Application Note
Food & Environment

The finest LC-EC Applications for Food & Beverage analysis

Phenols
Bisphenol A
Catechins
Flavonoids
Phenols
Antioxidants
Resveratrol
Epicatechin
Quercetin
Other polyphenols

Carbohydrates
Monosaccharides
Lactose
Other oligo- and polysaccharides

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

Mono-, di- and trisaccharides in plants

- **Carbohydrates in plant ecology**
- **Analysis of plant tissue extracts & root exudates**
- **Pulsed Amperometric Detection (PAD)**
- **SenCell with Au working electrode**

Summary

In this application note the analysis of mono-, di- and trisaccharides in plants (leafs, root exudates) is demonstrated using the DECADE Elite electrochemical detector and SenCell, in combination with an Agilent 1260 Infinity Bio-Inert LC system. The method is based on separation by High Performance Anion Exchange Chromatography (HPAEC) in combination with Pulsed Amperometric Detection using a 4-step potential waveform.

The method development and all data presented in this application note are courtesy of Mrs. Ciska E. Raaijmakers, Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands. NIOO-KNAW is a research institute focused on fundamental and strategic research in the field of animal -, plant - and microbial ecology in terrestrial and freshwater environments. The scientists of the department of Terrestrial Ecology are experts in the ecology, physiology and chemistry of plants, and in soil ecology, entomology and behavioral biology. They investigate the interactions between plants, the animals and diseases that damage plants above and below the ground, and the natural enemies of these attackers [1].

Introduction

Plants, like all organisms, require energy for growth and this is achieved via photosynthesis. In photosynthesis light energy is converted into chemical energy in the form of sugars by the so-called chloroplasts (most abundant in leaf cells). These carbohydrates, or sugars, are essential for the plants metabolism (plants growth and quality) and also provide nutrition to natural enemies. Plants are the primary food source on earth for a wide range of above ground and below ground organisms. Plant roots release a wide range of carbon-containing compounds into its rhizosphere (an area of a few mm surrounding the root), the so-called root exudates. Among these components, sugars, amino acids and organic acids are released in the largest quantities. The level of sugars in plant tissue and the rhizosphere can be influenced by the plant response caused by shoot and /or root damage. Therefore, the measurement of changes in sugar levels in plants tissue and root exudates is important in terrestrial ecology [2].

Figure 1: Antec DECADE Elite detector (left) with SenCell (right) for the analysis of carbohydrates based on Anion-Exchange Chromatography with Pulsed Amperometric Detection.

High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) can be used for the compositional analysis and quantification of sugars in plant extracts and root exudate samples.

Method

The LC-EC conditions are listed in table 1. The analysis was performed using an Agilent 1260 Infinity Bio-Inert LC system, consisting of an quaternary low-pressure gradient pump, autosampler and thermostatted column compartment. For detection a DECADE Elite electrochemical detector with SenCell flow cell (see figure 1) was used in combination with an Agilent 35900E Series II Dual Channel Interface (A/D converter) to acquire the detector signal in the Agilent MassHunter software. The DECADE Elite was controlled via a PC using the Antec Dialogue Elite software. The DECADE Elite was interfaced with the Agilent 1260 Infinity Bio-Inert LC system using a dedicated connection solution for Agilent LC systems, consisting of an analog output-to-ADC and remote cable.

Table 1

LC-EC Conditions	
LC system	Agilent 1260 Infinity Bio-Inert LC system in combination with Agilent 35900E Series II Dual Channel Interface (A/D converter)
LC detector	Antec Scientific DECADE Elite electrochemical detector
Trap Column	Thermo Scientific BorateTrap 4 x 50 mm Inline trap column (placed between LC pump and injector)
LC Column	Thermo Scientific CarboPac PA1 250 x 2 mm ID analytical column + CarboPac PA1 50 x 2 mm ID guard column.
Mobile phase	Isocratic elution with 100 mM NaOH carbonate-free in water, the mobile phase is continuously sparged with Helium 5.0
Flow rate	0.25 mL/min
$V_{injection}$	5 µL
Temperature	Column at 20°C (separation), Flow cell at 30°C (detection)
Flow cell	Antec Scientific SenCell™ with Au WE and HyREF™ (Pd/H_2) RE, AST setting 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	1 - 2 µA
ADF	0.1 Hz
Range	2 µA/V

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. 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. The method for the analysis of sugars in plants is based on isocratic separation using an CarboPac PA1 250 x 2 mm ID anion exchange column and alkaline mobile phase (100 Mm NAOH, pH 13) followed by pulsed amperometric detection on a gold (Au) working electrode (SenCell). For optimal separation the column temperature was set to 20°C in the thermostatted oven compartment of the Agilent LC system.

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 deionized water (resistivity >18 MΩ.cm) which was sonicated and sparged with Helium 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 Helium (0.2 bar overpressure during the analysis to minimize the build-up of carbonate ions in the mobile phase and to assure a reproducible analysis. For more details about mobile phase preparation and precautions see the application note Carbohydrates in Food [3].

To eliminate the presence of Borate contaminants in the mobile phase a BorateTrap™ inline trap column was installed in the solvent line between pump and autosampler. Borate contamination in eluents can cause a significant loss of peak efficiency, especially for sugar alcohols (reduced monosaccharides) like Sorbitol and Mannitol. If borate is present in the mobile phase, it binds to both the anion-exchange stationary phase and carbohydrate molecules. A carbohydrate-borate complex is eluted less efficiently than the carbohydrate molecule itself, resulting in peak tailing or co-elution of the alcohol sugars. For the example described in this application, the installation of the Borate trap was crucial otherwise no separation and quantification of the alcohol sugars was possible.

Detection

For the pulsed amperometric detection [4] of the mono-, di and trisaccharides the Antec SenCell electrochemical flow cell is used, controlled by the DECADE Elite electrochemical detector. This novel flow cell [5] has a confined wall-jet design flow 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 is applied as shown in figure 2.

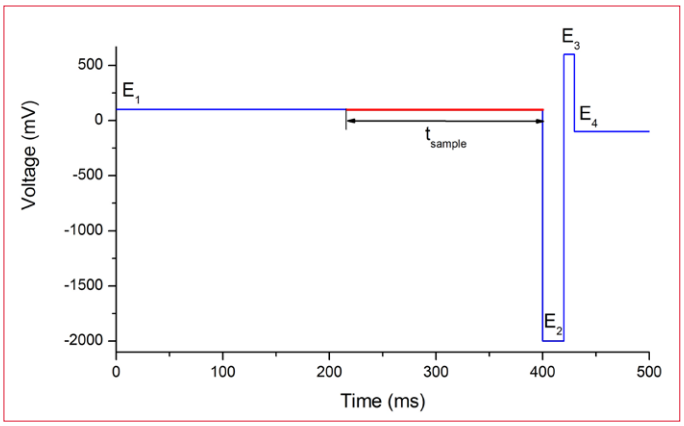

Figure 2: 4-step PAD potential waveform for the detection of carbohydrates.

This particular waveform resulted in an excellent reproducibility and minimal electrode wear [6]; i.e. resulting in less flow cell maintenance and system down time. The cell current was typical about 1 – 2 µA under the specified conditions. The temperature for detection was set to 30°C.

Sample preparation

In the section below the sample preparation procedures are described for [1] plant leaf samples and [2] root exudate samples.

[1] Plant samples: After collection plant samples were kept frozen until freeze-drying. Sample material was freeze-dried before analysis. After drying, samples were stored in a desiccator or air free bags until extraction, to avoid moist contamination. Extraction of the freeze-dried plant samples was performed in the following way:

- Dry sample material was crumbled and mixed in a dish.
- An Eppendorf tube containing 2 metal balls was filled with as much as possible crumbled dry sample material and ground using a Retch grinding machine (2 min 30 b/s).
- 50.0 mg of ground plant material was put into a 2 mL Eppendorf tube with perforated cap.
- First extraction:
  - 1 mL 70/30 % (v/v) MeOH/Water was added to the tube and the solution vortexed.
  - The solution was boiled for 5 minutes and subsequently sonicated for 15 min in an ultrasonic bath.
  - After sonication the solution was centrifuged for 10 min at 10000 rpm
  - The supernatant was transferred to a clean 2 mL Eppendorf tube using a pipette.
- Second extraction:
  - Again 1 mL 70/30 % (v/v) MeOH/Water was added to the original Eppendorf tube containing the remaining plant leaf sediment, and sonicated for 15 min in an ultrasonic bath.
  - After sonication the solution was centrifuged for 10 min at 10000 rpm.
  - The supernatant of the second extraction was transferred to the Eppendorf tube containing the supernatant of the first extraction.
- The total volume of the combined supernatant in the Eppendorf tube was adjusted to 2 mL by adding 70/30 % (v/v) MeOH/Water solution*.
- The obtained extracts were kept in the freezer (-20°C) until analysis.

For HPLC analysis the extracts were diluted 100x, by pipetting 10 µL into a HPLC vial and adding 990 µL MilliQ water, followed by capping and mixing. Subsequently, 5 µL of this solution was injected into the HPLC system for analysis.

*) Adjustment to 2 mL of the combined supernatant was done gravimetrically using the following procedure: three clean 2mL Eppendorf tubes were filled with 2 mL 70/30 % (v/v) MeOH/Water and weighted on an analytical balance. The average mass of the three tubes (at 0.001 g precise) was taken as reference and the weight of the tubes with combined supernatant were adjusted to that weight by adding 70/30 % (v/v) MeOH/Water.

[2] Root exudate samples: are obtained by flushing and filtering plant root cultures growing in glass-wool-water medium. 1 Liter of water extract used for flushing is subsequently freeze dried and concentrated to a volume of 1 mL, from which 5 µL is injected into the HPLC system for analysis.

Results

In figure 2 a typical chromatogram is shown of a 5 µL injection of a 10 ppm standard mix of 10 saccharides in water obtained with the HPAEC-PAD system using the specified conditions in table 1. All compounds elute within 30 minutes.

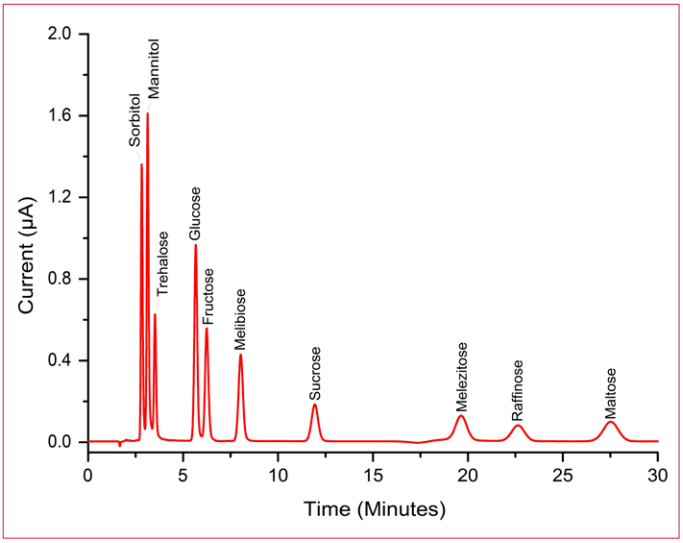

Figure 3: Chromatogram of a standard mix consisting of 10 ppm Sorbitol, Mannitol, Trehalose, Glucose, Fructose, Melibiose, Sucrose, Melezitose, Raffinose and Maltose in water (5 µL injection).

Linearity, Repeatability and Detection Limit

The linear response of the saccharides was investigated in the concentration range of 0.5 – 10 ppm. The obtained correlation coefficients were better than 0.999 for peak area for all compounds of interest (see table 2).

Table 2

Linearity, Repeatability and Detection Limit (LOD)

Compound	*Retention (min)*	*R (Corr. Coefficient) **	*RSD, Area (%) ***	*LOD (ppb) ****	*LOD (nmol/L) ****
Sorbitol	2.82	0.9999	1.41	27	148
Mannitol	3.13	0.9997	0.79	13	71
Trehalose	3.53	0.9999	1.70	21	61
Glucose	5.68	0.9999	1.58	16	89
Fructose	6.25	0.9997	1.97	21	117
Melibiose	8.05	0.9999	0.44	22	64
Sucrose	11.95	0.9999	0.81	38	111
Melezitose	19.68	0.9988	0.73	36	71
Raffinose	22.68	0.9999	1.78	26	52
Maltose	27.56	0.9998	0.53	29	85

*) The linearity was determined using a 5 point calibration curve based on a 0.5, 1, 2.5, 5 and 10 ppm standard.

**) The RSD of the peak area was determined with the 1 ppm standard. Population n=10.

***) the LOD's were calculated based on the response (area) of the lowest calibration standard of 0.5 ppm (n=10), where the LOD = 3.3 x standard deviation of the response / slope. See reference [7], ICH guideline.

The relative standard deviation (RSD) for peak area was determined for 10 replicate injections of the 1 ppm standard mix of the saccharides dissolved in water. The RSD for peak area was < 2% for all sugars (see table 2). The RSD's for the retention times of all components is typically ≤ 0.2%.

The Limit of Detection (LOD) for all saccharides are shown in table 2 in ppb and molar concentration. the LOD's were calculated based on the calibration curves (Area), as LOD = 3.3σ/S, where σ = the standard deviation obtained from the response (Area) of 10 repetitive injections of the 0.5 ppm standard, and S is the slope of the calibration curve [5]. The concentration detection limits for the sugars obtained were in the range of 50 – 150 nmol/L (10 – 40 ppb)., which is well below the concentration range typically found in such plant samples.

Plant samples

In the following section two examples are shown of the analysis of mono-, di- and trisaccharides in actual plant samples. Prior to injection into the LC system both samples were extracted following the procedure described in the sample preparation section. In figure 3 a chromatogram of a 5 µL injection of a leaf sample of the Brassica Oleracea (Brussels sprouts) is shown. In figure 4 a chromatogram of a root exudate sample of a tomato plant. The main peaks observed in both samples are originating from sucrose, fructose and glucose.

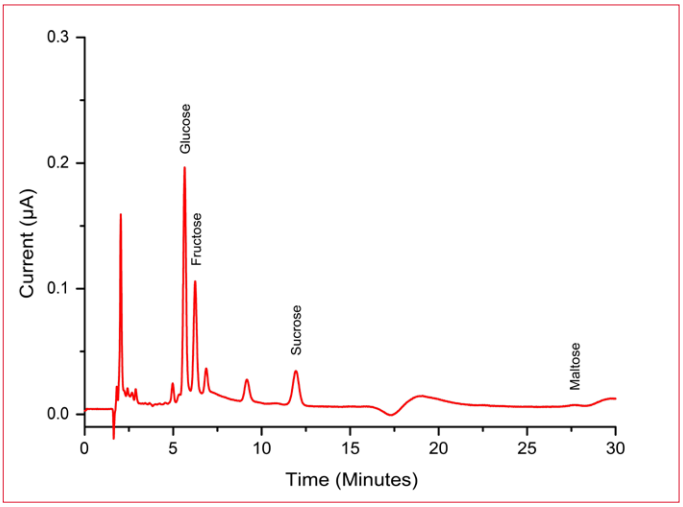

Figure 4: Chromatogram of a 5 µL injection of a Brassica Oleracea (Brussels sprouts) leaf sample, obtained using the extraction method described in the sample preparation section of this note.

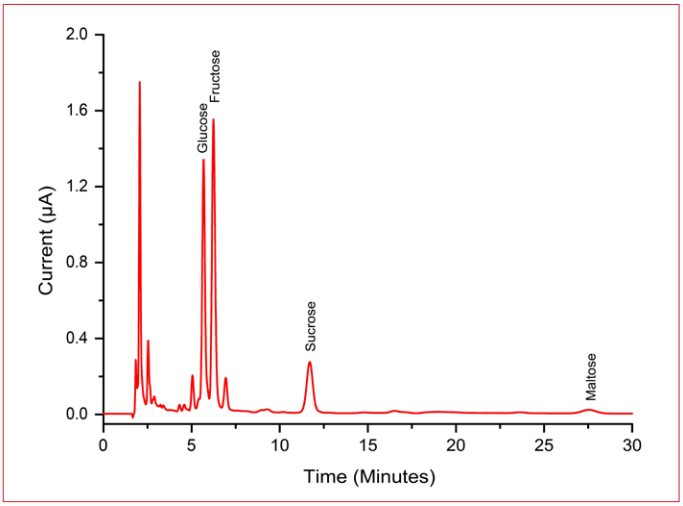

Figure 5: Chromatogram of a 5 µL injection of root exudate of a tomato plant sample obtained by flushing and filtering tomato root cultures growing in glass-wool-water medium.

The calculated carbohydrate contents (Glucose, Fructose, Sucrose and Maltose) for both samples are listed in table 3. These concentrations correspond to saccharide levels typically found in such plant samples.

Table 3

mono-, di- and trisaccharides contents in plant sample

Compound	*Concentration*	
	*(1) Leaf Brassica Oleracea (ng/mg)**	*(2) Root exudate tomato plant (mg/L)***
Glucose	3.7	1.5
Fructose	3.2	2.8
Sucrose	3.1	1.5
Maltose	0.2	0.2

*) concentration defined as ng sugar per mg freeze dried plant material.
**) sugar concentration in mg/L (ppm) in the 1 Liter of collected water extract used for flushing of the roots.

References

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7. ICH guideline, Validation of Analytical Procedures: Methodology, Q2(R1) (1994), 11 http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf

Conclusion

The DECADE Elite detector in combination with the SenCell flow cell offers a user-friendly and sensitive detection solution for the analysis of saccharides in plant extracts or root exudates using HPAEC-PAD. Easy integration of the DECADE Elite into a third-party LC system like the Agilent 1260 Infinity Bio-Inert LC system is provided by means of a (optional) dedicated connection solution for Agilent LC systems, consisting of an analog output-to-ADC and remote cable.

Below all part numbers related to the DECADE Elite detector, SenCell and cables for interfacing with an Agilent 1260 Infinity Bio-Inert LC system (+ Agilent 35900E Series II Dual Channel A/D converter) are listed.

Ordering information

176.0035	DECADE Elite SCC electrochemical detector, white
116.4321	SenCell 2 mm Au HyREF
250.0128A	DECADE Elite SCC output cable D9 - Agilent ADC - 3m
250.0129A	DECADE Elite remote cable D25 AZero - Agilent

047078*	BorateTrap Inline Trap Column, 50 x 4.0 mm ID
057178*	CarboPac PA1 analytical column, 250 x 2.0 mm ID
057179*	CarboPac PA1 guard column, 50 x 2.0 mm ID

*) The CarboPac and Borate trap columns used in this application are manufactured and sold by Thermo Scientific (https://www.thermofisher.com).

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the DECADE Elite electrochemical detector and SenCell. The actual performance may be affected by factors beyond Antec's control. Specifications mentioned in this application note are subject to change without further notice.