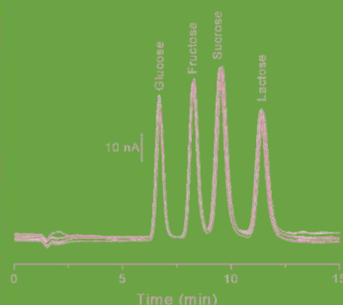
CARBOHYDRATES IN FOOD

THE FINEST LC-EC APPLICATIONS FOR FOOD & BEVERAGE ANALYSIS EVER PROCESSED

Bisphenol A
Catechins
Flavonoids and phenols
Phenols
Antioxidants

Polyphenols
Resveratrol
Epicatechin
Quercetin
other polyphenols

Carbohydrates
Iodide
Vitamins A, C, D, E, and K
Q10
ubiquinols



INTRODUCTION

Carbohydrates not only provide the most easily accessible energy source for our body, they also play an important role in many physiological processes. They are involved in intercellular recognition, infection processes, and certain types of cancer. Carbohydrates analysis is of interest to the food industry but also many fields in life sciences. Analytes of interest include simple mono- or disaccharides (such as glucose and sucrose), oligosaccharides (Maltodextrin), polysaccharides (starch, cellulose) and glycoproteins.

- Carbohydrates in food & life sciences
- Pulsed Amperometric Detection
- Robust & reproducible Analysis
- Flexcell with exchangeable working electrode

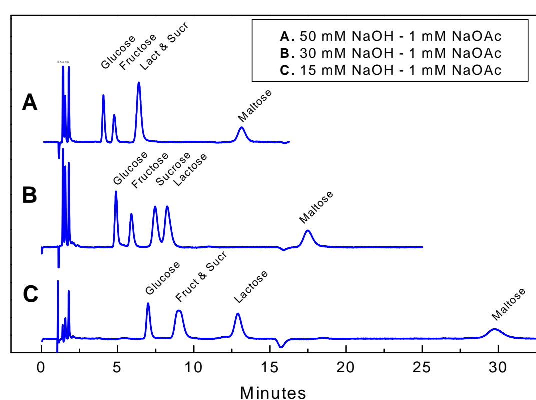
Summary

The ALEXYS Carbohydrates Analyzer is a dedicated LC solution for the analysis of sugars and oligosaccharides in a variety of samples.

Fig. 1. ALEXYS Carbohydrates Analyzer.

Method

Under alkaline conditions (pH > 12) carbohydrates can be separated by means of Anion-Exchange Chromatography. Carbohydrates are weak acids with pK_a values ranging between 12 and 14. At high pH they will be either completely or partially ionised 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. The elution order of carbohydrates on such anion-exchange columns is usually as follows: sugar alcohols elute first, followed by mono-, di-, tri-, and higher oligosaccharides.



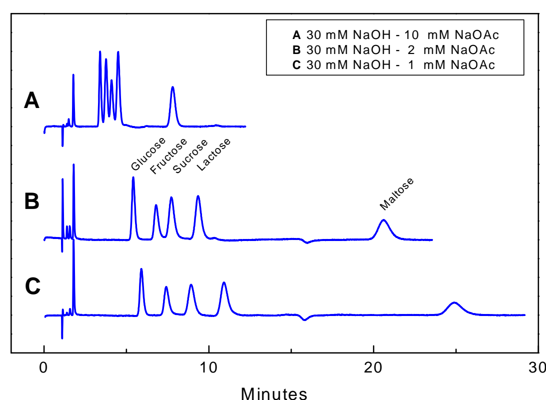


Fig. 2. [A] Retention times of common food carbohydrates as a function of sodium hydroxide (top) and [B] sodium acetate (bottom) concentration of the mobile phase.

The retention behaviour of the carbohydrates can be controlled by the concentration of sodium hydroxide and sodium acetate in the mobile phase.
An increase of the sodium hydroxide concentration [OH-] has a dual effect on the retention of carbohydrates. The increase in ionic strength of the eluent causes a decrease in analyte retention, while the higher pH will increase the degree of dissociation resulting in an increase in analyte retention. If the pH > pKa (full dissociation) then the ionic strength will dominate the separation process and the retention decreases. This is illustrated in Fig. 2A.
Sodium acetate is commonly used as a 'modifier' to decrease the elution time of higher molecular weight carbohydrates allowing faster analysis.
Pulsed amperometric detectors are relatively insensitive to ionic strength changes of a sodium acetate gradient, as long as the sodium hydroxide concentration remains constant during the gradient run. High purity grade sodium acetate should be used for the preparation of the mobile phase (impurities can cause large baseline shifts during a gradient run).

Pulsed amperometric detection

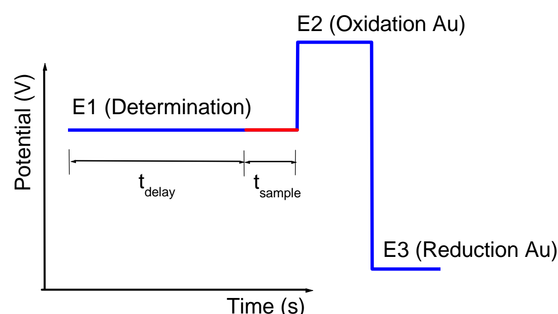


Fig. 3. Three-step potential waveform for the pulsed amperometric detection of Carbohydrates.

Pulsed Amperometric Detection (PAD) with a gold (Au) working electrode is applied for carbohydrate analysis. A series of electrode potential steps (pulses) enable frequent (typically 1 - 2 Hz) renewal of the working electrode (WE) surface [1,2]. The repeating three-step potential waveform is schematically shown in Fig. 3.
E_1 is the actual detection potential. During t_1 the carbohydrates are oxidized at the Au electrode surface. The delay time t_{delay} before the start of the actual current measurement assures that the contribution of the electrode charging current caused by the potential steps is small.
A large positive potential E_2 is applied to achieve anodic formation of surface oxides in order to clean the electrode surface.
With a negative potential E_3 the oxidized electrode surface is reduced back to a reactive Au surface, the so-called reactivation step.
Detailed information about waveform optimisation for pulsed amperometric detection of carbohydrates can be found in several papers published by LaCourse and Johnson [3, 4].

Mobile phase preparation

He-degassing is required in carbohydrate analysis for reproducible retention times.

Carbon dioxide gas present in air will dissolved as CO_3^{2-} ions in the strong alkaline eluent. The dissolved carbonate ions will increase the ionic strength of the mobile phase resulting in a shortening of the retention times of the carbohydrate analytes. Therefore, keeping the mobile phase free of carbonate is one of the key factors towards reproducible carbohydrate analyses via Anion-Exchange Chromatography.

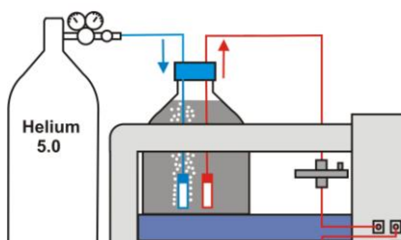


Fig. 4. Schematic representation of mobile phase bottle with Helium sparging assembly.

Take the following precautions to assure carbonate-free mobile phases

- Prepare the NaOH mobile phase using a 50% w/w carbonate-free NaOH stock solution (commercially available) and thoroughly degassed deionised water (≥ 18 MΩ). Commercially available NaOH pellets are not acceptable for eluent preparation, because they are always covered with a thin adsorbed layer of sodium carbonate.

- The mobile phase should be stored in plastic containers instead of glass. NaOH is a strong etching agent and will react with the inner glass wall resulting in the release of silicates and borates.

- Most carbon dioxide dissolved in deionised HPLC grade water (> 18 Mohm-cm) can be removed by degassing the water in an ultrasonic bath for 10 – 15 minutes, and subsequent sparging with Helium 5.0 gas.

- Subsequently add the appropriate amount of 50% w/w NaOH solution to obtain the final eluent. Always pipette the necessary amount of NaOH from the top part of the 50% NaOH stock solution. Any carbon dioxide present in the solution will precipitate as sodium carbonate to the bottom of the flask, leaving the top part of the solution virtually carbonate free.

- High-purity grade sodium acetate should be used for the preparation of the mobile phase (impurities can cause large baseline shifts during a gradient run).

It is advisable to prepare fresh mobile phase daily. The mobile phase should be sparged continuously with Helium during the analysis to obtain reproducible retention times.

Column regeneration

Especially, during the isocratic analysis of carbohydrates with weak eluents ([NaOH] < 50 mM) a gradual loss of retention is observed due to the slow build up of interfering anions on the column. If during the isocratic analysis of carbohydrates a loss of retention is observed, regeneration of the column is necessary. Regeneration of the column can be achieved by flushing the column with a volume of 30 – 60 mL of carbonate-free 0.2 M NaOH. After regeneration, the column should be allowed to re-equilibrate again with mobile phase. Stabile retention times (RSD < 0.4%) can be achieved again after flushing the column for 5 hour with eluent at a flow rate of 2 mL/min.

Part I - Isocratic analysis of sugars

Mixtures of simple sugars, such as mono- and disaccharides can be determined using PAD under isocratic conditions with high sensitivity and good reproducibility.

Table 1

LC-EC Conditions	
HPLC	ALEXYS Carbohydrates Analyzer
Sample	2 µmol/L Glucose, Fructose, Lactose and Sucrose in water
Mobile phase	30 mM NaOH and 1 mM NaOAc, the mobile phase is continuous sparged with Helium 5.0
Flow rate	2 mL/min
Vinjection	20 µL
Temperature	30°C, column and flow cell
E-cell	E1, E2, E3: 0.05, 0.75, -0.80 Volt ts, t1, t2, t3: 0.06, 0.5, 0.13, 0.12 seconds
I-cell	300 - 500 nA

This method is particularly attractive for the analysis of sugars in a wide range of food products such as beverages, fruit juices, milk products and beer.

Reproducibility

The performance of the ALEXYS Analyzer is demonstrated using a standard mixture of Glucose, Fructose, Lactose and Sucrose in water. In figure 6 an overlay is shown of 23 consecutively recorded chromatograms.

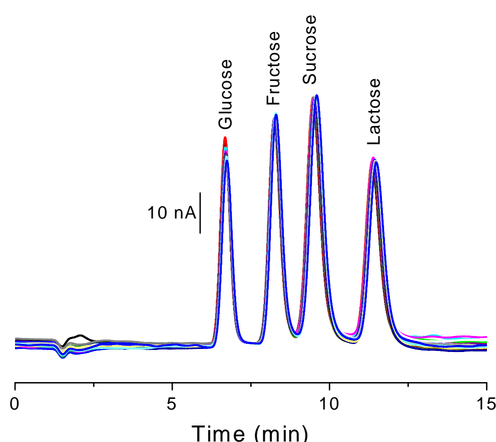
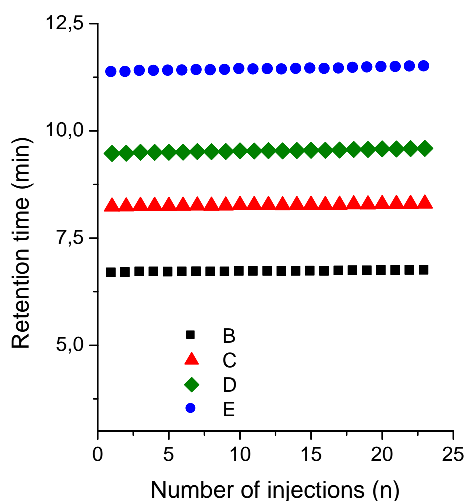


Fig. 5. Top: chemical structure of Glucose, Fructose, Lactose and Sucrose. Bottom: overlay of 23 chromatograms of a standard mixture of 2 µM Glucose, 2 µM Lactose, 4 µM Fructose and 4 µM Sucrose in water (20 µl injected), ADF 0.01 Hz. The theoretical plate number for the components are N ~ 14.900, 10.900, 12.000 and 17.300 plates/meter, respectively.

The relative standard deviations (RSD) of the retention times and peak areas were determined of 23 consecutive injections of the sugar mixture (see Fig. 6). The reproducibility of the method is evident from the obtained RSD values of < 0.4% and < 3% for the retention times and peak areas of the sugars, respectively.



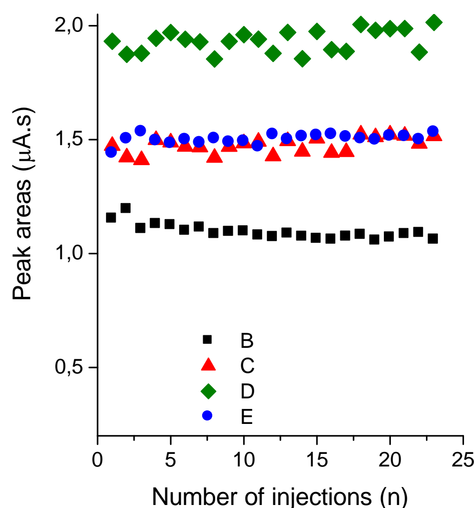


Fig. 6. Reproducibility (n=23) of 20 µL injections of a standard mixture of 2 µM Glucose, 2 µM Lactose, 4 µM Fructose and 4 µM Sucrose in water. Top: retention time, Bottom: peak area.

During the reproducibility experiments the mobile phase was sparged continuously with Helium 5.0 to prevent the formation of carbonate ions and subsequent loss in retention.

Linearity and Detection Limits

For the standard mixture the concentration detection limits (CLOD) were determined to illustrate the sensitivity of PAD detection of carbohydrates at a gold working electrode. The detection limits are displayed in Table 2. The CLOD here is based on a 20 µL injection and defined as the concentration that gives a signal that is three times the peak-to-peak noise.

Table 2

Concentration detection limits, standards in water

	CLOD (nM)	LOD (pmol)
Glucose	10	0.2
Fructose	15	0.3
Sucrose	15	0.3
Lactose	10	0.2

With the ALEXYS Carbohydrate Analyzer a Limit of Detection of 0.2 pmol (on column) for glucose and Lactose could be reached under the specified conditions. In Fig. 7 an example chromatogram of a 20 nM sugar mixture is shown to demonstrate the

sensitivity of the method. The analysis of these carbohydrates at the 100 pmol range can be achieved routinely.

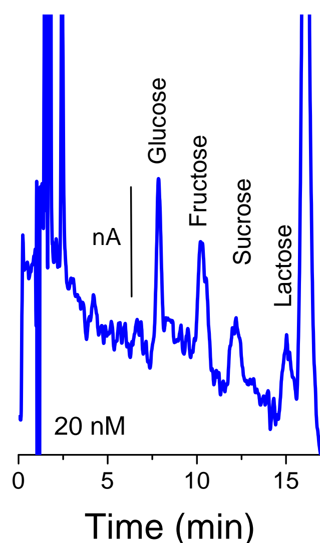


Fig. 7. Chromatogram of a 20 µL injection of 20 nM Glucose, Fructose, Sucrose and Lactose in water.

An excellent linear detector response in the concentration range between 20 nM and 10 µM was observed for the standard mixture of sugars.

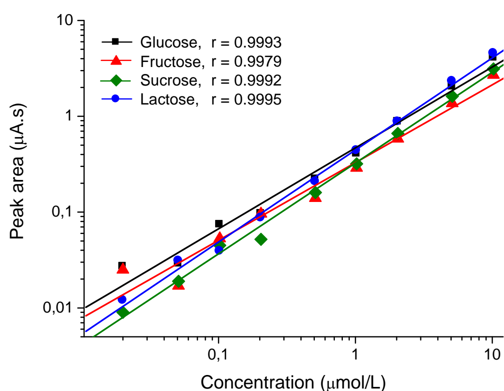


Fig. 8. Calibration plots of Glucose, Fructose, Sucrose and Lactose. Peak area as function of concentration (concentration range 20 nmol/L - 10 µmol/L, 20 µL injections, n=10 per concentration). R determined via weighted linear regression method.

Applications

In the following section several examples are shown of the analysis of mono- and disaccharides in apple juice, buttermilk and malt beer.

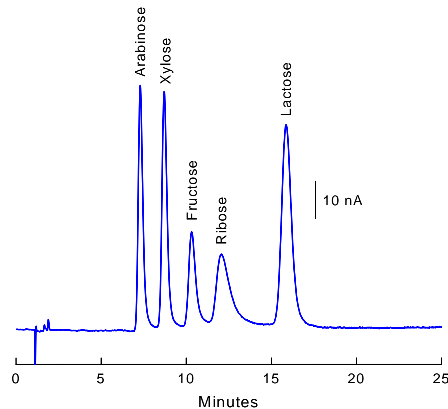


Fig. 9. 10 µM Arabinose, Xylose, Fructose, Ribose and Lactose standard in de-ionized water. Injection volume 10 µL, Flow rate 2 mL/min, mobile phase 20 mM NaOH, isocratic elution.

Analysis of sugars in apple juice

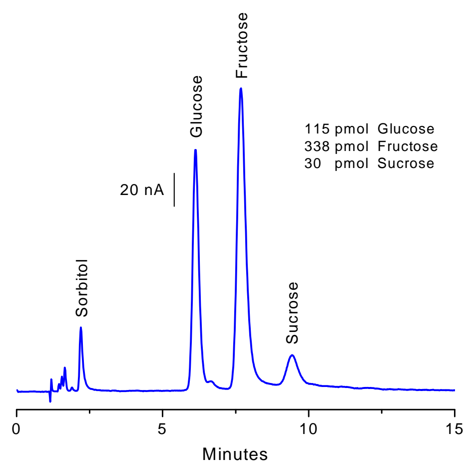


Fig. 10. Apple juice. Sample diluted 10,000 x in de-ionized water. Injection volume 10 µL, Flow rate 2 mL/min, mobile phase 30 mM NaOH, isocratic elution.

Analysis of Carbohydrates in Malt Beer

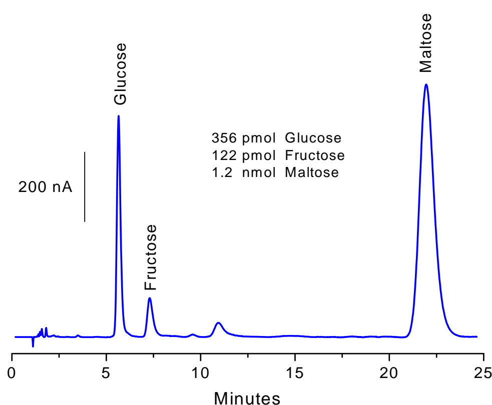


Fig. 11. Malt beer. Sample was degassed for 10 minutes (ultrasonic bath) to remove dissolved CO2 and diluted 1000 x in de-ionized water. Injection volume 10 µL, Flow rate 2 mL/ min, mobile phase 30 mM NaOH – 2 mM NaOAc, isocratic elution.

Analysis of Carbohydrates in Buttermilk

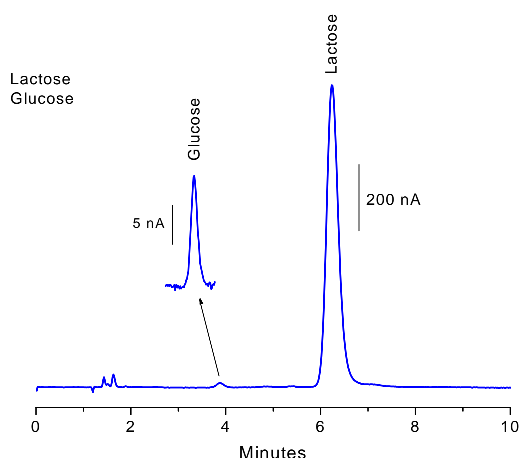


Fig. 12. Buttermilk. Sample was diluted 1000 x in de-ionized water and solution was filtered over a 0.2 µm filter Injection volume 10 µL, Flow rate 2 mL/ min, mobile phase 100 mM NaOH, isocratic elution.

Aqueous samples, such as shown in the examples above only require little sample preparation. These samples only need to be sonicated, diluted and filtered prior to injection. Other food products contain carbohydrates that are physically associated or chemically bound to other components, *e.g.,* nuts, cereals, fruit, breads and vegetables need more intensive sample preparation to isolate the carbohydrate from the rest of the food before it can be analyzed.

Part II - Gradient analysis of oligosaccharides

The HPLC analysis of oligo- and polysaccharides is increasingly important in the study of human nutrition. Starch, a polymer (polysaccharide) based on glucose subunits is one of the important base materials used in the food industry nowadays. The food and beverage industry uses the hydrolysis products of corn- or potato starch in a wide variety of food products. Starch can be depolymerised into smaller chains (oligosaccharides) resulting in syrups or maltodextrin. Corn syrup is widely used as a food and beverage sweetener. Maltodextrins, which are non-sweet nutritive oligomers, are often used as a filler or binder in food products.

The ALEXYS Carbohydrates Analyzer (binary gradient) is particular suitable for the more demanding analysis of complex carbohydrate mixtures such as oligosaccharides. With this high-pressure gradient system based on pulsed amperometric detection "fingerprints" of oligosaccharides and other complex carbohydrate mixtures can be recorded. It can serve as a tool for estimating the chain length (DP) distribution.

The performance and sensitivity of the ALEXYS gradient system is demonstrated by means of the determination of Maltodextrin in a commercially available artificial sweetener.

Table 3

Conditions for the analysis of Maltodextrin

HPLC	ALEXYS Carbohydrates Analyzer
Sample	10 mg of artificial sweetener dissolved in 100 mL de-ionized water
Mobile phase	A) 60 mM NaOH B) 60 mM NaOH – 500 mM NaOAc Mobile phases are continuous sparged with Helium 5.0
Gradient	t = 0 min: 90 % A, 10 % B t = 15 min: 10 % A, 90 % B
Flow rate	2 mL/min
$V_{injection}$	20 µL
Temperature	30°C, column and flow cell
E-cell	E1, E2, E3: 0.05, 0.75, -0.80 Volt ts, t1, t2, t3: 0.06, 0.5, 0.13, 0.12 seconds
I-cell	-0.5 - 1 µA

In Fig. 13 an overlay is shown of 25 chromatograms of a filtered solution of 100 mg/L artificial sweetener in de-ionized water. The chromatograms were recorded using the settings summarized in Table 3. In Maltodextrins (see structural formula in Fig. 13) the glucose subunits are joined by α1,4 linkages (y) with occasional branches of α1,6 linked glucose (x).

In the chromatograms the different chain lengths can be identified ranging from Maltose (DP 2) , Maltotriose (DP=3) up to DP = 14.

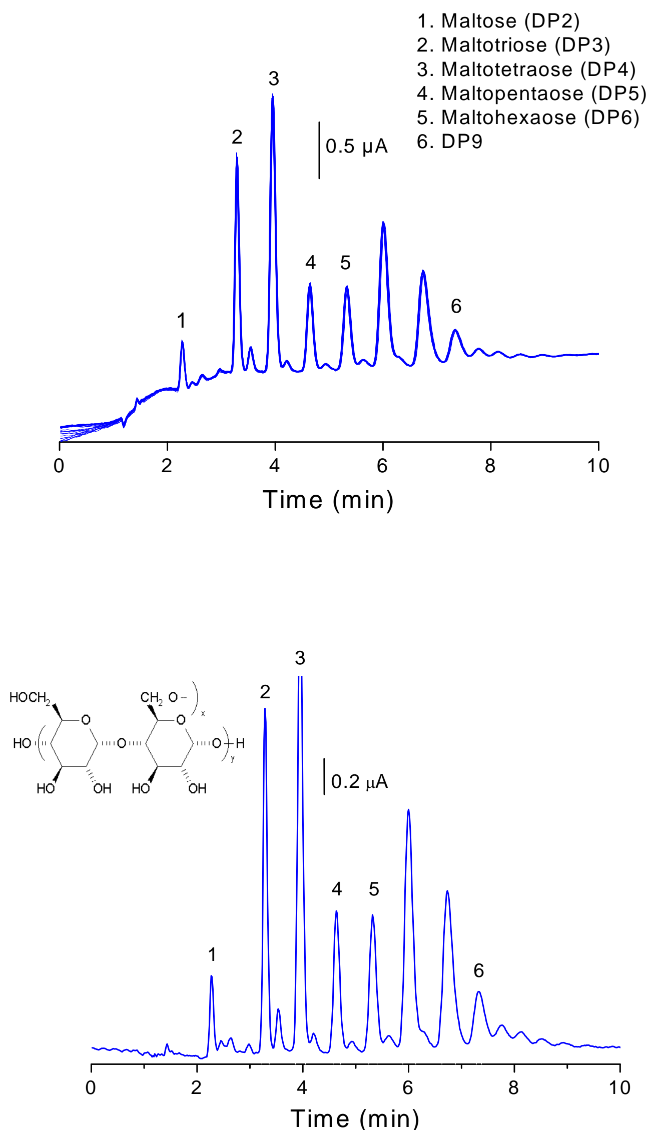


Fig. 13. [a] Top: Overlay of 25 chromatograms of 20 µL injections of a 100 mg/L solution of artificial sweetener, containing Maltodextrin. For conditions see Table 3. [b] Bottom: One chromatogram of [a] corrected for the gradient baseline.

Reproducibility

The relative standard deviations (RSD) of the retention times and peak areas were determined for DP2 to DP6 of 25 consecutive injections of the artificial sweetener (see Fig. 14).

The good reproducibility of the method is evident from the obtained RSD values (n=25) of < 0.3% and < 1.5% for the retention times and peak areas of the different oligomers, respectively.

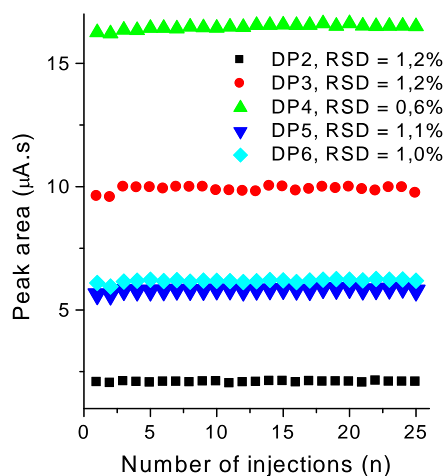


Fig. 14. Reproducibility (n=25) of 20 µL injections of a 100 mg/L solution of artificial sweetener containing Maltodextrin. Top: retention time, Bottom: peak area.

Linearity and Sensitivity

For DP3, DP5 and DP9 the linearity in detector response (peak area) was checked by diluting the 100 mg/L solution of artificial sweetener in the concentration range 2.5 mg/L - 100 mg/L.

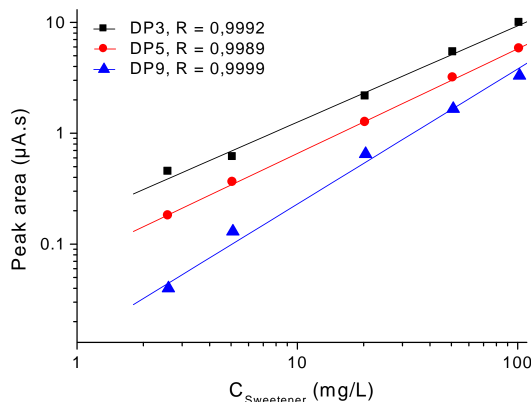


Fig. 15. Peak area of DP3, DP5 and DP9 as function of the concentration of artificial sweetener, concentration range: 2.5 mg/L - 100 mg/L. (n=2 per concentration). R determined via weighted linear regression method.

The oligomers with DP3, DP5 and DP9 showed an excellent linear respons in the specified concentration range. Amounts of 1 ng (on-column) of artificial sweetener gave well detectable peaks for the three oligomers (S/N > 15 for DP9) demonstrating the sensitivity of the method.

Conclusion

The ALEXYS Carbohydrates Analyzer provides a reliable solution for the routine analysis of carbohydrates in food. Difficult sample matrices can be analysed using the binary gradient system. Excellent reproducibility and detection sensitivity have been demonstrated.

References

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PART NUMBERS AND CONFIGURATIONS	
180.0054A	ALEXYS Carbohydrates Analyzer
250.1080	ALC-525 column, 250x4.6mm, 7um
250.1082	ALC guard column starter kit

Selectivity ALC-525 column

In the Table 1 the capacity factor for a series of carbohydrates are given as a function of the pH of the mobile phase. The capacity factors were determined using carbohydrate standards dissolved in water. This table can serve as a rough guide line to determine if the ALC-525 anion-exchange column has potentially enough selectivity for your specific application.

Table 1. Capacity factor as function of pH for a series of carbohydrates analysed on an ALC-525 column.

	k'			
C_{NaOH} (mM)	20	50	100	200
pH	12.3	12.7	13	13.3
Inositol	0.38	0.33	0.32	0.3
Xylitol	0.73	0.65	0.61	0.59
Arabitol	0.85	0.7	0.68	0.63
Dulcitol	0.98	0.76	0.76	0.69
Adontiol	1.03	0.98	0.93	0.8
Sorbitol	1.14	0.93	0.86	0.79
Mannitol	1.32	1.03	0.98	0.83
Galactosamine	5.59	2.88	1.98	1.17
Fucose	5.82	3.45	2.68	1.72
Glucosamine	6.15	3.22	2.03	1.18
Arabinose	6.24	3.17	2.2	1.34
Galactose	6.53	3.49	2.42	1.43
Glucose	6.83	3.39	2.33	1.35
Mannose	7.69	3.67	2.19	1.25
Xylose	7.97	4.14	2.48	1.4
Sorbose	8.99	4.42	2.67	1.51
Fructose	9.20	4.13	2.77	1.6
Sucrose	9.50	7.43	5.4	4.43
Melibiose	9.66	5.65	3.25	1.85
Ribose	11.52	5.5	3.33	1.89
Lactose	13.48	7.92	4.45	2.18
Raffinose	13.49	10.89	7.33	3.73
Stachinose	15.05	11.06	8.1	4.35
Rhamnose		3.3	1.93	2.2
Cellobiose		11.25	6.41	3.28
Maltose		17.8	9.74	4.39
Maltotriose				11.02