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
Sucralose
Sugars & sucralose

Sugars & sweetener in sugar-free beverages and chewing gum

- **ALEXYS carbohydrate analyzer**
- **SweetSep™ AEX200 anion-exchange column**
- **High-resolution separation of sugars & sucralose**
- **Sugar-free beverages and chewing gum**

Summary

Sugar-free products emerged in response to health concerns over sugar consumption. The discovery of saccharin in 1879 marked the beginning of artificial sweeteners [1]. Sucralose, a popular modern sweetener, was discovered in 1976. It is about 600 times sweeter than sugar and contains no calories [2]. Sucralose is widely used in sugar-free products, including diet sodas, sugar -free candies, and baked goods to help achieve the desired sweetness. In the regulations (EC) No 1924/2006 and specific directive 2003/115/EC the amount of sugar in sugar-free products and the amount of sucralose in products are specified [3,4]. To check if the sugar-free products meet the requirements, a fast and reliable method is necessary to quantify the sugars and sweeteners.

This application note is complementary to the previously published application note “220-031 Sucralose in beverages and chewing gum”. In this application note a method is presented to quantify both common sugars and sucralose in sugar-free products using the ALEXYS carbohydrate analyzer in combination with the SweetSep™ AEX200 column. Various sugar-free products were analyzed using the presented method, demonstrating the performance of this HPAEC-PAD system solution.

Electrochemistry Discover the difference

Introduction

The increasing consumer awareness regarding the health impacts of dietary sugars has led to a significant shift towards sugar-free products [5,6]. The market now offers a variety of alternatives to traditional sugar, including sucralose an artificial sweetener, that plays a distinct role in providing sweetness without the associated caloric intake of sugars like sucrose. Sucralose is particularly appealing for individuals seeking to manage their weight, reduce their risk of chronic diseases such as diabetes, and maintain overall health [2].

Sucralose, derived from sucrose, is notable for its intense sweetness, which is approximately 600 times that of sucrose, allowing it to be used in minimal amounts without contributing calories[2]. Sugars like glucose, fructose and sucrose are naturally present in for example vegetables and fruits, but they are also commonly added to processed food and drinks, as well as sugar-free drinks. Glucose is a monosaccharide. It is a simple sugar, although not calorie-free, is often used in products where rapid energy supply is needed but must be regulated due to its impact on blood sugar levels [7]. Sucrose, commonly known as table sugar, remains a widely used sweetener, but its high caloric content and association with obesity and other metabolic disorders have made its alternatives more popular. [8] Sucrose is a disaccharide composed of glucose and fructose. Fructose, a natural sugar found in many fruits, is sweeter than sucrose and glucose but has a different metabolic pathway [9]. As it is sweeter than sucrose and glucose in equal amounts, it is commonly used as bulk sweetener.

Fig. 1. *ALEXYS Carbohydrate Analyzer.*

In the European Union, the marketing and labeling of sugar-free products, a nutrition claim, are governed by stringent regulations to ensure consumer safety and accurate information. The Regulation (EC) No 1924 / 2006 of the European Parliament and of the Council lays down the legal framework for the use of nutrition and health claims on foods. This regulation ensures that any nutrition claims made regarding the health benefits or nutritional value of sugar substitutes are scientifically substantiated and not misleading to consumers. The sugar-free claim can only be made whether a product contains no more than 0.5 g of sugar per 100 g or 100 mL [3]. Sucralose is regulated in specific directive 2003/ 115 /EC of the European Parliament and of the Council. This describes the sweeteners that are permitted in the different categories of food products together with the maximum permitted doses.[4] To check if the products meet the requirements there is a need for a sensitive and selective analytical method to separate and quantify sugars and sweeteners in food and beverages.

In this application note a method is presented to separate and quantify the sugars glucose, sucrose and fructose and the artificial sweetener sucralose using High-Performance Anion Exchange Chromatography (HPAEC) combined with Pulsed Amperometric Detection (PAD). For the separation of the sugars and artificial sweetener the new high-resolution anion exchange column, the SweetSep™ AEX200 based on highly monodisperse 5 µm particles is used.

Method

The HPAEC-PAD analysis was conducted using the ALEXYS Carbohydrate Analyzer, consisting of the ET210 eluent tray for nitrogen blanketing, a P6.1L quaternary LPG pump, an AS6.1 autosampler, CT2.1 column thermostat and DECADE Elite electrochemical detector (Figure 1). The SenCell with Au working electrode and HyREF (Pd/H_2) reference electrode was selected for sensitive detection of the carbohydrates. The HPAEC-PAD system was operated using the settings listed in Table 1. A few precautions were made to guarantee method reproducibility and system stability, particularly when working with ion exchange chromatography using a mobile phase at a high pH.

Carbonate ions

CO_2 present in the air can get easily dissolved in the mobile phase and form carbonate ions (CO_3^{2-}). These carbonate ions interfere with carbohydrate retention on anion exchangers due to their strong binding properties as a divalent ion. This will

Table 1

HPAEC-PAD conditions	
LC system	ALEXYS Carbohydrates Analyzer - quaternary LPG (Antec Scientific)
Detector	DECADE Elite electrochemical detector
Columns	SweetSep™AEX200, 4 x 200 mm column, 5 µm SweetSep™AEX200, 4 x 50 mm precolumn, 5 µm Borate ion trap, 4 x 50 mm column, 10 µm (all columns Antec Scientific)
Mobile phase (MP)	MP A: 49.5 mM NaOH + 60 mM NaOAc MP B: DI water MP C: 50 mM NaOH MP D: 100 mM NaOH + 100 mM NaOAc (resistivity > 18 MOhm.cm and TOC<10ppb) Eluents blanketed with Nitrogen 5.0
Flow rate	0.7 mL/min
System pressure	About 220 bar (during isocratic elution)
Injection	10 µL
Temperature	30 °C for separation, 45 °C for detection
Flow cell	SenCell with Au WE, stainless steel AUX and HyREF (Pd/H_2) reference electrode , 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	0.5 Hz
Range	2 µA/V

Table 2

Step-gradient program		
Time (min)	*Mobile phase*	*Description*
0 - 10	10 mM NaOH	Isocratic elution and detection
10 - 20	49.5 mM NaOH + 60 mM NaOAc	
20 - 25	100 mM NaOH + 100 mM NaOAc	Column clean-up and regeneration
25 - 60	10 mM NaOH	Equilibration, starting conditions

*) Prepared by proportioning 80% B and 20% C.

lead to shorter retention times, decreased column selectivity, loss in resolution, and poor reproducibility. To minimize the introduction of carbonate ions in the mobile phase the eluents were carefully prepared manually using a commercially available carbonate-free 50% w/w NaOH solution. The diluent was DI water (resistivity >18 MΩ.cm, TOC < 5 ppb). During analysis, the ET210 eluent tray is used to pressurize the headspace of the mobile phase with inert Nitrogen gas (0.2— 0.4 bar N_2 overpressure).

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. The retention time of carbohydrates is inversely correlated with the pKa value and increases significantly with molecular weight. Only polymeric anion-exchange columns are suitable for carbohydrate separation in this alkaline condition. Therefore, a strong anion-exchange column SweetSep™ AEX200 was chosen for the separation of the sugars and sweeteners. The high uniformity and monodispersity of the AEX200 resin allow for fast and high-resolution separation of carbohydrates.[10] The use of a pre-column filter is advised when using samples that might contain particulate matter.

The analysis of sugars and sweeteners is based on gradient elution outlined in Table 2. The initial condition was set to 10 mM NaOH for 10 minutes. Under these conditions the elution of the sugars glucose, sucrose and fructose took place. A step gradient to 49.5 mM NaOH + 60 mM NaOAc was applied for 10 minutes, during which the elution of sucralose took place. Subsequently, a column clean-up step (100 mM NaOH + 100 mM NaOAc) was executed for 5 minutes, followed by 35 minutes of re-equilibration to starting conditions, resulting in a total analysis time of 60 minutes. During the clean-up and regeneration step, all late eluting interferences and carbonate ion build-up will be removed from the column, ensuring reproducible analysis. The separation temperature was set at 30°C. Note, that it usually takes a few runs to equilibrate the HPAEC-PAD system and get stable retention times.

Detection

The Antec SenCell was used for the pulsed amperometric detection of carbohydrates. This flow cell [11] has a confined wall-jet design and consists of a Au working electrode (WE), a HyREF (Pd-Hydrogen) reference electrode (RE), and a stainless-steel auxiliary electrode (AE). For detection, a 4-step potential waveform was applied. 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 [12], resulting in less flow cell maintenance and system down time. [12]. The oven temperature was set at 45° C. Under the specified conditions the cell current was typically about 0.2-0.4 µA.

Preparation of standards, reagents and samples

Standard: Individual sugars and artificial sweetener (glucose, sucrose, fructose and sucralose) were purchased from Sigma or Carbosyth. The 10 and 1 mM stock standards of the individual sugars and artificial sweetener were prepared in 95/5 (v/v%) water acetonitrile. To prevent fast degradation and suppress bacterial and fungal growth a small amount of acetonitrile is added. The stock standard is stored in the freezer at -20 °C and is stable for more than a month. The working standard mixes were prepared by serial dilution of the stock standard with ID water. The working standard mixes were prepared in a concentration range of 0.02 - 35 mg / L (0.1 - 200 µM) for glucose and fructose, 0.03 - 70 mg / L (0.1 - 200 µM) for sucrose and 0.04 - 80 mg / L (1 - 200 µM) for sucralose. Prior to use these working standard mixes were stored in the fridge at 4 °C.

Samples

Three different sugar-free beverages and one chewing gum were analyzed, all commercially available and purchased from a local supermarket in the Netherlands. The following samples were investigated:

- Royal Club mocktails gin tonic 0%
- Red Bull zero
- Red Bull sugarfree
- Ahold Fresh mint chewing gum

The sugar-free beverages were prepared by adding 25 mL of the drinks in a beaker, which was allowed to stand in the ultrasonic bath for 30 minutes to remove dissolved CO_2 gas. The filtrate was diluted to the desired concentration with DI water. Subsequently, the solutions were filtered through a 0.2 µM polyethersulfone (PES) syringe filter. Subsequently, 10 µL of the filtered solution was injected in the LC system and analyzed.

The chewing gum was frozen at -20 °C overnight and cut/ chopped into small pieces. 250 mg of chopped chewing gum was transferred into a 50 mL centrifuge tube and 25 mL of DI water added. The centrifuge tube was sonicated to promote the dissolution process. After sonication, the solution was stirred at 50°C for 1.5 hours. This is followed by stirring at room temperature for 1 hour. 6 aliquots of 1 mL were pipetted into Eppendorf vials and then centrifuged for 5 minutes at 6000 rpm. The supernatant was collected and filtered twice over Whatman 589/1 filter paper. The filtrate was diluted to the desired concentration and as final step the diluted solution was filtered through a 0.2 µM polyethersulfone (PES) syringe filter. Subsequently, 10 µL of solution was injected in the LC system and analyzed.

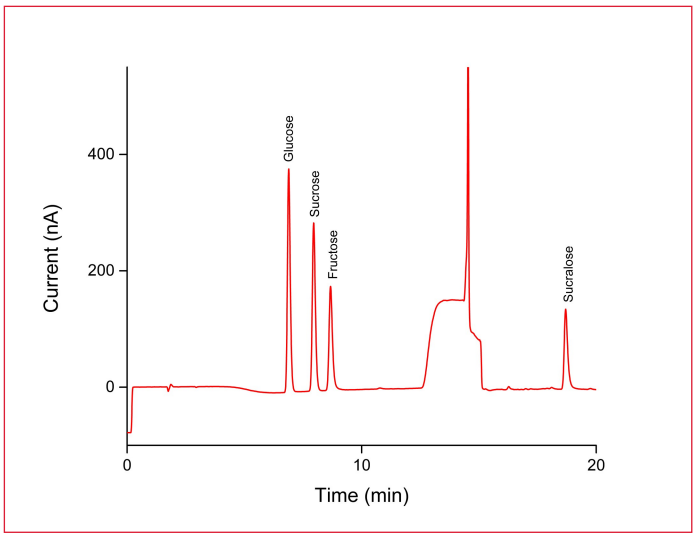

Fig. 2. *The chromatogram obtained from a 10 µ injection of the 10 µM standard mix with glucose (2 mg/L), sucrose (3 mg/L), fructose (2 mg/L) and sucralose (4 mg/L) in DI water.*

Results

A chromatogram of a 10 µL injection of a 10 µM standard mix is shown in Figure 2. The standard mix consist of the three sugars: glucose, sucrose and fructose and one artificial sweetener: sucralose. All compounds in the standard mix eluted within 19 minutes and are baseline-separated (resolution > 1.5).

The peak efficiency for the three sugars was approximately 58.000-65.000 theoretical plates/meter. The tailing factor for these three sugars was between 1.1 - 1.3. For sucralose the peak efficiency was approximately 300.000 theoretical plates/ meter with a tailing factor of 1.4.

Linearity

The linearity was investigated in the concentration range of 0.02 - 35 mg / L (0.1 - 200 µM) for glucose and fructose, 0.03 - 70 mg / L (0.1 - 200 µM) for sucrose and 0.04 - 80 mg / L (1 - 200 µM) for sucralose. In Figure 3 the calibration curves of the four sugars are shown. All four sugars had an excellent correlation coefficient greater than 0.999. The calibration curves in Figure 3 were used for the quantification of the beverage samples.

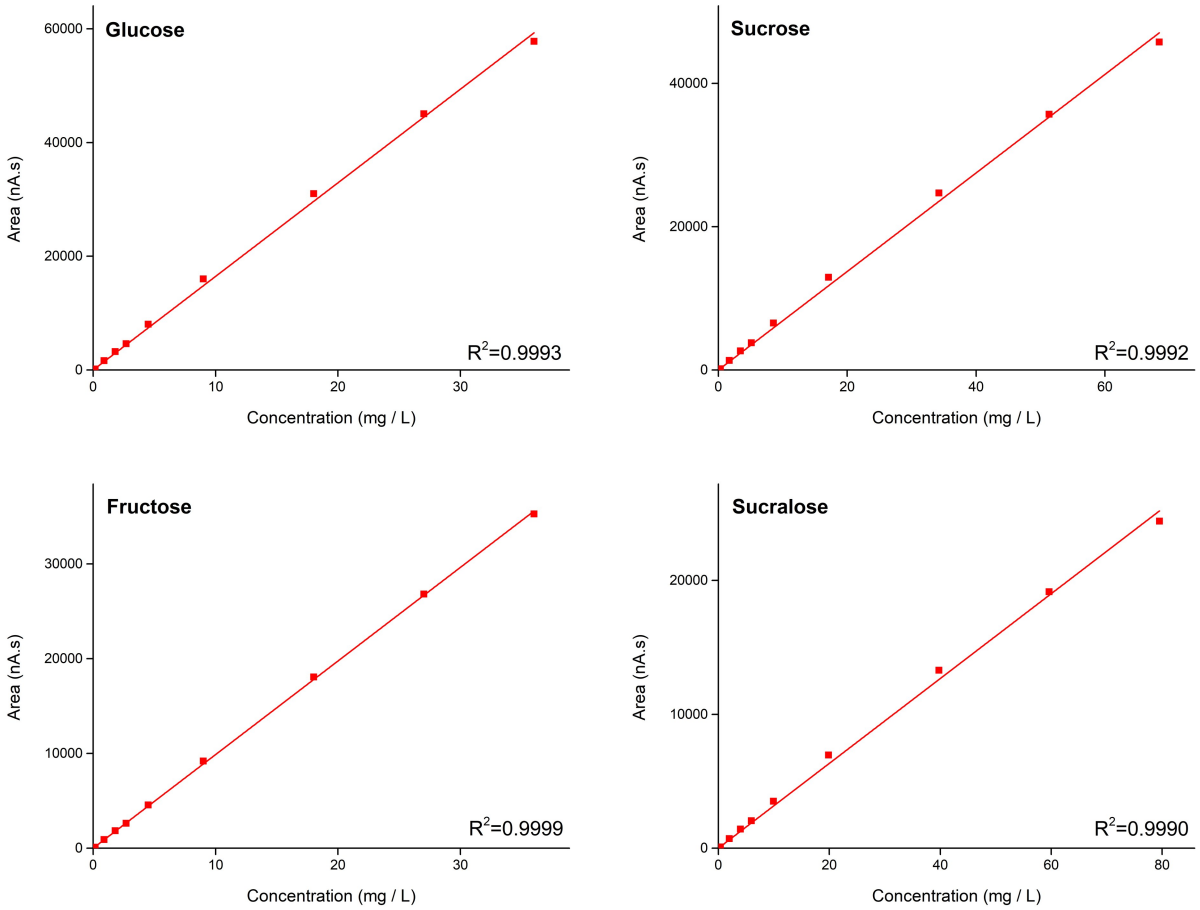

Fig. 3. *Calibration curves of the 3 sugars and sweetener in the concentration range of 0.02—35 mg/L for glucose and fructose, 0.03—70 mg/L for sucrose and 0.04—80 mg/L for sucralose. The fitted lines are forced through the origin (0,0).*

Repeatability

The repeatability was assessed by 10 repetitive injection of a 10 µM standard mix. This corresponds to a concentration of 2 mg/L for glucose and fructose, 3 mg/L for sucrose, and 4 mg/L for sucralose. The relative standard deviation (RSD) was determined for the retention time and peak area and listed in Table 3. The RSDs for retention time were below the 0.3% for all sugars. The RSDs for the peak area were below 0.9%, respectively. These data demonstrate that with this method reproducible analysis of all the analytes of interest can be achieved at this concentration.

LODs and LOQs

The limit of detection (LOD) and limit of quantification (LOQ) were determined for all sugars and sweetener in the standard mix. The LODs were calculated as the analyte response corresponding to 3x the ASTM noise (average peak-to-peak baseline noise of 20 segments of 0.5 min). The noise was based on a 10 minute section of the baseline. For glucose, sucrose and fructose the peak response of the 0.1 µM (glucose and fructose 0.02 mg/L, sucrose 0.03 mg/L) standard mix injection was obtained and used to calculate the LODs and LOQs. Considering that sucralose cannot be measured at a concentration of

Table 3

Repeatability of 10 µL injections of a 10 µmol/L standard mix in DI water (n=10)

	RSD (%)	
Compound	t_R	*Area*
Glucose	0.14	0.13
Sucrose	0.29	0.17
Fructose	0.11	0.82
Sucralose	0.09	0.12

Table 4

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

	Limit of Detection (LOD)	*Limit of Quantification (LOQ)*
Compound	*µg/L (ppb)*	*µg/L (ppb)*
Glucose	5	18
Sucrose	14	45
Fructose	11	37
Sucralose	306	1019

0.1 µM with AST 2 due to its lower response factor, the peak response of the 1 µM (0.40 mg/L) standard mix injection was used to calculate the LOD and LOQ for sucralose. The LODs and LOQs are listed in µg/L (ppb) in Table 4. The detection limit found for sucralose with the step-gradient approach is a factor 3 higher than found for the target analysis described in application note 220_031 based on isocratic elution [13]. This is due to the larger noise level observed with the step-gradient method. However, the typical concentrations found in the sugar-free beverages was at least a factor 10 higher than the LOQ, assuring accurate quantification of the sucralose contents.

Sample analysis

For the analysis of the sugars and sweetener with the presented method, three commercially available sugar-free beverages and one brand of chewing gum are purchased from the supermarket in the Netherlands. The chromatograms of all the products are shown in Figures 4 – 7.

Table 5

Sugar and sweetener contents (mg / 100 mL)

Product	*Glucose*	*Sucrose*	*Fructose*	*Sucralose*
Gin tonic	0.03		0.02	10.1
Red Bull zero	15.8	0.4	0.5	15.7
Red Bull sugarfree	1.8		0.4	15.0
Ahold fresh mint gum *	0.19	0.06		0.66

*) Contents in mg / 100 g product

The nutrition label on the products do not disclose detailed information about the exact content of sugar and sweeteners in the beverages or chewing gum. The content of sugar and sweetener in the products was quantified using the external calibration curves shown in Fig 3. The sugar and sweetener contents found in the products are listed in Table 5.

Red Bull zero and sugarfree are both sugar-free products. They contain the same main ingredients, caffeine, taurine and B-group vitamins, but have a different taste. This might be attributed to the difference in sugar and sweetener content in these Red Bull beverages. Red Bull zero contains significantly more sugars.

In Regulation (EC) No 1924/2006 of the European Parliament and of the Council it is stated that sugar-free products may not contain more than 0.5 g of sugar per 100 mL or 100 mg of product [3]. It is evident from table 5 that the total content of the main sugars glucose, sucrose and fructose found in all sugar -free products are well within the specified limit. Nevertheless it should be noted that other minor amounts of sugars which might be present in such samples were not quantified.

The amount of sucralose in food stuff is regulated in the directive 2003/115/EC of the European Parliament and of the Council [4]. In non-alcoholic drinks the maximum allowed amount is 30 mg sucralose per 100 mL, while for chewing gum it is 150 mg sucralose per 100 mg. The sucralose content for all beverages and chewing gum fall within the specified limits (see table 5).

When measuring sucralose in combination with the common sugars glucose, sucrose, and fructose, a primary challenge is the

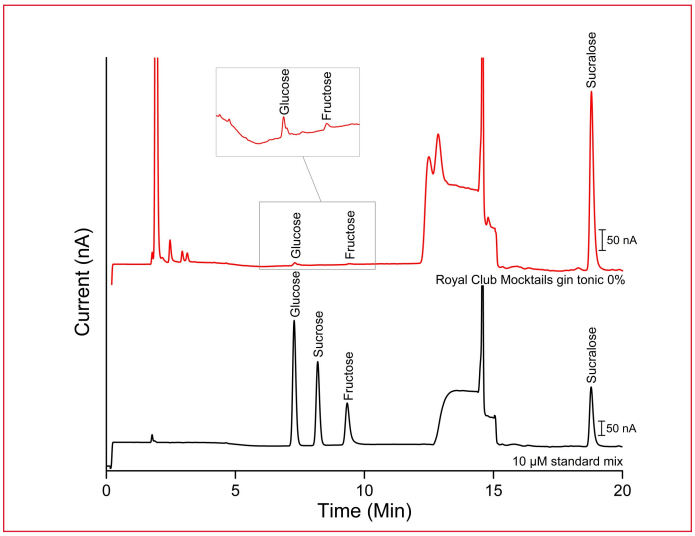

Fig. 4. *Chromatogram obtained from a 10 µL injection of the gin tonic (red) and a 10 µM standard mix in DI water (black)*

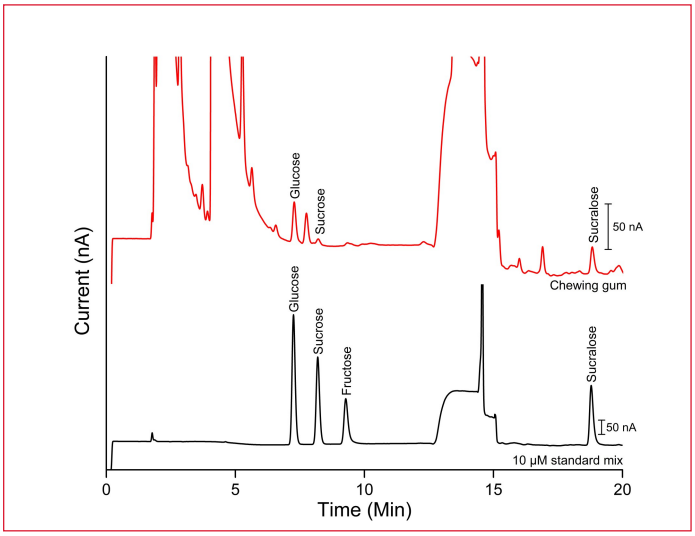

Fig. 5. *Chromatogram obtained from a 10 µL injection of the chewing gum sample (red) and a 10 µM standard mix in DI water (black).*

difference in response factor of sucralose compared to the sugars. The response factor of sucralose is significantly lower, which puts restrictions on the maximum sample dilution factor which can be applied to get signal responses for sucralose above its LOQ. For samples with a relatively large concentration of sugars this might be suboptimal for quantification, because their responses might fall outside the linear range for detection. For such samples the common sugars and sucralose should be quantified in separate runs with different sample dilution factors.

For the sugar-free samples analyzed in this study the concentration of sugars was sufficiently low relative to that of sucralose that all components of interest could be quantified in one run. However note that for different products different dilution factors were used for optimal quantification. The gin tonic and chewing gum samples were measured with a 10-fold sample dilution, whereas for the Red Bull samples a 20-fold diluted sample was used.

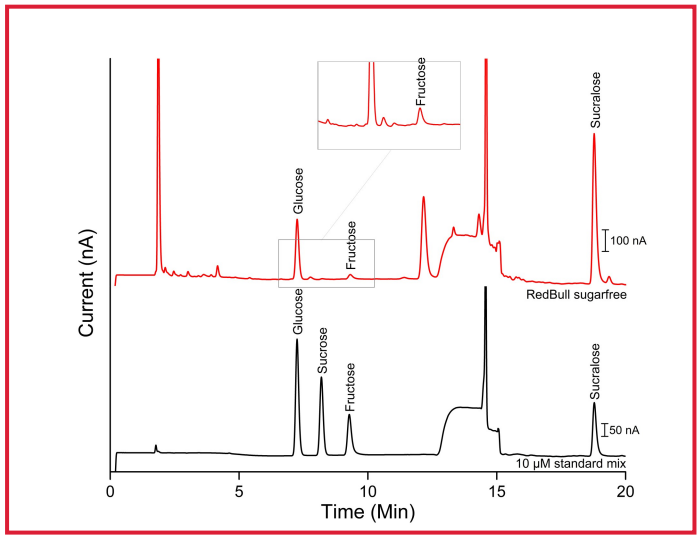

Fig. 6. *Chromatogram obtained from a 10 µL injection of the Red Bull sugarfree sample (red) and a 10 µM standard mix in DI water (black).*

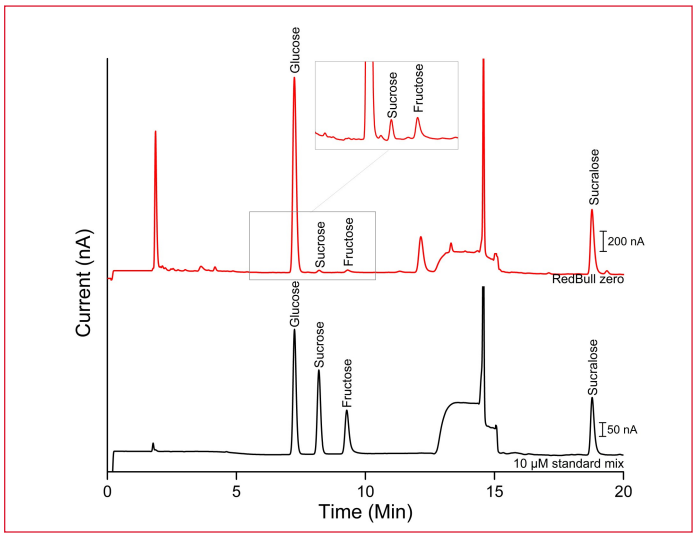

Fig. 7. *Chromatogram obtained from a 10 µL injection of the Red Bull zero sample (red) and a 10 µM standard mix in DI water (black).*

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 10 µM standard.

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

The sample recovery found for all sugars and sucralose in the samples ranged between 85% - 105%.

Conclusion

The ALEXYS Carbohydrate Analyzer in combination with the SweetSep AEX200 anion exchange column offers a tailor-made solution for the selective and sensitive analysis of common sugars and sucralose in sugar-free products based on HPAEC-PAD. The use of a high-resolution IC column based on 5 µm particle technology enables fast separation of the three relevant sugars (Glu, Fru, Suc) and sucralose within 19 minutes. The method applicability was demonstrated by successful analysis of different commercially available sugar-free beverages and chewing gum products.

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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 precolumn, 5 µm
260.0015	SweetSep™ AEX200, 4 x 50 mm precolumn, 5 µm
260.0030	Borate ion trap, 4 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.

*) 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.

Antec Scientific (USA)
info@AntecScientific.com
www.AntecScientific.com
T 888 572 0012

Antec Scientific (worldwide)
info@AntecScientific.com
www.AntecScientific.com
T +31 172 268888

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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