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Analysis of carbohydrates in plants by high-performance anion-exchange chromatography coupled with electrospray mass spectrometry

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Abstract

A mass spectrometer was coupled to high-performance anion-exchange chromatography (HPAEC) with the help of electrochemical neutralization of the eluent and post-column addition of lithium chloride for carbohydrate analysis. Parallel selective channels (single ion monitoring) were used to decrease the detection limits and separate unresolved peaks. The mass specific detection allowed the simultaneous analysis of a wide range of sugar alcohols, mono-, di- and oligosaccharides. Carbohydrates extracted from leaves of poplar submitted to drought stress were analyzed using pulsed amperometric detection (PAD), then mass spectrometry. It allowed the confirmation of peak attribution and the identification of salicin as a major compound in the extracts. Different responses to water deficit and re-hydration were obtained for several carbohydrates, suggesting different roles in osmoprotection processes.

Keywords: Ion chromatography; Anion-exchange; Mass spectrometry; Carbohydrates; *Populus euphratica*; Salicin

1. Introduction

Among the major constituents in plants, carbohydrates are known to vary with season, light availability and vegetative stage, reflecting growth and daily variation of photosynthesis. Carbohydrates are also generally involved in osmoprotection during abiotic stress like hyperosmotic stress. The carbohydrates involved consist in hexoses (mostly fructose and glucose), disaccharides (trehalose, sucrose), sugar alcohols (inositol, mannitol, etc.), and complex carbohydrates (raffinose, stachyose, etc.) [1].

Number of difficulties can be encountered during the chromatographic analysis of carbohydrates, especially due to the high diversity of carbohydrates occurring in nature. In addition, the wide range of functional groups (hydroxyl, amino, acetamino, phosphate) increases the chemical diversity thus greatly complicating the choice of stationary and mobile phases. Moreover, due to the absence of chromophoric or fluorescent groups, a derivatization step is often required to analyze carbohydrates with conventional detectors. Gas chromatography with FID or MS detection has been employed to separate and identify carbohydrates [2–6]. However, due to their high polarity, hydrophilicity and low volatility, carbohydrates have to be converted into acetate or TMS derivatives before GC analysis. For these reasons, HPLC is often preferred for carbohydrates quantification. Although UV detection can be used [7], underivatized carbohydrates are commonly detected by refractive index detection [8–9]. Recently, a fluorescence detector has been used to detect carbohydrates at the picomole level, after labeling with 2-aminobenzamide [10]. Size-exclusion chromatography, capillary electrophoresis and capillary electrochromatography are less used techniques to analyze carbohydrates [11–14].

High-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) is an efficient method to quantify carbohydrates in natural samples and food products [15–21]. In fact, due to their weak acidic properties ($pK_a \approx 12$), carbohydrates can

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be ionized in strong basic conditions. Thus, an accurate separation of carbohydrates can be achieved by using a strong anion exchange stationary phase and hydroxide solutions as eluent. PAD is an electrochemical method based on the oxidation of carbohydrates on a gold electrode by application of a positive potential. It provides high sensitivity and selectivity, without any time-consuming derivatization step. However, in plant extracts, as in many natural samples, the high complexity of the matrix may lead to coelution and excessive noise. Consequently, interferences between the matrix components and the target compounds can strongly affect the results by hindering peak attribution and integration. The mass spectrometric detection is highly selective and sensitive, and can therefore solve a lot of chromatographic troubles. However, MS is commonly considered to be incompatible with the highly alkaline eluents used in HPAEC. Technical improvements allowing the MS analysis of carbohydrates by HPAEC and results obtained on drought-stressed poplar are described in this work.

2. Experimental

2.1. Samples

Populus euphratica seedlings were grown for one year under natural daylight in greenhouse conditions, in 5 l pots containing a peat/sand mixture (50/50; v/v). About 80–100 cm high plants were then assayed for water deficit tolerance. Imposed drought was applied during 18 days until soil humidity reached 5 ± 2% and the plants were then maintained for 10 days under these water deficit conditions before re-hydration. Control plants were irrigated in order to maintain a soil humidity of 23 ± 3%. *P. euphratica* leaves were harvested during water deficit conditions at day 18, after at least 7 days of maintenance at 5% soil humidity (from Day 25 to 28) and after soil re-watering (Day 32). Generally, four or five different plants were used for leaf sample harvesting, nevertheless only two were used in control conditions at Day 32. Collected samples were directly frozen in liquid nitrogen and stored at −80 °C for carbohydrate analyses.

2.2. Carbohydrates extraction

About 150 mg of fresh matter were finely crushed in liquid nitrogen and extracted with 1.5 ml of an ethanol/water mixture (80/20; v/v) during 1 h. The samples were centrifuged at 3000 × *g*, at 4 °C, during 10 min. The supernatant was recovered and evaporated at reduced pressure. The extract was finally dissolved in 1 ml water, filtered at 0.45 μm and analyzed.

2.3. Analysis of carbohydrates using HPAEC-PAD

HPAEC-PAD analyses were conducted on a Dionex DX-500 chromatograph (Sunnyvale, USA) constituted by a Spark Midas autosampler, a GP-40 gradient pump and an ED-40 electrochemical detector. The analytical column was a Dionex Carbopac PA-10 (4 mm × 250 mm) with a PA-10 guard column (4 mm × 50 mm). The eluents were pure water and 300 mM NaOH. Percentages of both eluents were calculated to obtain the desired hydroxide concentration: 12 mM during 25 min, 12–150 mM in 1 min, 5 min at 150 mM, 150–12 mM in 1 min and column equilibration at 12 mM during 8 min. The flow rate was 1 ml min^{-1} and the column was kept at 30 °C. The PAD detection was achieved with a gold working electrode and an Ag/AgCl reference electrode, with a data collection rate of 2 Hz. The potential was set to 0.10 V during 0.41 s (integration between 0.1 and 0.4 s), −2.0 V during 20 ms, 0.6 V during 10 ms and −0.10 V during 60 ms. Carbohydrates were quantified using five-points calibration curves, ranging from 1 to 100 μmol l^{-1}.

2.4. Analysis of carbohydrates using HPAEC-MS

HPAEC-MS experiments were performed on a Dionex BioLC chromatograph, with an AS-50 autosampler, a GS-50 gradient pump and an EG-50 eluent generator. The mobile phase was on-line generated KOH at 0.25 ml min^{-1}. The hydroxide concentration was programmed as follows: 10 mM during 22 min, 10–100 mM in 1 min, 100 mM during 7 min, 100–10 mM in 0.5 min and column equilibration at 10 mM during 10 min. The analytical column was a Dionex Carbopac PA-20 (3 mm × 150 mm) with a PA-20 guard column (3 mm × 50 mm) at 30 °C. The desalter was an ASRS-Ultra with a constant current of 65 mA. The post-column addition of 0.5 mM LiCl was done with a Dionex AXP-MS auxiliary pump. MS data were acquired on a Finnigan MSQ quadrupole spectrometer with an electrospray interface (ESI). The probe temperature was fixed at 500 °C with a needle voltage of 3.5 kV.

3. Results and discussion

3.1. HPAEC-MS coupling

Due to their high salinity and low volatility, hydroxide eluents cannot be directly injected through the MS interface, where it can cause electrical shortcuts and capillary plugging by salt crystallization. The usual solution is to dilute alkaline eluents by a post-column addition of water. However, the concentrations of the analytes, and consequently the sensitivity of the detector, are reduced as well with this method. On the contrary, electrochemical desalting, also known as suppression, is an efficient way to convert hydroxide eluents into pure water. The neutralization of KOH is obtained by a combination of water electrolysis and ion-exchange: H^+ are produced by the oxidation of water and are then exchanged with K^+ ions of the eluent, which is therefore neutralized without diluting or modifying the analytes. In these conditions, background noise is significantly decreased