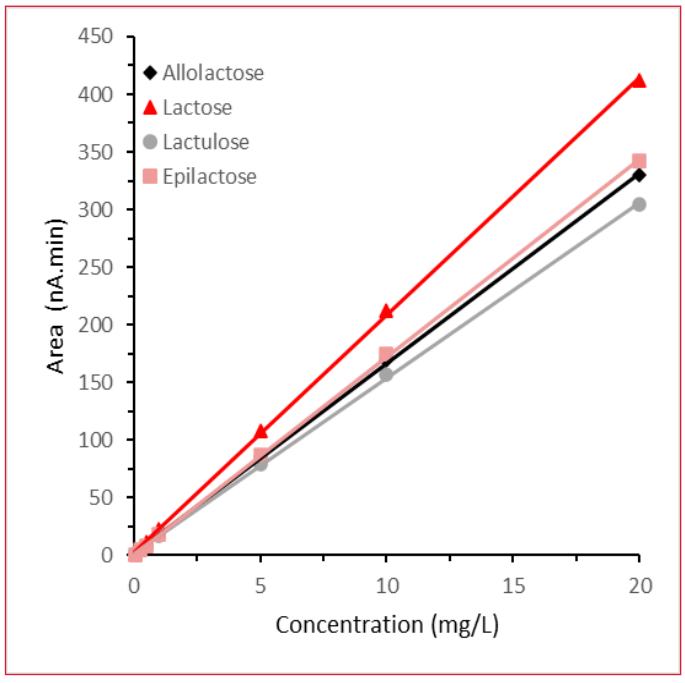

Figure 7: Calibration curve of Allolactose, Lactose, Lactulose and Epilactose in the concentration range of 0.05 - 20 mg/L.

are shown in figure 7. The linear correlation coefficient was 0.9997 or better for all 4 sugars.

Dairy sample analysis

In total 4 commercially available 'Lactose-free' labelled products were purchased from supermarkets in the Netherlands and analyzed using the presented method.

Table 7

'Lactose-free' labelled dairy products

Product	*Lactose content according to label*
Semi-skimmed milk	< 0.01 g/100 mL
Cream cheese	< 0.01 g/100 g
Yoghurt	< 0.01 g/100 mL
Whipping cream	< 0.1 g/ 100 g

The lactose contents specified on the product labels ranged from < 10 mg to < 100 mg Lactose per 100 g or mL of product. All samples were prepared as described in the method section.

The contents of lactose and lactose isomers in the samples was determined in two different ways, using:

- Calibration curve based on standards (0.05 - 20 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 amount of a standard containing allolactose, lactose, lactulose and epilactose. The spike concentration was 1 ppm (1 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 ppm standard.

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

In figure 8 the chromatograms of the samples are shown. The sample is shown in red, the spiked in black and the 1 ppm standard containing allolactose (1), lactose (2), lactulose (3) and epilactose (4) is shown in grey. In every 'lactose-free' product tested allolactose and lactose was found, the values are listed in table 8 . Notably, in the semi-skimmed milk lactose was

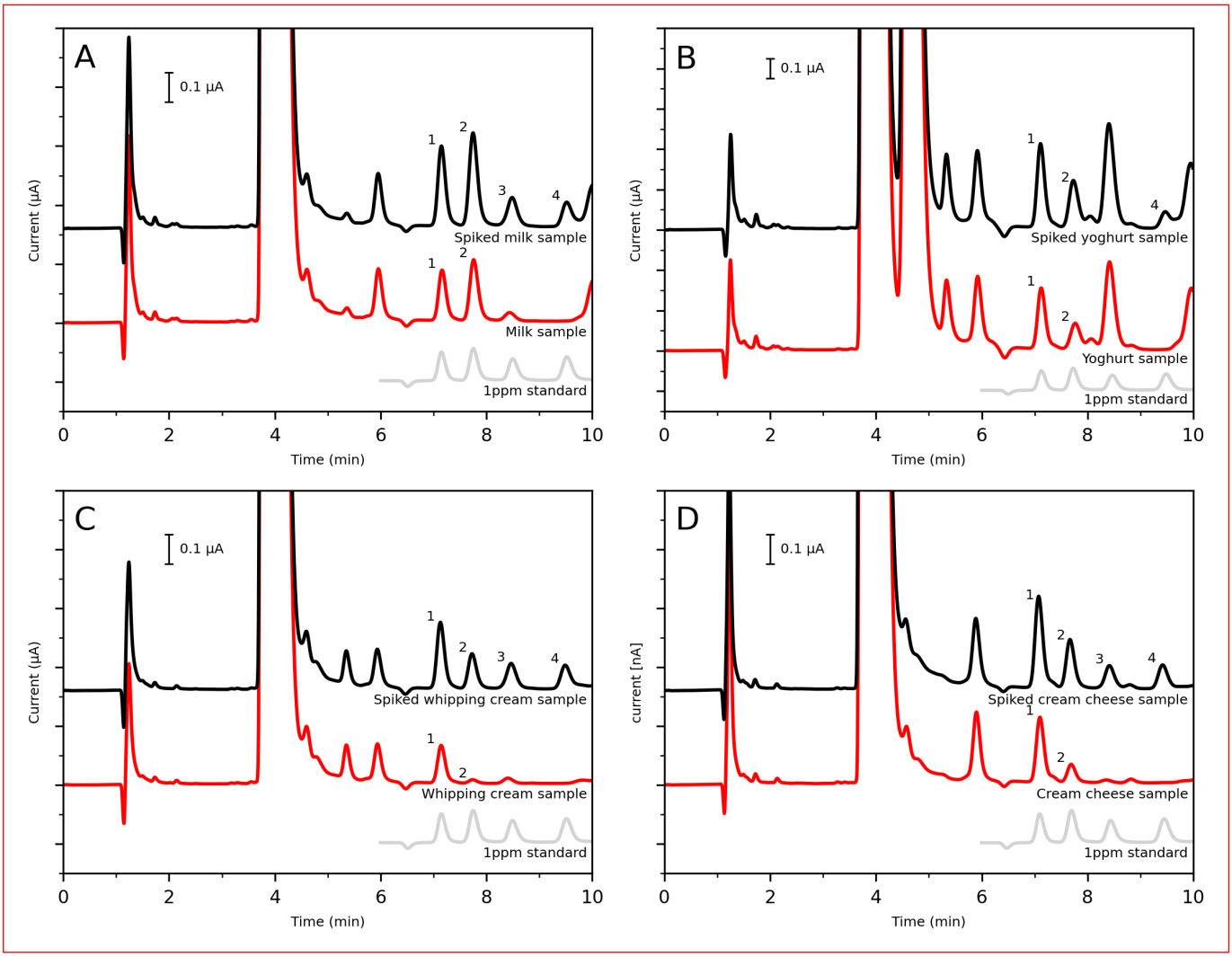

Figure 8: Chromatograms of the dairy samples. A: Milk sample (red), spiked milk sample (black), and 1 ppm standard (grey). B: Yoghurt sample (red), spiked yoghurt sample (black), and 1 ppm standard (grey). C: Whipping cream sample (red), spiked whipping cream sample (black), and 1 ppm standard (grey). D: Cream cheese sample (red), spiked cream cheese sample (black), and 1 ppm standard (grey). Labeled peaks: 1. Allolactose, 2. Lactose, 3. Lactulose and 4. Epilactose.

present at a concentration of 19 mg/100 mL, which is more than specified on the product label (10 mg/100 mL).

In all samples a significant amount of allolactose was present, ranging from 14 to 30 mg/100 mL. This underlines the need for selectivity to differentiate between lactose and allolactose to accurately quantify the amount of lactose. None of the samples contained a detectable amount of lactulose or epilactose. However, it has to be noted that under the selected mobile phase conditions (14 mM NaOH) unknown peaks are eluting closely at the retention times of lactulose, making quantification difficult. By using a higher concentration of KOH (> 20 mM) as eluent it is possible to separate lactulose from the interferences for better identification, see reference [8].

The concentrations in this table 8 were calculated using the standard addition method (single point calibration). There was a good correlation between the values in table 8 and the concentrations calculated based on the calibration curve. The sample recovery found for lactose, ranged between 90% - 97%.

Table 8

Lactose & allolactose, contents and sample recovery

Product	*Lactose*		*Allolactose*	
	mg/ 100 g product	*Recovery*	*mg/ 100 g product*	*Recovery*
Semi-skimmed milk	19	95%	17	99%
Cream cheese	5.8	90%	25	92%
Yoghurt	9.7	97%	30	102%
Whipping cream	5.8	93%	14	92%

Meat and meat analogue samples

For the analysis of lactose in meat and meat analogues three commercial meat products were analyzed, see table 9.

Table 9

Meat and meat analogues

Sample	*Product description*
A	Vegan meat analogue based on pea-protein
B	Breaded chicken with processed-cheese filling
C	Dry-cured ham

It should be noted that the analyzed meat samples are products which are not specifically labelled as 'lactose-free' products.

The samples were prepared using the sample preparation procedure described below:

1. The sample was homogenized using a turrax.
2. 1 gram of sample was weighted in a 100 mL volumetric flask and 50 mL DI water added.
3. the sugars were extracted by placing the flask in hot shaking water bath (60°C) for 20 minutes
4. Subsequently, 100 µL Carrez I and 100 µL Carrez II reagent was added. Followed by addition of DI water up to a total volume of 100 mL.
5. The solution was allowed to stand for 30 minutes and vortexed again to obtain a homogeneous turbid suspension.
6. The turbid sample solution was centrifuged 10 min at 4000 xG
7. The supernatant was collected, diluted 40 times, and filtered over a 0.20 µm Polyethersulfone (PES) syringe filter.
8. 10 µL of the filtered supernatant was injected into the LC system and analyzed.

See table 1 and 10 for the LC conditions and step-gradient program used for the analysis of the meat samples. Whereas for the dairy samples 100 mM hydroxide was sufficient to regenerate the column, the meat samples required a stronger column clean-up solution. A solution consisting of 100 mM hydroxide and 100 mM acetate was sufficient to remove all late-eluting compounds. Column regeneration was also more effective with a column clean-up solution containing NaOAc, leading to increased retention, hence a longer isocratic elution time of 12 minutes was used for the meat samples.

Table 10

Step-gradient program meat samples

Time (min)	*Mobile phase*	*Description*
0 - 12	15 mM NaOH	Isocratic elution and detection
12 - 17	100 mM NaOH, 100 mM NaOAc	Column clean-up and regeneration
17 - 30	15 mM NaOH	Equilibration, starting conditions

The chromatograms of the samples and a 0.5 µM standard are shown in figure 9 in red and grey, respectively. With the gradient program used in this method raffinose (10) elutes after lactulose (9) and before epilactose (11). The sugar contents of the food products are summarized in table 11. The concentrations shown were calculated using a calibration curve based on standards ranging from 0.5 - 10 µmol/L.

Table 11

Carbohydrate content meat and meat analogues

Carbohydrate	*Sample A (mg/ 100 g product)*	*Sample B (mg/ 100 g product)*	*Sample C (mg/ 100 g product)*
Arabinose	8	-	7
Galactose	15	6	11
Glucose	543	367	39
Sucrose	964	17	-
Fructose	404	89	20
Lactose	-	61	-
Raffinose	56	-	-

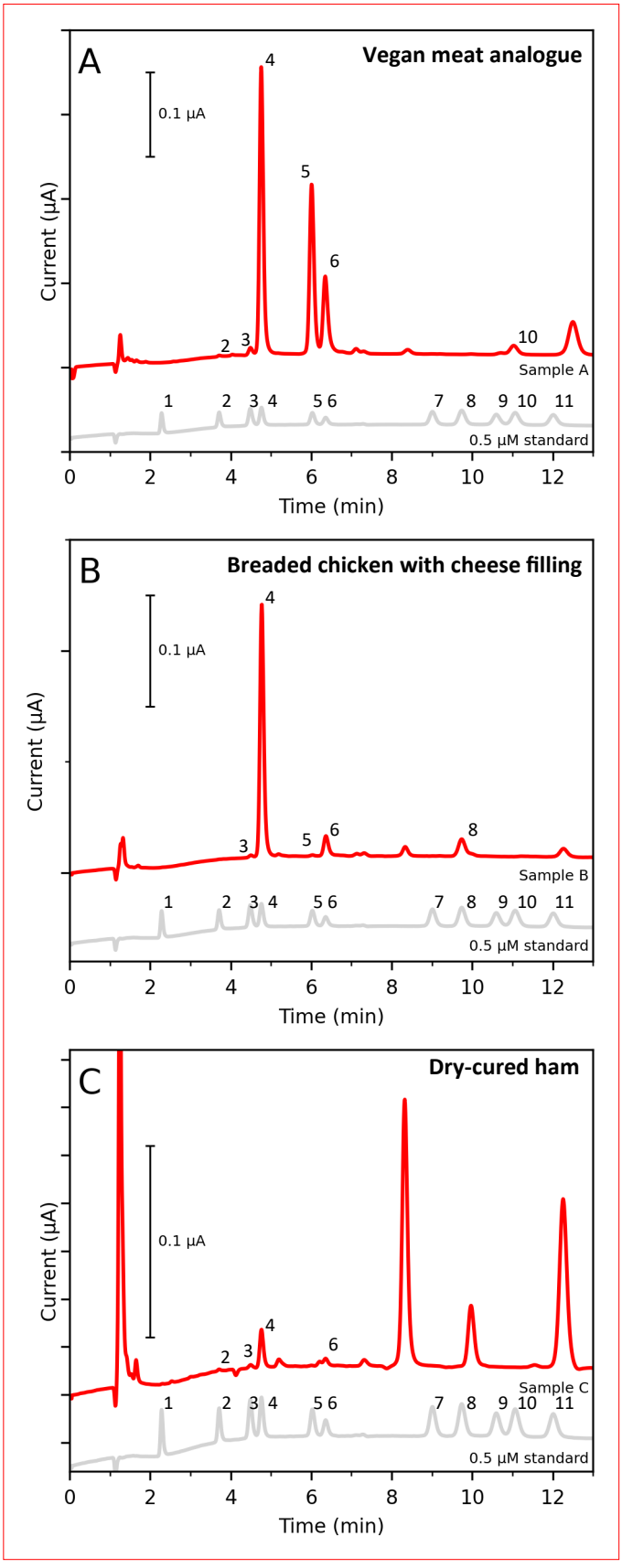

Figure 9: Chromatograms of samples A to C (red) and 0.5 µM standard (grey). Labeled peaks: 1. Fucose, 2. Arabinose, 3. Galactose, 4. Glucose, 5. Sucrose, 6. Fructose, 7. Allolactose, 8. Lactose, 9. Lactulose, 10. Raffinose , 11. Epilactose.

The highest amount of carbohydrates was found in sample A, the vegan meat analogue. Lactose was only present in sample B, the chicken-based product with processed-cheese filling. The lactose concentration in this product was 61 mg per 100 g product which evidently originates from the cheese filling. Sample C, the dry-cured ham, contained the lowest amount of carbohydrates of the three samples.

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Conclusion

The ALEXYS Carbohydrates Analyzer, in combination with the SenCell flow cell offers an dedicated and optimized analysis solution for the fast and sensitive quantification of lactose and isomers in dairy, meat and other products. All lactose isomers are eluting within 10 minutes using the selected LC conditions. The total run time including column clean-up and equilibration is 30 min. In addition to lactose, also other major sugars in processed food, such as galactose, glucose and sucrose can be quantified using the presented method. Optionally, an stronger column cleaning step with sodium acetate can be programmed, for sample matrices containing large concentrations of strongly retaining contaminants.

Ordering information

System	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quartenary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT 2.1 Column thermostat
Software*	
195.0035	Clarity CDS single instr. incl LC, AS module

*) The ALEXYS Carbohydrates Analyzer can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

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