SeQuant Technical Note TN-006

Separation of Carbohydrate-related Metabolites from Leaf Tissue via ZIC®-HILIC Chromatography and on-line Electrospray Mass Spectrometry

Carbohydrates and other polar compounds in the plant metabolome are complicated to retain with traditional chromatographic techniques, but can easily be separated using a ZIC®-HILIC stationary phase. Combined with mass spectrometric detection, LOD's from 0.2-2.0 µM for neutral oligosaccharides, sugar alcohols, and sugar phosphates can be achieved.

Introduction

Carbohydrates and carbohydrate-related compounds make up a significant portion of the plant metabolome and can be determined at trace levels mass spectrometrically with electrospray ionization (ESI-MS). Since a large number of compounds in the metabolome are polar and hydrophilic (e.g. carbohydrates, organic acids, amino acids, and sugar phosphates) reverse phase (RP) HPLC is not a suitable technique to separate the compounds of interest.

Alternatively, high performance anion-exchange chromatography (HPAEC) coupled to pulsed amperometric detection (PAD) has been used for the separation of these compounds. While suitable separations can be obtained using HPAEC, the high salt content required for the separation makes MS detection difficult. Similarly, porous graphitic carbon (PGC) has been used for the separation of oligosaccharides and water soluble sugars. While the mobile phase used for PGC is compatible with MS, these separations may require extensive method development and the use of significant levels of carboxylic acids in the eluents in order to elute the most tightly-bound components.

Hydrophilic interaction liquid chromatography (HILIC) is a very powerful technique for the separation of complex mixtures of polar compounds. HILIC separates compounds using a mostly organic hydrophobic mobile phase with a hydrophilic stationary phase. The solutes elute in order of increasing hydrophilicity, which is the opposite of the elution order in reverse phase chromatography, and is especially useful for the separation of polar compounds that are poorly retained on reverse phase media.

ZIC®-HILIC chromatography is a unique form of HILIC that involves the bonding of zwitterionic sulfobetaine groups to a silica or polymer backbone of the stationary phase and thus allows for a significant aqueous fraction in the mobile phase. This allows greater solubility of polar analytes in the mobile phase and provides greater sensitivity. Typically, acetonitrile is used as the organic modifier with ZIC®-HILIC chromatography.

In this note, we discuss the separation and detection of a range of hydrophilic compounds (neutral sugars, sugars and sugar phosphates) which are found in the plant metabolome.

Experimental

Reagents and Eluents: Standards and reagents were reagent grade and eluents were HPLC grade. All were obtained from commercial sources. Polar metabolites were extracted (chloroform/methanol) from *Arabidosis thaliana* Col-0 wild-type or the *pgm1* starchless mutant leaf tissues, and reconstituted in acetonitrile/water.

Instrumentation: LC/MS analysis was performed on a Thermo Finnigan Surveyor HPLC system coupled to an ion trap MS (LCQ DECA XP Plus™, Thermo Scientific), equipped with an orthogonal electrospray interface.

Separation: The chromatographic separations were performed using a ZIC®-HILIC stationary phase (3.5 µm, 150 mm x 2.1 mm ID, mobile phase A was acetonitrile with 0.1 % formic acid (FA) (v/v), and mobile phase B was 5 mM ammonium acetate with 0.1 % FA (v/v) pH 4. A linear gradient from 10 % to 90 % B in 19 minutes was employed.

Results

Mixtures of standards were separated by the ZIC®-HILIC column including a mixture of a mono-, di-, tri-, tetra- and penta-saccharides, a mixture of sugar alcohols and inositol, and a mixture of different polar compounds found in plants. In each instance, good separation and retention for all compounds were obtained; neutral compounds were detected as formylated molecules, and sugar phosphates as deprotonated molecules. A typical series of ion chromatograms corresponding to the mixture of polar compounds is presented in Figure 1.

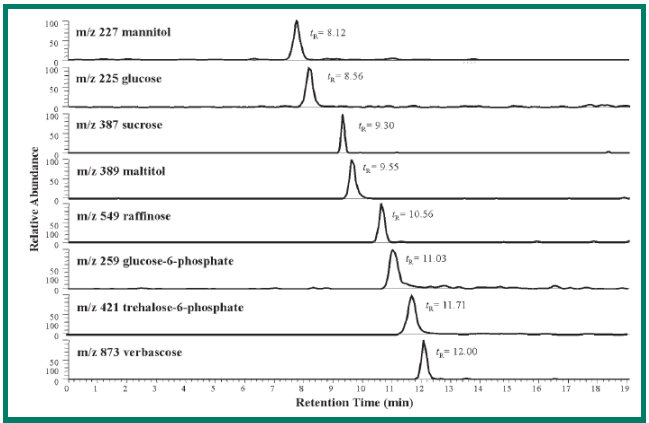

Figure 1: Extracted ion chromatograms of a standard solution of neutral sugars, sugar alcohols and sugar phosphates using ZIC®-HILIC column and gradient described in experimental.

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In the same vein, the ZIC®-HILIC column was capable of separating the isomeric monosaccharides Suc and trehalose (Tre), isomeric monosaccharide phosphates Glc1P and Glc6P, and the isomeric disaccharide phosphates Suc6P and Tre6P. The separation of the isomeric monosaccharides is shown in Figure 2.

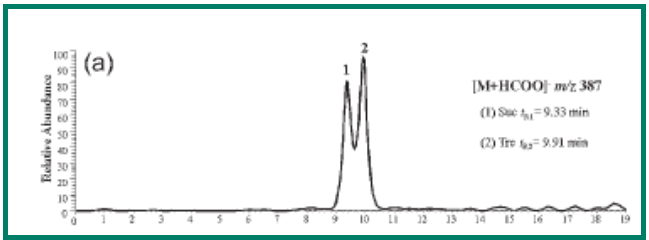

Figure 2: Separation of isomeric disaccharides Suc (*t*=9.33 min) and Tre (*t*=9.91 min) using ZIC®-HILIC column and gradient described in experimental.

Intra-day (3 x in a day) and inter-day (5 days) retention time studies were performed and provided RSDs of less than 2% for all compounds. The LODs ranged from 0.2 µM for neutral oligosaccharides to 1.0 µM for monosaccharides and sugar alcohols, and 2 µM for sugar phosphates, which is similar to those, obtained using PGC/ESI-MS.

The HILIC/ESI-MS method described above was used to separate and characterize the carbohydrate-related metabolites present in extracts of *A. thaliana*. The researchers found four metabolites present in the extract (Glc, Suc, raffinose and Glc6P) by comparison of the retention time, masses and ion trap tandem mass spectra with those of standards. The linearity of the method was determined by recording the response of five standards (0-100 µM) with R^2 >0.99. The metabolite levels of these four compounds in chloroform/methanol extracts of the two plants (Col-0 and *pgm1)* are shown in Figure 3; these levels were very similar to those obtained using a PGC stationary phase.

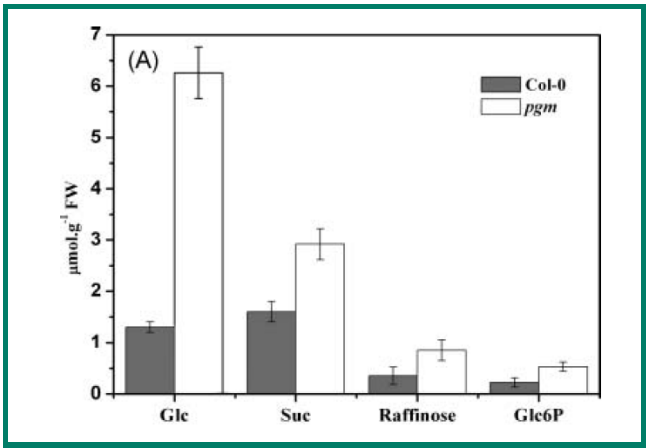

Figure 3: Metabolite levels determined in *A. thaliana* chloroform/methanol leaf extracts using ZIC®-HILIC stationary phase and the mobile phase described in the experimental section. Values are mean ± SD (n=3), each sample contained leaves from 3 rosettes.

Conclusion

Plant extracts contain a complex mixture of polar compounds including monosaccharides, oligosaccharides, sugar alcohols and sugar phosphates. It is difficult to separate these compounds via reverse phase and it is likewise difficult to detect them via MS if HPAEC is employed. However the use of the ZIC®-HILIC stationary phase enables their rapid and sensitive detection. As an example, a mixture of eight authentic standard compounds including four saccharides, two sugar alcohols and two sugar phosphates were readily separated in less than 15 min. The analytical methodology can be used to determine the level of various carbohydrate-related metabolites from extracts from plant tissue over the range 0 to 100 µM with LODs ranging from 0.2 µM for neutral oligosaccharides, 1.0 µM for sugar alcohols and 2.0 µM for sugar phosphates. The ZIC®-HILIC stationary phase allows for simple method development while the PGC gradient method may require extensive method development and strong conditions for the elution of highly polar compounds.

About ZIC®-HILIC Chromatography

The ZIC®-HILIC stationary phase is based on the covalently-bonded permanent zwitterionic sulfobetaine group shown in Figure 4. It is available with a silica support in 3.5, 5 and 10 µm particle sizes in various column dimensions from capillary to semi-preparative (75 µm up to 20 mm ID). In addition, it is available with a polymeric support in 5 µm particles (ZIC®-*p*HILIC). Merck SeQuant also publishes the tutorial booklet A Practical Guide to HILIC, which is available free.

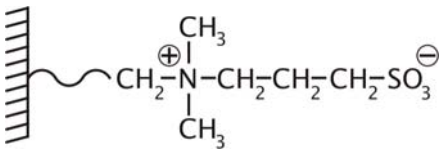

Figure 4: The Bonded Zwitterionic Sulfobetaine Group of ZIC®-HILIC.

About SeQuant

Merck SeQuant is a Swedish company that develops a broad range of innovative products for separation and purification of complex samples via chromatography. The company was founded in 1987 by researchers from Umeå University. Products include ZIC®-HILIC columns for HPLC, Membrane Suppressors and Suppressor Regeneration Systems for Ion Chromatography, Proteomic Calibration Kits, Post Column Reaction Delay and Mixing Coils which are marketed and supported around the world by a series of distributors.

Reference

Note: This application note is condensed from the scientific paper "Hydrophilic interaction chromatography /electrospray mass spectrometry analysis of carbohydrate-related metabolites from *Arabidosis thaliana* leaf tissue by Antonio, C., Larson, T., Gilday, A., Graham, I., Bergström and Thomas-Oates, J. *Rapid Commun. Mass Spectrom.* 2008, **22:** 1399-1407.

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