Separation and Identification of Polar Metabolites by Ion Chromatography Coupled with High-Resolution Accurate-Mass Mass Spectrometry

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Executive Summary
Analyzing small metabolites is necessary to identify biomarkers of certain disease states. The separation and identification of different classes of metabolites requires multiple analytical techniques. Ion chromatography (IC) is an effective technique for separating polar metabolites as it uses both a polar stationary phase and a polar mobile phase. Additionally, High-Resolution Accurate-Mass (HR/AM) mass spectrometry can confirm the identity of the given metabolite. When IC is coupled with HR/AM mass spectrometry, a large quantity of polar metabolites can successfully be determined at low concentrations. This further complements other analytical techniques used for metabolite analysis including Ultra High Performance Liquid Chromatography (UHPLC), Hydrophilic Interaction Liquid Chromatography (HILIC), and HR/AM mass spectrometry.

Keywords
Metabolomics, isobaric compounds, Ion Chromatography, capillary RFIC, mass spectrometry

Metabolomics Analysis
The study of metabolomic phenotypes quantifies numerous small molecule components called metabolites found in biological fluids and cell extracts. The relative quantitation of an entire complement of low molecular weight metabolites in plant or animal samples is compared during the process known as metabolic profiling. Differences in the up or down regulation of metabolites in a disease state relative to that of a normal state allow for the identification of many potential biomarkers in a given sample. This facilitates identifying potential causes and cures of the disease. To fully understand these metabolomic changes in the disease state, samples are prepared in controlled experiments: controls (non-disease state or non-aggressive disease state), disease state (or aggressive disease state), and samples with gene-modifications associated with the disease (knock-down). These samples typically require lengthy generation and sample preparation times before analysis to separate and identify the metabolomic differences of the control, disease state, and knock-down samples.

The preparation and analysis of these typically small volume samples can be quite challenging. Careful handling and storage of these precious biological samples are important as a large amount of information must be obtained from limited samples. The quality of samples is affected by factors including but not limited to collection time, containers used, and the types of preservatives and additives.

These biological samples also tend to include complex mixtures of cell materials and small organic molecules including but not limited to organic acids, inorganic anions and cations, sugars, and sugar phosphates. In addition, these molecules are found in disparate concentrations within the samples and exhibit a broad range of polarities. The variety of these small metabolites requires the use of more than one analytical technique to effectively separate and identify isobaric compounds.
Separation and Identification of Metabolites

Common analytical techniques utilized to separate and identify metabolites includes the use of traditional reversed-phase chromatography, UHPLC or HILIC. Traditional reversed-phase columns using a non-polar stationary phase are most effective for hydrophobic metabolites. The use of UHPLC or HILIC is generally a viable option, but can be limiting for resolving polar compounds. Additionally, many of these compounds are isobaric which can prevent sole identification by mass spectrometry. However, ion chromatography is an alternative technique that provides improved quantification and sensitivity of polar compounds. An application example will be highlighted and discussed later in Figure 3. A typical IC system is shown in Figure 1.

IC Coupled with High-Resolution Accurate-Mass Mass Spectrometry

When utilized in combination, IC coupled with HR/AM mass spectrometry can successfully separate and identify a larger quantity of polar metabolites at low concentrations compared to UHPLC or HILIC. Direct coupling of IC with HR/AM mass spectrometry is possible with Reagent-Free™ IC (RFIC™) systems as the hydroxide mobile phase (eluent) is converted to pure water by the electrolytic eluent suppressor. RFIC systems have automated eluent generation to electrolytically create the required eluents used for IC applications, thereby increasing consistency and reproducibility.

IC with HR/AM Mass Spectrometry Interface

Continuous in-line desalting

In traditional mass spectrometry, the use of a hydroxide mobile phase (eluent) may be of concern due to its non-volatile and corrosive nature. However, in anion-exchange chromatography, electrolytic eluent suppression converts hydroxide mobile phase back to pure water. This occurs via an ion-exchange substitution of K+ with H+ ions. The analyte is simply converted to its acid form.
Figure 2 illustrates the chemistry used in an anion-electrolytic suppressor. The suppressor removes potassium from the eluent and replaces it with hydronium ions formed by the electrolysis of the water. These hydronium ions combine with the hydroxyl ions from the eluent to form water.

![Chemical Diagram](image)

Figure 2. Dionex electrolytically regenerated anion suppressor.

**Highlighted Application**

The introduction of capillary format RFIC systems further improves direct coupling with HR/AM mass spectrometry, especially in metabolomic research applications where sample size is commonly limited. A common approach using capillary RFIC systems is to perform a large-volume direct injection, which is suitable for samples with low levels of matrix ions. For example, a 10 μL injection onto a 0.4 mm i.d. column in a capillary IC system is equivalent to a 1000 μL injection onto a 4 mm i.d. column. Therefore, capillary RFIC systems are beneficial when sample volumes are limited.2

Coupling a capillary RFIC system with a HR/AM mass spectrometry has been demonstrated in profiling metabolic biomarkers (sugar monophosphates) of oral squamous cell carcinoma (OSCC) metastasis in cell lysates.1 Although sugar phosphates are generally difficult to separate by traditional LC methods, sugar phosphates are routinely separated by IC. Figure 3 demonstrates how a capillary RFIC system coupled to HR/AM mass spectrometry provides the superior resolution and analytical sensitivity of these isobaric, polar metabolites as compared to UHPLC and HILIC. Please note that in the capillary RFIC system to mass spectrometer interface, a polar desolvation solvent (methanol) was used for maximizing sensitivity.

![Graph](image)

Figure 3. Capillary IC-HR/AM results had higher resolution and higher peak intensities over UHPLC- and HILIC-HR/AM spectroscopy using the Thermo Scientific™ Q Exactive™ Mass Spectrometer. Eleven sugar monophosphates (m/z \( = 259.0225 \)) were resolved by capillary IC-HR/AM with ~100-fold higher intensities as compared to three sugar phosphates by HILIC-HR/AM.

Peaks:
1. myo-Inositol-1 phosphate or myo-inositol-3 phosphate*  
2. Closest to myo-inositol monophosphates*  
3. α-D-Glucose-1 phosphate**  
4. myo-Inositol-1 phosphate or myo-inositol-3 phosphate***  
5. α-D-Manose-1 phosphate***  
6. myo-Inositol-4 phosphate*  
7. α-D-Fructose-1 phosphate**  
8. α-D-Galactose-1 phosphate**  
9. D-Fructose-6 phosphate**  
10. α-D-glucose-6 phosphate*  
11. D-Mannose-6 phosphate*

The sugar monophosphates were assigned identification using MS/MS fragmentation spectra (MS²) and Metlin database,* MS² with retention time,** or only MS***
Conclusion
IC with HR/AM mass spectrometry provides additional sensitivity and resolving abilities needed to identify and quantify polar compounds at lower concentrations in metabolomics analysis. Inline desalting by electrolytic suppressors converts the mass spectrometer-incompatible hydroxide mobile phase (eluent) to pure water, thereby making the flow from the IC compatible with the HR/AM mass spectrometer. Additionally, capillary IC-HR/AM mass spectrometry minimizes the consumption of precious biological samples.

References