



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

Sucralose in beverages & chewing gum

- **ALEXYS Carbohydrate analyzer**
- **SweetSep™ AEX200 anion-exchange column**
- **High-resolution separation of sucralose**
- **SenCell™ with Au working electrode**
- **Artificial sweeteners in food & beverages**

Summary

Sucralose, discovered in 1978, is an artificial sweetener about 600 times sweeter compared to normal table sugar, sucrose. In humans the majority of ingested sucralose is not metabolized in the body and is excreted. Sucralose is therefore used in many foodstuff and beverages now-a-days as non-caloric sugar substitute. Sucralose was first approved for use in 1991 in Canada, followed by the US in 1998 and the EU in 2004 and is now used globally in more than 80 countries as sweetener. The use of sweeteners, like sucralose in food products in the EU is regulated in a specific directive 2003/115/EC, describing which sweeteners are permitted in the different categories of food products together with the maximum permitted doses. To check if products meet these requirements there is a need for fast, sensitive, and selective analytical methods to quantify sucralose in food and beverage.

Sucralose is a carbohydrate derivative which can be separated and detected using High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). In this application note a HPAEC-PAD method is presented for the fast and sensitive analysis of sucralose in food and beverages using the ALEXYS carbohydrate analyzer in combination with the new SweetSep™ AEX200 column. The separation in this method is based on the new SweetSep™ AEX200 column, a strong anion-exchange column with highly monodisperse 5 µm particles. Various sucralose containing products were analyzed to demonstrate the versatility of the method.



Introduction

Sucralose has attained global approval for its use as a non-caloric sweetener in both food and beverage products. It is synthetic disaccharide produced by chlorination of sucrose, selectively replacing three of the hydroxy groups—in the C1 and C6 positions of the fructose portion and the C4 position of the glucose portion [1,2]. The sweetness potency of sucralose is approximately 600 times greater than that of sucrose (table sugar). Consequently, only small amounts of sucralose are necessary to increase the sweetness in foods and beverages [2]. Each country has its own rules concerning the use of sucralose. Generally, Europe enforces a stricter regulation on sugar content in beverages and food compared to the United States [3]. Beverages in the United States primarily contain high fructose corn syrup (HFCS) as sole caloric sweetener. The use of HFCS in beverages may play a role in the epidemic of obesity and diabetes [4]. In Europe the Union of European Soft Drinks Associations (UNESDA) has committed to reduce the added sugar in beverages across the EU and UK by substituting sugars by artificial sweeteners, like sucralose, to encourage consumers towards healthier drink choices. The acceptable daily intake (ADI) of sucralose also varies for Europe and the United States. In the United states, the ADI is set at 5 mg/kg body weight/day, whereas in Europe it is higher, 15 mg/kg body weight/day[5,6]. Despite various benefits of sucralose as a sugar substitute, it also present several negative side effects. For instance, individuals sensitive to artificial sweeteners may experience gastrointestinal discomfort, including symptoms like bloating, diarrhea, or stomach cramps. Moreover, excessive consumption of sucralose may lead to adverse effects on weight management [1,2].

The use of sweeteners, like sucralose in food products in the EU is regulated in a specific directive 2003/115/EC, describing which sweeteners are permitted in the different categories of food products together with the maximum permitted doses [7]. To check if products meet these requirements there is a need for fast, sensitive, and selective analytical methods to quantify sucralose in food and beverage.

Sucralose can be quantified using for example HPLC in combination with UV or refractive index detection [8]. However, due to the lack of a strong chromophore in sucralose the sensitivity of UV detection is poor. HPAEC-PAD combines superior selectivity with sensitive detection. The hydroxyl groups on carbohydrates can be oxidized, therefore, carbohydrates can be detected without derivatization using pulsed amperometric detection with pico- and femtomol sensitivity [9-12].

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

Method

The HPAEC-PAD analysis of sucralose in beverages and chewing gum is performed using an ALEXYS carbohydrate analyzer consisting of a quaternary low-pressure gradient (LPG) pump, autosampler, column thermostat and DECADE Elite electrochemical detector with SenCell flow cell (see Figure 1) was used. The ALEXYS carbohydrate analyzer used in this study is an older version of the system shown in Figure 8 on the last page. The conditions for this analysis are listed in Table 1. The HPLC system was controlled using the DataApex Clarity Chromatography Data System (CDS) software version 9.0.1.19.



Figure 1: Left: SenCell with Au working electrode and Pd/H₂ (HyREF) reference electrode. Right: DECADE Elite electrochemical detector.

Separation

Carbohydrates can be separated using anion-exchange chromatography under alkaline conditions (pH >12). Weak acids, like carbohydrates, have pKa values ranging between 12 and 14. At high pH, compounds will either be completely or partially ionized based on their pKa value. For carbohydrate separation under alkaline condition only a polymeric anion-exchange column is suitable. The retention time of carbohydrates is inversely correlated with the pKa value and increases significantly with molecular weight. The SweetSep™ AEX200, a strong anion-exchange column, was chosen for the separation of sucralose (Table 1). This column is based on a monodisperse 5 μm resin coated with quaternary amine functionalized nanoparticles. The monodispersity and high uniformity of the resin allows for fast and high-resolution separation of carbohydrates. The AEX200 column (4 x 200 mm) was used in combination with a precolumn (4 x 50 mm).



Table 1

HPAEC-PAD conditions	
LC system	ALEXYS Carbohydrate 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 + 80 mM NaOAc MP B: 200 mM NaOH + 100 mM NaOAc MP C: 200 mM NaOAc MP D: deionized (DI) water (resistivity > 18 MOhm.cm and TOC<10ppb) Eluents blanketed with Nitrogen 5.0
Flow rate	0.7 mL/min
System pressure	About 210 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, 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 - 20	49.5 mM NaOH + 80 mM NaOAc*	Isocratic elution and detection
20 - 30	200 mM NaOH + 100 mM NaOAc	Column clean-up and regeneration
30 - 45	49.5 mM NaOH + 80 mM NaOAc	Equilibration, starting conditions

*) Prepared by proportioning 33% A, 40% B and 27% D.

A borate ion trap column (4 x 50 mm) was installed in the solvent line between the pump and autosampler. The temperature for separation was set at 30°C. The analysis is based on a step-gradient with sodium acetate as modifier, see Table 2. Addition of sodium acetate (NaOAc) to sodium hydroxide reduces the retention time of all carbohydrates. Sucralose is strongly retained on a anion-exchange column and therefore NaOAc is required to establish fast elution. Carbonate ions (CO_3^{2-}) present in alkaline hydroxide eluent will bind strongly to the active sites of the stationary phase,

resulting in loss of retention and column efficiency. A column clean-up /regeneration step after isocratic elution with 200 mM NaOH and 100 mM NaOAc is therefore necessary to remove the bound carbonate ions and strongly retained compounds present in food and beverage samples. The gradient program shown in Table 2, with a total run time of 45 minutes, was used to analyze the samples. The 10 minute regeneration step assures reproducible retention behavior for each run.

To minimize the introduction of carbonate ions in the mobile phase the eluents were carefully prepared manually using a carbonate-free 50% w/w NaOH solution (commercially available). The diluent was DI water (resistivity >18 MΩ.cm) which was sonicated and sparged with Nitrogen 5.0 prior to use. The mobile phase should be prepared in plastic bottles instead of glass. NaOH is a strong etching agent and will react with the inner glass wall resulting in the release of silicates and borates. The appropriate amount of 50% w/w NaOH solution was carefully pipetting into the diluent under gently stirring and Helium sparging to prepare the required the mobile phase solutions. The bottles with mobile phase and column clean-up solution were blanketed with Nitrogen (0.4 bar overpressure) during the analysis to minimize the build-up of carbonate ions in the mobile phase and to assure a reproducible analysis.

Detection

For the pulsed amperometric detection of sucralose the Antec SenCell electrochemical flow cell is used. This flow cell [15] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd/H₂) reference electrode (RE), and stainless steel auxiliary electrode (AE). The applied 4-step potential waveform is shown in figure 2. The detection temperature was set at 45 °C. Under the specified conditions, the cell current was typically about 0.2 - 0.4 µA with these PAD

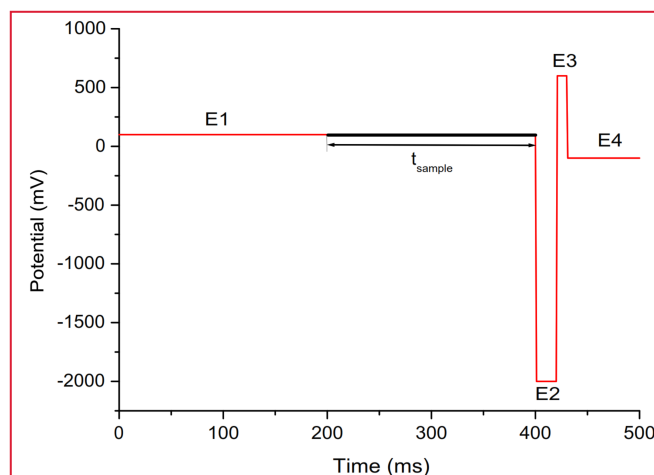


Figure 2: 4-step PAD potential waveform for the detection of monosaccharides and other carbohydrates.



settings. The 4-step potential waveform has several benefits: (1) consistent long-term peak area response and (2) less flow cell maintenance and system downtime as results of minimal electrode wear [16].

Preparation of standards, reagents and samples

Standards: Sucralose was purchased from Carbosynth. A 1 mM stock standard of sucralose was prepared in 95/5 (v/v%) water/acetonitrile. To minimize bacterial and fungal growth 5% Acetonitrile is added to prevent fast degradation. The stock standard was stored in the freezer at -20°C . At 4°C the stock standard is stable for more than a month. Working standards in a concentration range of 1 - 20 μM (0.4 - 8 mg/L) were prepared by dilution of the stock standards with DI water.

Sample preparation beverages: The following sucralose products were prepared and analyzed using the method described below:

- AH Ice tea zero
- Fanta orange NL
- Fanta orange USA

Procedure:

1. 25 mL of beverage sample was added to a beaker. Which was allowed to stand in the ultrasonic bath for 30 minutes to remove dissolved CO_2 gas.
2. The filtrate was diluted with DI water to the desired concentration.
3. Subsequently, the solutions are filtered through a 0.2 μm polyethersulfone (PES) syringe filter
4. 10 μL of the filtered solution was injected into the LC system and analyzed.

Sample preparation chewing gum: The following chewing gum, with sucralose, was prepared and analyzed using the method described below:

- AH fresh mint chewing gum

Procedure:

1. The chewing gum was frozen at -30°C overnight and then cut/chopped into small pieces.
2. 250 mg of the chopped chewing gum was weighted and transferred to a 50 mL centrifuge tube, followed by addition of 25 mL of DI water.
3. To help the dissolution process, the centrifuge tube was sonicated for 30 minutes.

4. After sonication, the solution was stirred at 50°C for 1.5 hours.
5. Following stirring in 50°C for 1.5 hours, the solution is stirred at room temperature for 1 hour.
6. 6 aliquots of 1 mL was centrifuged in Eppendorf vials for 5 minutes at 6000 rpm.
7. The supernatant was collected and filtered twice over Whatman 589/1 filter paper.
8. The filtrate was diluted with DI water to the desired concentration.
9. As a final step the diluted solution was filtered through a 0.2 μm polyethersulfone (PES) syringe filter.
10 μL of the filtered solution was injected into the LC system and analyzed.

Results

The chromatogram of an 10 μL injection of a 10 μM sucralose standard in DI water is shown in Figure 3. 10 μM corresponds to a mass concentration of 4.0 mg/L (4 ppm). Sucralose elutes approximately at a retention time of 6.8 minutes. The total run cycle time is 45 minutes due to the wash and equilibration step. The peak efficiency found for sucralose was 46,326 theoretical plates/meter with a tailing factor of 1.6.

Linearity

The linearity was investigated in the concentration range of 1 - 500 μM . This concentration range corresponds with a mass concentration range of 0.4 - 199 mg/L. Within the range of 1 - 450 μM the linearity was excellent (0.999), above 450 μM the curve became non-linear. For the actual quantification of sucralose in the samples a calibration curve in the range of 1 - 20 μM (0.4—8 mg/L) was used, see Figure 4 on the next page.

Repeatability

The repeatability of the method and system was evaluated by repetitive injections with the 10 μM sucralose standard, corresponding to a mass concentration of 4.0 mg/L (4 ppm).

Table 3

Repeatability of 10 μL injections of a 10 μM sucralose standard in DI water (n=10)

Compound	RSD (%) 10 $\mu\text{mol/L}$	
	t_R	Area
Sucralose	0.13	0.50

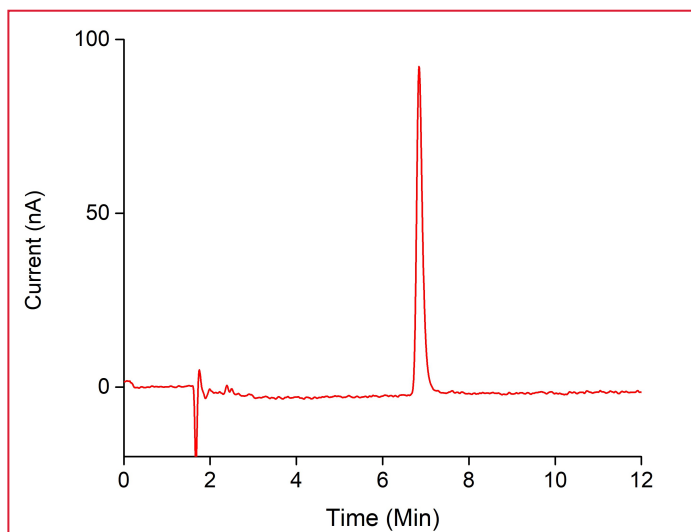


Figure 3: Chromatogram obtained with a 10 μ L injection of 10 μ M sucralose standard in DI water.

(average peak-to-peak baseline noise of 14 segments of 0.5 min). The noise was calculated based on a 7 minute section of the baseline. The LOD and LOQ were calculated based on the injection of the 4 μ mol/L sucralose standard recorded in the 2 μ A/V range.

Table 4

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

Compound	Limit of Detection (LOD)		Limit of Quantification (LOQ)
	μ g/L (ppb)	nmol/L	μ g/L (ppb)
Sucralose	97	245	324

Sucralose has a concentration detection limit of about 245 nmol/L. The calculated LOQ (10x S/N) for sucralose is about 324 μ g/L.

Sample analysis

In total 4 products, three beverages and a chewing gum, were purchased from supermarkets in the Netherlands and analyzed using the presented method, see Table 5.

Table 5

Sugar content in samples

Product	Sucralose listed on the product label	Sugar content in g per 100 mL product
AH Ice tea zero	Yes	0.0
Fanta orange NL	Yes	5.6
Fanta orange USA	No	12.4
AH Fresh mint gum	Yes	0*

*) total sugars in g / 100 gram product.

Three of the products (Fanta orange NL, ice tea and chewing gum) list sucralose as artificial sweetener on the product label without specifying the exact content. Fanta orange USA does not specifically state sucralose as ingredient. The Ice tea beverage and chewing gum are both labelled as sugar-free product. All samples were prepared following the sample preparation procedure described in the method section. The content of sucralose in the samples was quantified using an external calibration curve based on sucralose standards in the concentration range of 0.4 - 8 mg/L. In addition, all samples were spiked with 10 μ M (4mg/L) sucralose for peak identification.

The chromatograms for all samples are shown in Figure 5-8. All figures contain an overlay of the chromatograms of the sample

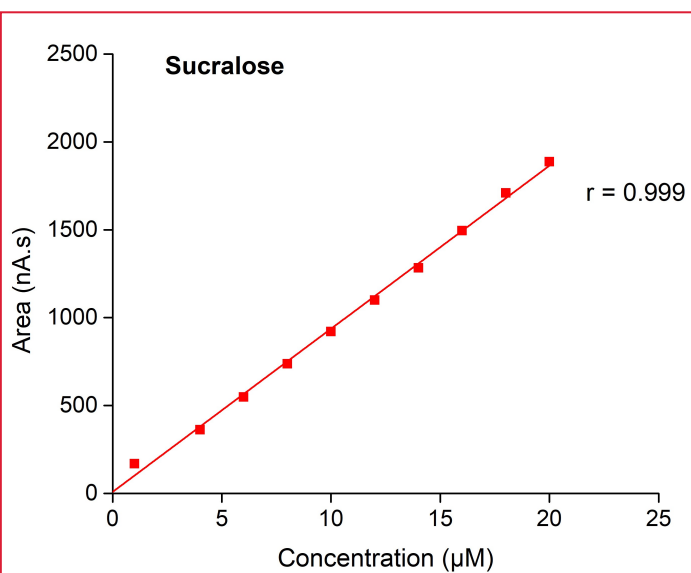


Figure 4: Calibration curve of sucralose standards in DI water in the concentration range of 1 - 20 μ M (0.4 - 8 mg/L).

The relative standard deviations (RSD) for retention time and peak area for sucralose are listed in Table 3. The good repeatability of the method is evident from the low RSD values obtained. The RSD for retention time and peak area were 0.13% and 0.50%, respectively. This data demonstrates that reproducible analysis of sucralose can be achieved with this method.

LOD and LOQ

The Limit of Detection (LOD) and Limit of Quantification (LOQ) for sucralose are shown in Table 4 . The LODs were calculated as the analyte response corresponding to 3x the ASTM noise



(red), spiked sample (blue), and the 10 μM sucralose standard (black). The sucralose peaks in the chromatograms are marked with a grey box.

Sucralose was identified in AH Ice tea zero, Fanta orange NL and AH fresh mint gum (Figures 5, 6, and 8). Fanta orange USA does not contain any sucralose as sweetener (Figure 7). There are no apparent interferences that coelute with sucralose in the chromatograms of the samples and the spiked samples. It is evident from Table 5 that Fanta orange produced in the USA contains a significant higher amount of sugar compared to the Fanta orange beverage produced in the Netherlands.

More than two times more sugar per serving of 100 mL. In addition, in US beverages often high fructose corn syrup (HFCS) is used as a sweetener instead of sucrose. Fructose is sweeter than sucrose (table sugar). Therefore, Fanta orange USA has a noticeable sweeter taste. In Europe the Union of European Soft Drinks Associations (UNESDA) has committed to reduce the added sugar in beverages across the EU and UK to encourage consumers towards healthier drink choices. One of the tools to reduce added sugars in regular beverages like Fanta, while keeping the same level of sweetness, is by addition of low- or no-calorie sweeteners like sucralose.

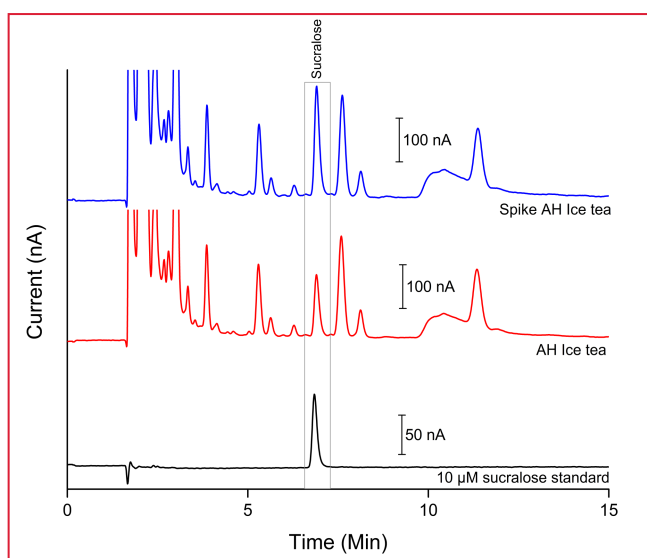


Figure 5: Chromatogram obtained with a 10 μL injection of the AH Ice tea sample (red), spiked Ice tea sample (blue) and 10 μM standard mix in DI water (black).

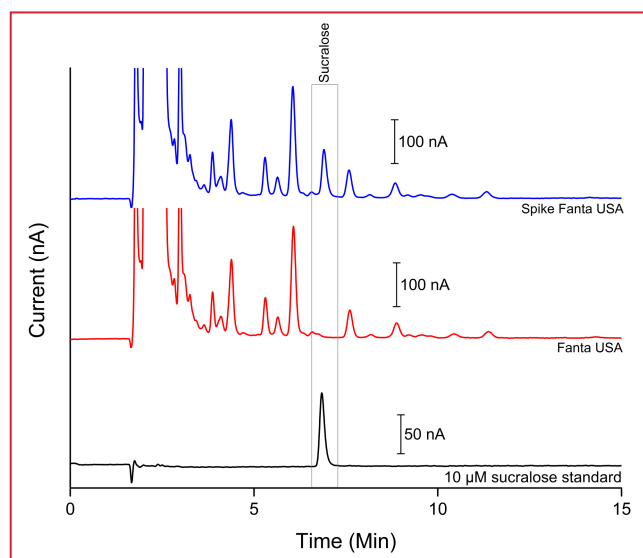


Figure 7: Chromatogram obtained with a 10 μL injection of the Fanta orange USA sample (red), spiked Fanta USA sample (blue) and 10 μM standard mix in DI water (black).

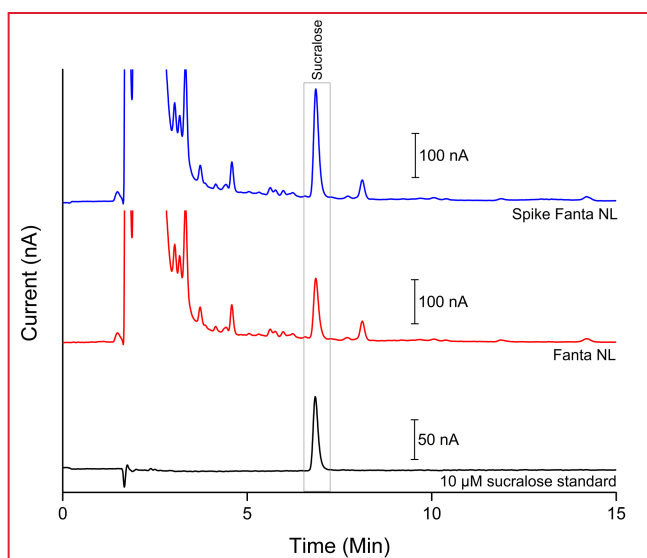


Figure 6: Chromatogram obtained with a 10 μL injection of the Fanta orange NL sample (red), spiked Fanta NL sample (blue) and 10 μM standard mix in DI water (black).

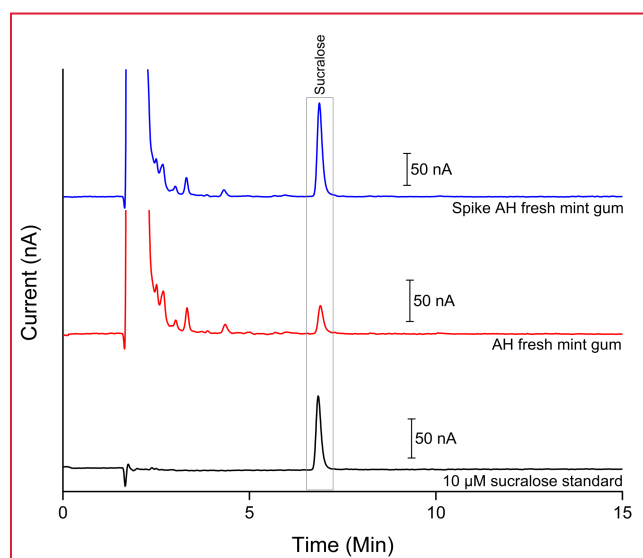


Figure 8: Chromatogram obtained with a 10 μL injection of the AH Fresh mint gum sample (red), spiked AH fresh mint gum sample (blue) and 10 μM standard mix in DI water (black).



The sucralose content in the analyzed samples are listed in Table 6. The sucralose values shown in Table 6 are based on quantification using the external calibration curve (Fig 4).

Table 6

Sucralose contents

Product	Sucralose	
	Content mg per 100 mL product	Maximum permitted dosage mg per 100 mL (2003/115/EC)
Ah Ice tea zero	5.5	30
Fanta orange NL	5.6	30
Fanta orange USA	-	-
AH fresh mint gum *	127.1	150

* Contents in mg / 100 g product.

For comparison, the sucralose content in the samples were also calculated based on standard addition using the 10 μ M sucralose spiked samples as single point calibrator (data not shown). There was a good correlation between the values in Table 6 and the contents calculated based on the standard addition method. The sample recoveries calculated for sucralose based on the responses of the analyte in the sample, spiked sample and 10 μ M standard, ranged between 109 - 115%.

The sucralose content quantified by HPAEC-PAD analysis were compared to the maximum permitted dosage listed in the EU directive 2003/115/EC [7]. The maximum allowed amount of sucralose according to the directive are 30 mg per 100 mL for non-alcoholic drinks and 150 mg per 100 g for chewing gum with no added sugar. It is evident from table 6 that the sucralose content found in all beverages and chewing gum are within the maximum allowed EU limits. The presented HPAEC-PAD method has an LOQ of about 0.3 mg/L, which is approximately a factor 50–100 x below the upper limit of the sucralose concentration expected in the samples, allowing accurate quantification of sucralose at such concentration levels.

Conclusion

The ALEXYS Carbohydrate Analyzer in combination with the SweetSep™ AEX200 anion exchange column offers a selective and sensitive analysis solution for the quantification of sucralose in food and beverages.

The presented HPAEC-PAD method based on the new SweetSep™ AEX200 column demonstrates fast separation of sucralose (within 8 min) with high resolution in several food & beverage samples containing sucralose.



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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
Columns	
260.0010	SweetSep™ AEX200, 4 x 200 mm column, 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 also control drivers are available in Waters Empower™, Agilent OpenLab CDS and Agilent OpenLab CDS Chemstation Edition. Please contact Antec for more details.

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

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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