Application Note

Environment

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Carbohydrates
Monosaccharides
Other oligo- and polysaccharides

Phenols
Bisphenol A
Catechins
Flavonoids
Antioxidants
Resveratrol
Epicatechin
Quercetin
Other (poly)phenols
Chloro- and nitrophenols

Herbicides
Glyphosate
Aminomethylphosphonic acid (AMPA)

Carbohydrate Analysis in Plant Ecology

- **Mono-, di-, and trisaccharides in plants**
- **Samples: leaf extracts and root exudates**
- **Pulsed Amperometric Detection (PAD)**
- **SenCell with Au working electrode**
- **Sensitive & selective analysis**

Summary

In this application note the analysis of mono-, di and trisaccharides in plants (leaves and 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 in combination with Pulsed Amperometric Detection (HPAEC-PAD) 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].

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 HPAEC-PAD conditions that were used are listed in Table 1. The analysis was performed with an HPLC system with a quaternary low-pressure gradient pump, autosampler, a thermostatted column compartment, and the DECADE Elite electrochemical detector with SenCell flow cell (Table 1).

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 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

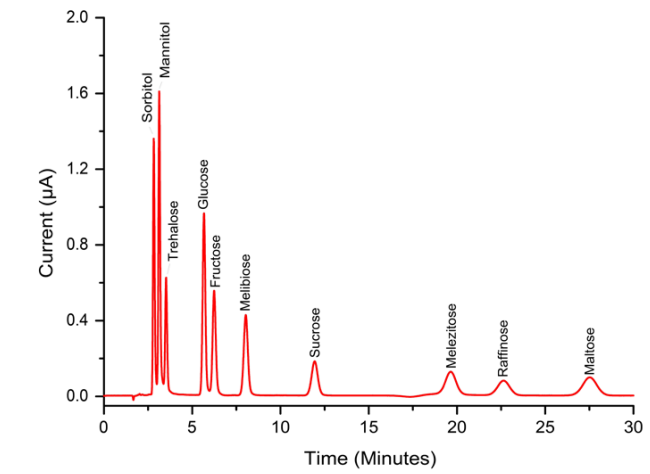

Figure 1: Chromatogram of a standard mix consisting of 10 ppm sorbitol, mannitol, trehalose, glucose, fructose, melibiose, sucrose, melezitose, raffinose and maltose in water (5 µL injection). Conditions as in Table 1.

temperature was set to 20 °C in the separate thermostatted oven compartment of the LC system (Agilent).

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

Table 1

Conditions

LC system	HPLC system with quaternary LPG mixer (for automated column cleaning)
Detector	DECADE Elite electrochemical detector
Columns	BorateTrap™ Inline Trap Column, 50 x 4.0 mm ID, 20 µm (between LC pump and injector) CarboPac™ PA1 IC column, 50 x 2.0 mm ID, 10 µm + 250 x 2.0 mm ID, 10 µm All columns: Thermo Scientific™ Dionex™
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/mL
Temperature	20 °C for separation 30 °C for detection
$V_{injection}$	5 µL
Flow cell	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
Range	2 µA/V
ADF	0.1 Hz
I-cell	1 - 2 µA

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 pipetted into the diluent under gentle stirring and helium sparging to prepare the required 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 Products[3].

To eliminate the presence of borate contaminants in the mobile phase an inline trap column was installed in the solvent line between pump and autosampler (Table 1). 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 trap was crucial, otherwise no selection 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 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 (Figure 2). This particular waveform results 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.

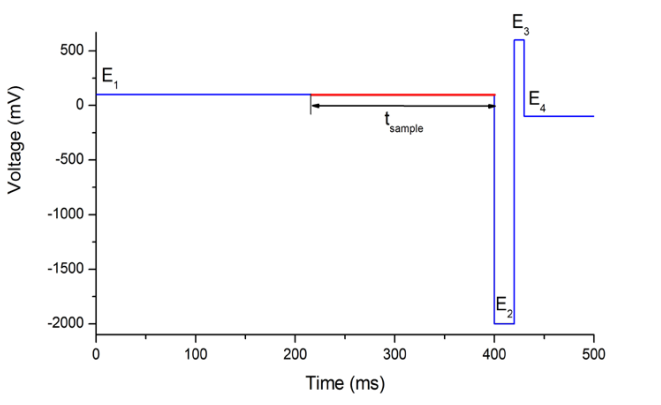

Figure 2: 4-step PAD potential waveform for the detection FDG and impurities.

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 2 mL 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:

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 1 a typical chromatogram is shown of a 5 µL injection of a 10 ppm standard mix of 10 saccharides in water. All compounds elute within 30 minutes.

Table 2

Linearity, repeatability and detection limit (LOD)

	Ret. Time (min)	*Corr. coeff. R (-) **	*RSD, Area (%) ***	*LOD (ppb) ****	*LOD (nM) ****
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 (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 .

Linearity, repeatability and detection limit (LOD)

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).

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 concentrations. 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).

Plant samples

This section shows two examples of the analysis of mono-, di and trisaccharides in actual plant samples. Figure 3 shows a chromatogram of a 5 µL injection of a leaf sample of the *Brassica oleracea* (Brussels sprouts), and Figure 4 shows 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.

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 trisaccharide content in two plant samples

Compound	*Concentration*	
	*(1) **Leaf** from Brassica oleracea (ng/mL) **	*(2) **Root exudate** from 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.

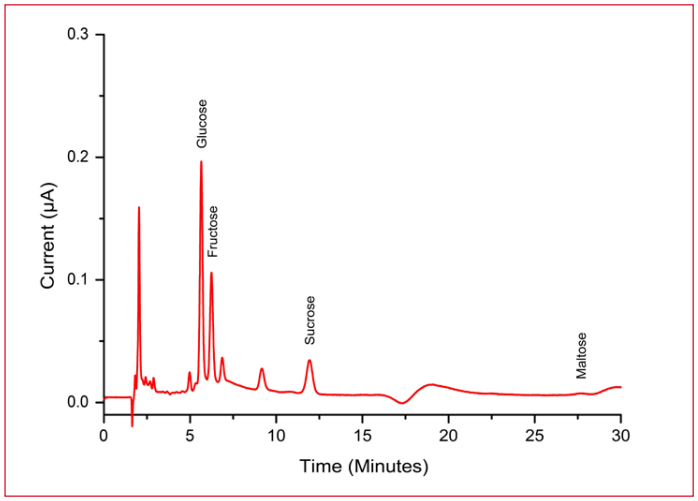

Figure 3: Chromatogram of a 5 µL injection of a *Brassica oleracea* (Brussels sprouts) leaf sample, obtained using the extraction method described in the sample preparation section of this note.

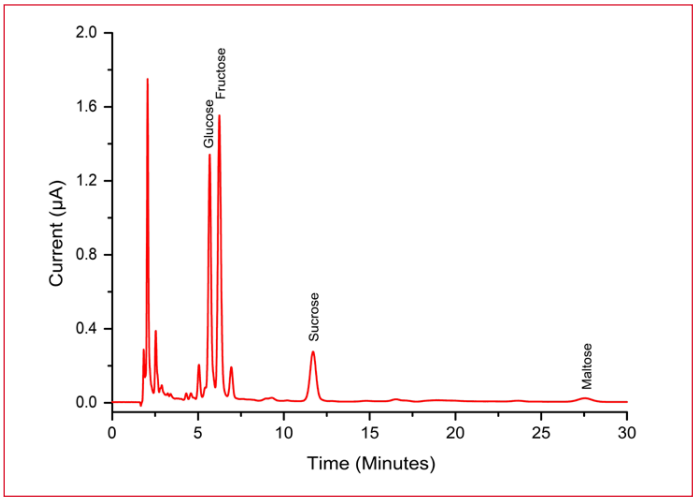

Figure 4: Chromatogram of a 5 µL injection of root exudate of a tomato plant sample obtained by flushing and filtering tomato root cultures growing in glass -wool-water medium.

References

1. Netherlands Institute of Ecology (NIOO-KNAW), https://nioo.knaw.nl/en/about-nioo
2. M. Van Geem, Y. A. Harvey, A.M. Cortesero, C.E. Raaijmakers, R. Gols, Interactions Between a Belowground Herbivore and Primary and Secondary Root Metabolites in Wild Cabbage, J. Chem. Ecol., 41 (2015) 41, 696–707 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4568014/pdf/10886_2015_Article_605.pdf 41:696–707. doi:10.1007/s10886-015-0605-7
3. Antec, Carbohydrates in Food Products, Application note, 220_002
4. W.R. Lacourse, Pulsed Electrochemical Detection In High Performance Liquid Chromatography, John Wiley & Sons, New York, 1ed,1997.
5. H. Louw, H.J. Brouwer, N. Reinhoud, Electrochemical flow cell, (2016) US patent 9310330
6. R.D. Rocklin, A.P. Clarke, M. Weitzhandler, Improved longterm reproducibility for pulsed amperometric detection of carbohydrates via a new quadruple-potential waveform, Anal. Chem, 70 (1998), 1496 – 1501
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 systems like the Agilent 1260 Infinity Bio-Inert LC system is provided by means of a software drivers for the DECADE Elite or dedicated analog output-to-ADC and remote cables.

Figure 5: Recommended instrument configuration for this application: the ALEXYS Carbohydrate Analyzer with Solvent Switch Valve.
The system consists of a P6.1L pump with integrated degasser and Solvent Switch Valve (SSV) for the option to run step gradients, an AS6.1L autosampler, an ET 210 Eluent tray for helium blanketing, and the DECADE Elite electrochemical detector. A CT 2.1 column oven with broad temperature range can be added optionally for separations under near-ambient temperatures. The ALEXYS Carbohydrate Analyzer can be operated under different Chromatography Data System (CDS) software: DataApex™ Clarity™ CDS (version 8.3 and up) or Thermo Scientific™ Chromeleon™ CDS (version 7.2 SR 5 and up).

Ordering information

Detector only - for connection to 3rd party HPLC	
176.0035A	DECADE Elite SCC electrochemical detector
116.4321	SenCell 2 mm Au HyREF
Recommended ALEXYS analyzer	
180.0055W	ALEXYS Carbohydrate Analyzer with Solvent Switch Valve
186.A05852	CT 2.1 column thermostat
116.4321	SenCell 2 mm Au HyREF
Software#	
195.0035	Clarity CDS single instr. incl LC, AS module

#) other option: Antec ECD drivers are available for use with Chromeleon CDS, OpenLAB CDS or OpenLAB Chemstation CDS. The ALEXYS Carbohydrates Analyzer can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

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