

# A New Approach to the Analysis of Anionic Metabolites by CESI-MS with Negative Ion Electrospray Ionization

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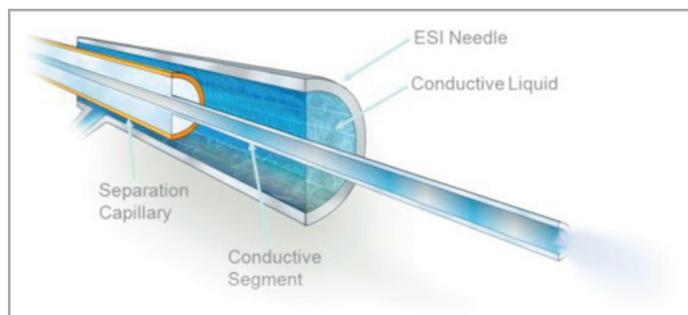
## Introduction

Despite recent developments in hyphenated techniques and column technology, the analysis of relatively small and highly polar analytes (e.g., small organic acids, phosphorylated sugars and underivatized amino acids) remains challenging. These small charged metabolites are difficult to retain on standard reversed-phase liquid chromatography (RPLC) columns and usually require HILIC/Hypercarb columns or ion chromatography. Ion chromatography runtimes are usually long, and Hypercarb columns usually need extensive conditioning before samples are run and to maintain peak shape. HILIC chromatography often requires that extracts contain organic solvents such as acetonitrile, which may not be the optimal solvent for highly polar compounds. Moreover, different mobile phases or LC columns are needed for the analysis of cationic and anionic metabolites, respectively.

Capillary electrospray ionization (CESI) is the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single process in a single device (Figure 1). CESI-MS operates at low nL/min flow rates and offers several advantages. These include increased ionization efficiency and a reduction in ion suppression. CESI-MS separates analytes by their charge and size and therefore is a complementary separation mechanism to more traditional techniques, such as RPLC. The purpose of this study was to investigate the use of CESI-MS to analyze highly polar and charged metabolites at physiological levels without the need for derivatization. This work evaluates how this technique can be used to separate isobaric and structurally similar metabolites, including phosphorylated sugars, and shows the utility of CESI-MS in negative ion mode for the analysis of metabolites representing various chemical families, such as sugar phosphates and organic acids.

## Materials and methods

**Chemicals:** All chemicals were reagent grade and were purchased from Sigma Aldrich.



**Figure 1.** The OptiMS cartridge provides an ultra-low flow ESI interface.

**Sample preparation:** Standards were prepared by dissolving the pure solids into water and serial diluting into water. The plant sample was provided by the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) in Germany and resulted from an extraction of wheat using 50% aqueous methanol.

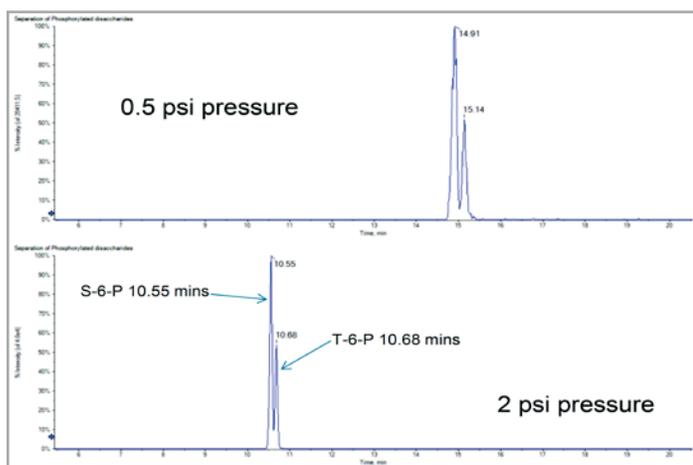
**CESI-MS method:** Sample was injected by pressure (5 psi, 5 s) in a bare fused-silica OptiMS cartridge (30  $\mu$ m ID x 91 cm), which was thermostatted using recirculating liquid coolant regulated at 25 °C. For this analysis, the SCIEX TripleTOF® 5600+ LC-MS/MS System was fitted with the NanoSpray® III Ion Source. Gas 1, gas 2 and temperature were not applied (set to 0, 0 and 50, respectively) because ionization at these very low flow rates occurs by simply applying the ion-spray voltage set at -2000 V. The curtain was set to 5 psi. The CE separation used the conditions shown in Table 1 with a background electrolyte of 10% acetic acid. Data was acquired by a looped experiment, where a single TOF-MS scan was acquired over the mass range 600–2000 amu together with 50 product ion TOF scans for potential metabolites. Where standards were available, the CE voltage was optimized for the product ion scan for the individual metabolite. The total cycle time for the analysis was less than 1.5 s and was optimized to give greater than 8 points across an analyte peak.

Action	Time (min)	Pressure (psi)	Direction	Voltage (kV)	Solution
Rinse	3	75	Forward	0	Methanol
Rinse	1	50	Forward	0	Water
Rinse	2	100	Forward	0	10% acetic acid
Rinse	2	75	Reverse	0	10% acetic acid
Injection	5s	5	Forward	0	Sample vial
Injection	10s	0.5	Forward	0	10% acetic acid
Separation	30	2	Forward	30	10% acetic acid
Voltage	3	5	Forward	1	10% acetic acid

**Table 1.** CESI separation method used for the detection of anionic metabolites.

## Results

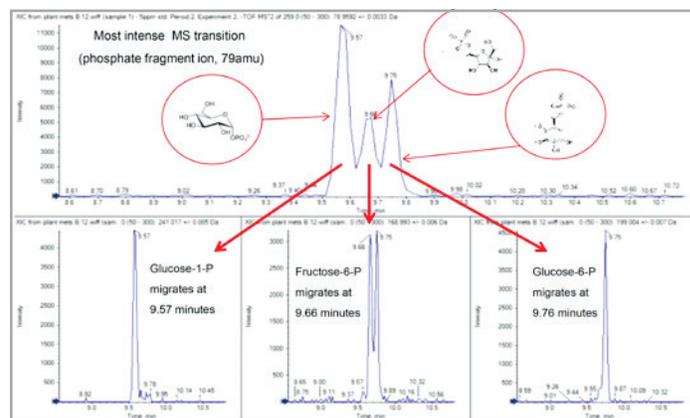
In CE, compounds are separated according to differences in their intrinsic electrophoretic mobilities (i.e., on their charge-to-size ratios). A way to speed up the separation in CE is to apply a pressure to the electrophoretic analysis. In this context, it is important to assess whether the use of such a pressure does not compromise the high separation efficiency of CE. Therefore, one of the first experiments used in the development of this method was the evaluation of pressure on the migration times and resolution. For this test, 2 closely related and isobaric phosphorylated disaccharides were used. Figure 2 shows the effect of pressure on the separation of sucrose-6-phosphate (S-6-P) and trehalose-6-phosphate (T-6-P).



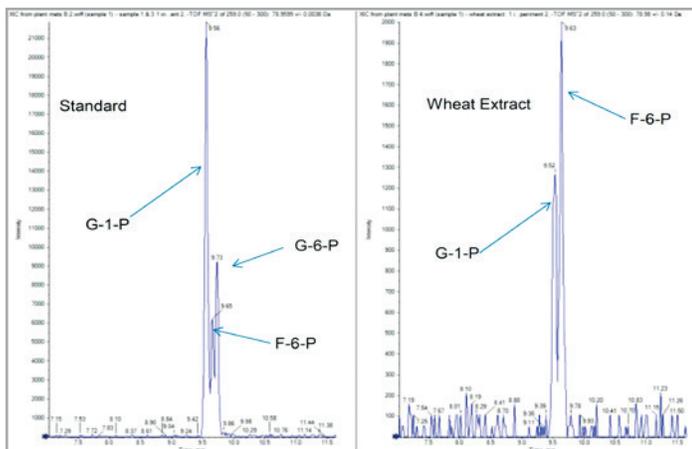
**Figure 2.** The effect of pressure on the separation of sucrose-6-phosphate (S-6-P) and trehalose-6-phosphate (T-6-P).

In this experiment, increasing the pressure during the separation from 0.5 to 2 psi speeded the analysis without a significant effect on the resolution. The 2 disaccharides are isobaric with very similar fragmentation patterns and their major fragment ion, resulting from the loss of the phosphate group, is the same for both compounds. Therefore, separation is essential for the selective identification of these analytes by MS. In this method, the polarity used for separation is reversed and was needed to push the metabolites to the MS detector. Some pressure is required to help counteract the residual electroosmotic flow on the bare fused-silica OptiMS cartridge, but due to the use of a low-pH separation buffer (10% acetic acid), only a low pressure was required.

When glucose enters an organism, it is either broken down to release energy or stored as fat via the intermediate, dihydroxyacetone phosphate. In this process of cellular glycolysis, phosphorylated forms of monosaccharides are very important intermediaries in the pathway. One of these major intermediate products in this cycle is glucose-6-phosphate. Isobaric to fructose-1- and glucose-1-phosphate, the separation of this intermediate from these other structural isomers is often difficult to achieve. Using the same method, separation of these 3 isobaric metabolites is possible, as illustrated in Figure 3. As with the phosphorylated disaccharides, the product ion spectra for each of these compounds are very similar and therefore separation is critical for a confident identification.



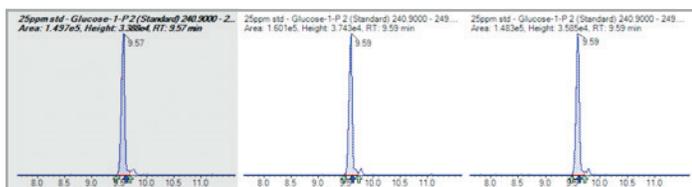
**Figure 3.** CESI-MS Analysis of Phosphorylated Monosaccharides.



**Figure 4.** Comparison of a standard with a wheat extract for the analysis of phosphorylated monosaccharides.

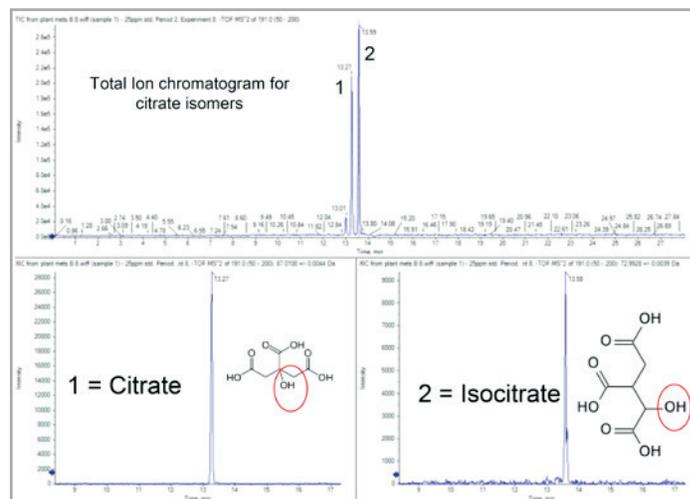
Figure 4 shows how this method was applied to a wheat extract. What can clearly be shown is the separation and detection of glucose- and fructose-1-phosphate in the absence of glucose-6-phosphate, even though the original sample that had been previously prepared for HILIC chromatography had to be diluted into water 8-fold. What can also be observed is the reproducibility of the migration times of the standard when compared to the extracted sample.

In order to assess migration time reproducibility, a mixed standard containing over 15 compounds—including, among other compounds, phosphorylated mono and disaccharides, isocitrate, citrate, succinate, malate, pyruvate and cyclic adenosine monophosphate (cAMP)—was injected multiple times. In Figure 5, 100  $\mu$ Molar standard of glucose-1-phosphate was injected 3 times, and it clearly shows that the migration times observed are reproducible. This was repeated to give intraday RSD values  $\leq 1.1\%$  for 10 repeated injections and an interday migration time RSD  $\leq 1.3\%$  for 5 repeated injections using a series of anionic metabolites with different migration times.<sup>2</sup>



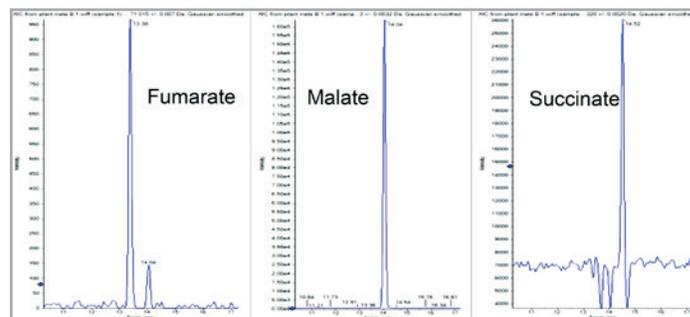
**Figure 5.** 100  $\mu$ Molar glucose-1-phosphate standard injected in triplicate to study migration time stability taken from MultiQuant™ Software 3.0.

Other isobaric compounds included in this screen were citrate and isocitrate, which form part of the citric acid cycle. Figure 6 shows the separation of these structural isomers. In this example, citrate and isocitrate differ by the position of the hydroxy group, which affects their pKa and therefore their charge and migration times.



**Figure 6.** Separation of isocitrate and citrate by CESI-MS.

Also included in this study were other intermediates of the citric acid cycle, such as succinate, malate, fumarate, oxaloacetate and pyruvate, and the analysis of 3 of these compounds is shown in Figure 7.

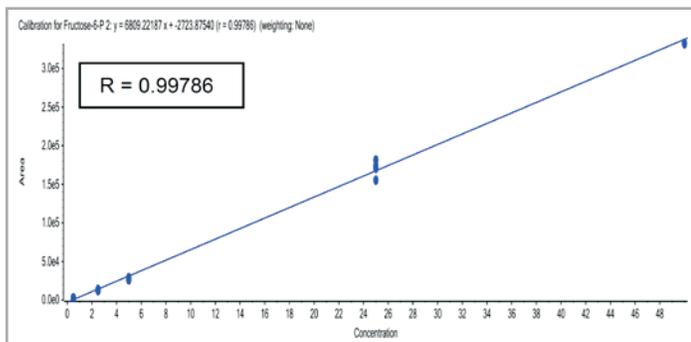


**Figure 7.** Detection of fumarate, malate and succinate by CESI-MS.

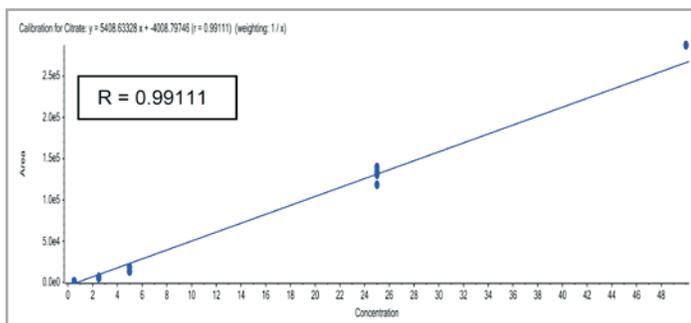
Some of these citric acid cycle metabolites are very small and MS fragmentation leads to only a few fragments. This gives limited options for selective detection by MS/MS, and therefore separation of these compounds is important.

To finally check the applicability of CESI-MS for quantification of these metabolites calibration standards were prepared in water. Standards were then injected multiple times and examples are shown in Figures 8 and Figure 9 which indicated that the responses are linear with R values  $\geq 0.99$  in all instances.

So far, we have evaluated the sensitivity of this approach for over 20 different anionic metabolites, and their limits of detection range from 100 nMolar for the phosphorylated monosaccharides down to 10 nMolar for cAMP,<sup>2</sup> an important secondary messenger derived from adenosine triphosphate, which is involved in the energy transfer in cells. These limits of detection mean that so far, the anionic metabolites tested can be detected at physiologically relevant levels.



**Figure 8.** Calibration curve for Fructose-6-phosphate (200 nMolar - 200  $\mu$ Molar).



**Figure 8.** Calibration curve for citrate (260 nMolar - 260  $\mu$ Molar).

## Conclusions

A fast, robust and reliable CESI-MS method for the highly efficient and selective analysis of more than 20 negatively charged polar metabolites has been developed. The electrophoretic separation is assisted by the application of a small pressure at the inlet and has been shown to provide reproducible migration times and separation of isobaric species and structural isomers of phosphorylated sugars, which is not possible by reversed-phase HPLC techniques.

In the future, work is planned to evaluate electrophoretic pre-concentration in order to increase the injection volume and therefore the sensitivity for anionic metabolites. We also plan to expand this analysis to include more metabolites for global metabolic profiling studies.

## References

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2. Ramautar, R. Probing a new volume regime in metabolomics with sheathless capillary electrophoresis-mass spectrometry. Conference proceedings, *HPLC 2015*, Geneva, Switzerland, June 21–25, 2015.

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