POST-COLUMN ADDITION AS A TOOL TO ENHANCE PERFORMANCE IN MICROFLOW LC-MS

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The need for improving electrospray ionization (ESI-MS) sensitivity has been the driving force behind many of the recent technological advances in the mass spectrometry field. A solution that has gained significant interest is microflow LC-MS, where the separation takes place at flow rates lower than 50 µL/min using columns with an internal diameter between 0.1 and 1.0 mm. The increased sensitivity observed with microflow LC-MS can be attributed in part to the enhanced ionization processes, but mostly to better sampling efficiencies obtained using lower flow rates.

With a standard ESI, the high flow and the large diameter of the spray tip produce a relatively broad and divergent plume of rather large droplets. As a result, only a very small fraction of the ions can be sampled by the sampling orifice. With micro and nanospray, smaller droplets are formed requiring fewer fissionable events and the spray tip produces a more convergent plume resulting in more ions being sampled.

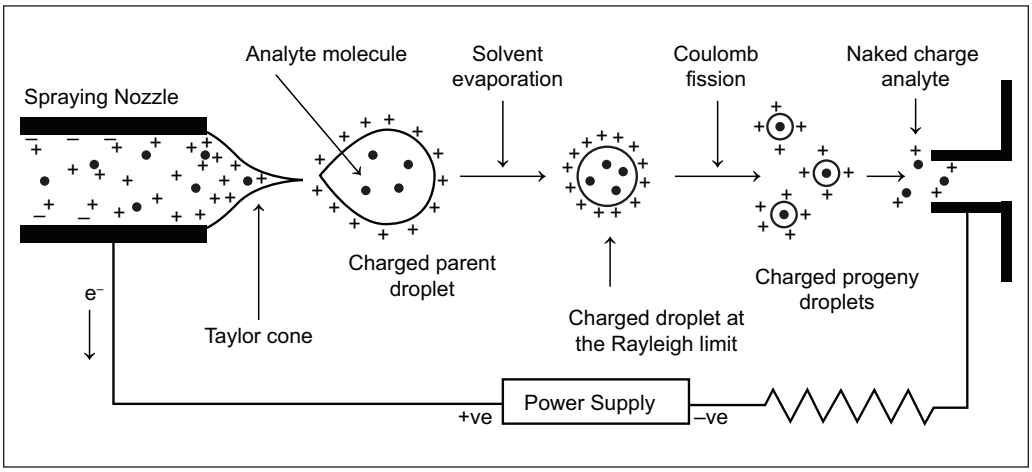

Figure 1. ***The electrospray process.*** *Ions present in a solution are transferred to a gas phase, which involves three major steps: (1) production of charged droplets at the ES capillary tip; (2) shrinkage of the charged droplets by solvent evaporation and repeated droplet dis-integrations, leading ultimately to very small highly charged droplets capable of producing gas-phase ions, and (3) the actual mechanism by which gas-phase ions are produced from the very small and highly charged droplets.*

In examining fundamental aspects of the electrospray ionization phenomenon, several solvent properties have proven to be important parameters in determining success of the electrospray process. These properties include: surface tension, conductivity, viscosity, and dielectric constant of the solution.

The surface tension of the solvent impacts the required electric field at the capillary tip for producing a stable electrospray.[1] For a given solvent, the required electrospray voltage increases with the square root of the surface tension. Solvents with low surface tension could be used to reduce the required voltage and decrease the likelihood of an electric discharge that could degrade the performance of electrospray ionization. Surface tension also plays a role in droplet shape. The radius of an initially formed charged droplet, as well as its Rayleigh limit, is a function of the surface tension of the liquid.[2,3] Water, having higher surface tension than organic solvents, produces larger initial droplets. In addition, the evaporation of water from the charged droplet is slower than the evaporation of the organic solvent (Figure 1). For these reasons, the disintegration of the charged droplets is less efficient with water than with organic solvents and the number of droplets emitting gas-phase ions is decreased. The surface tension of liquid mixtures has been investigated by several researchers.[4-6] In particular, Hassani[7] examined the surface tension of several alcohol-water mixtures. This study showed there is a nonlinear decrease in surface tension with the addition of alcohols. Among several alcohols investigated, isopropanol-water mixture exhibited the lowest surface tension and thus was selected as the model post-column addition modifier in our study.

The ideal solvent composition for ESI analysis is application dependent. Analysis in the positive ion mode requires different solvent characteristics than analysis in the negative ion mode, and the response of a given analyte can be enhanced or suppressed in different solvent systems.[8] However, it may be necessary to deviate from the ideal solvents in order to maintain non-covalent interactions and protein conformation or to interface with liquid chromatography.

The optimal conditions for liquid chromatography may not always be compatible with electrospray ionization mass spectrometry. The presence of non-volatile ionic species, such as phosphate and sulfate buffers in the ESI spray is deleterious. Strong acids, such as trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), and hydrochloric acid, which are used as ion-pairing agents in LC, also tend to surpress the analyte signal in ESI-MS.

Modification of the mobile phase (e.g. addition of buffers, changing the pH and solvent strength) may be required to enhance the compatibility of the mobile phases with the detector. Altering solvent properties by post-column addition of a modifier can be an effective technique to improve sensitivity without affecting the chromatographic separation.

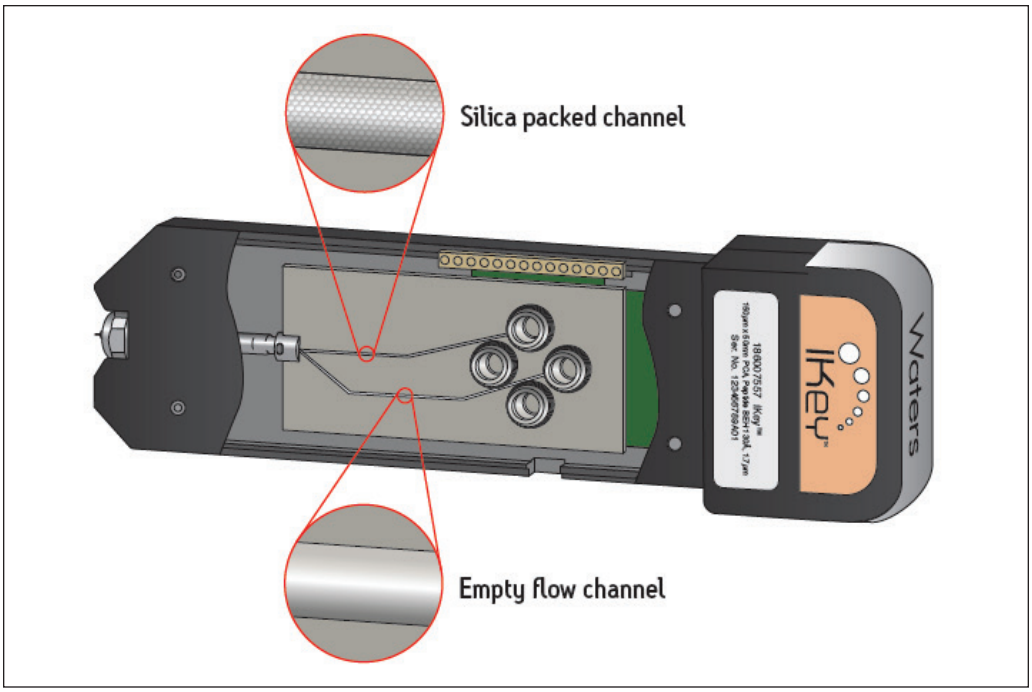

Figure 2. ***Post-column addition (PCA) iKey.*** *The analytical channel is connected to the upper port and the post-column addition channel is connected to the right port.*

The use of post-column addition is the best means to decouple the LC from the MS. In this way, an optimal ESI MS performance can be achieved by applying the post column addition without interfering with the previously optimized LC condition.

The plug-and-play design of the ionKey/MS™ System allowed the development of a post-column addition device eliminating all user-made fluidic and electronic connections.

The post-column addition (PCA) iKey contains two channels, a 150 µm I.D. channel packed with sub-2-µm particles, and an open channel used for post column addition of solvent (Figure 2). The two channels meet after the chromatographic separation completes and prior to the emitter leaving insignificant dead volume. The use of a PCA iKey™ requires an ACQUITY UPLC® M-Class Auxiliary Solvent Manager (ASM) to provide a stable and accurate flow to the post column addition channel.

The experiments were performed using the ionKey/MS system composed of the ACQUITY UPLC M-Class, the ionKey source, and Xevo® G2-XS QTof or Xevo TQ-S Mass Spectrometer. The modifier was introduced at flow rates ranging from 100 to 1000 nl/min.

1. IMPROVEMENTS IN NEGATIVE IONIZATION

First, the use of isopropanol to lower the surface tension to enhance the ionization process and sensitivity was evaluated.

Metabolite profiling of biological samples, such as urine, is a challenging task due to the chemical and structural diversity of the components. The chromatographic separation of the metabolites of a drug is usually performed using reversed-phase chromatography. Polar metabolites, such as glucuronides, sulfates, and glutathione conjugates, are typically analyzed in the negative-ion mode.

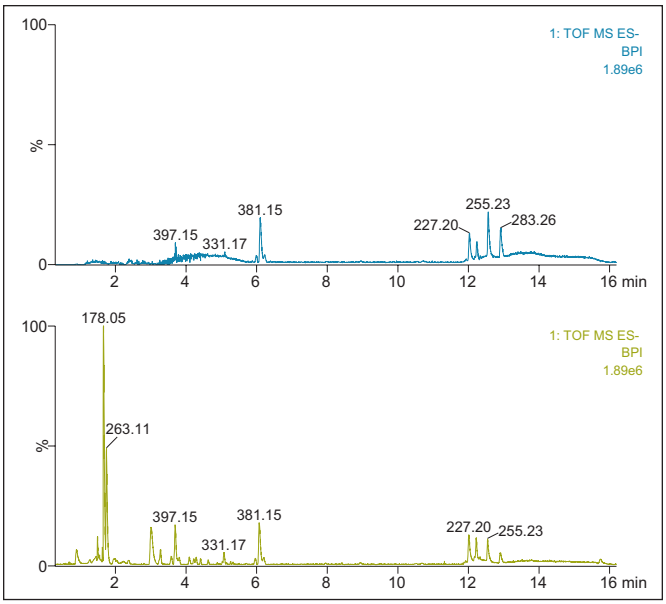

Figure 3. ***Chromatographic separation of human urine.*** *0 µL/min (top) and 1 µL/min (bottom) IPA was used as a post-column modifier. The chromatograms are scaled to the highest relative intensity among the two chromatograms.*

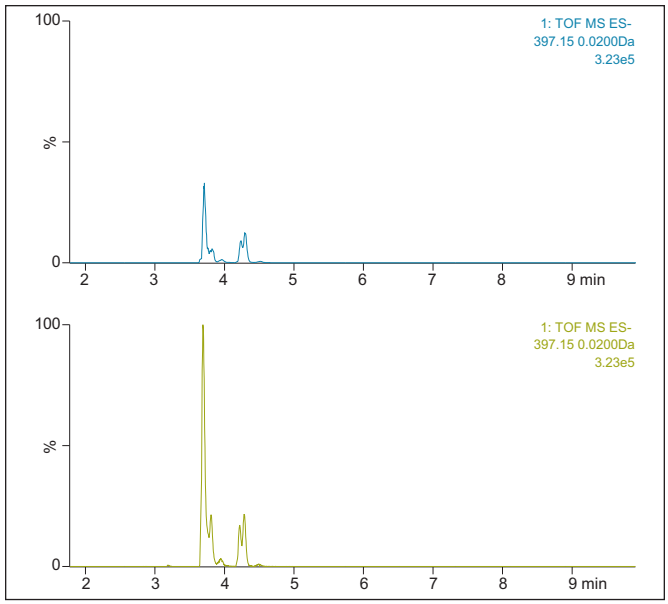

Figure 4. ***Extracted ion chromatograms of the hydroxylated glucuronide metabolites of ibuprofen;*** *top chromatogram in the absence of IPA; bottom chromatogram with IPA.*

In the negative ion mode ESI-MS, the analyte sensitivity is dependent upon the nature of the analyte, as well as the mobile-phase properties, such as organic solvent and electrolyte contents.

The analysis of human urine in negative ionization mode using post-column addition of isopropanol showed a significant increase in sensitivity. Pre-dose urine and ibuprofen-metabolite-containing urine (3 h after the oral administration of 200 mg) samples were collected from a healthy male volunteer. The urine samples were directly injected after a 1:50 dilution in water.

The separation was performed by applying a gradient from 5% to 65% ACN in 10 minutes at a flow rate of 3 µL/min. Under standard gradient conditions, in the absence of isopropanol, not all compounds were detected (top chromatogram in Figure 3). Post-column addition of isopropanol enabled the detection of the more hydrophilic compounds, including ibuprofen metabolites. Also, the addition of isopropanol reduced the required capillary voltage for producing a stable electrospray, and therefore, minimized the possibility of electric discharges that can generate undesired background noise.

We examined the sample for evidence of ibuprofen metabolites by acquiring the data using the MSe acquisition mode (using combination of low (5 eV) and high (25 eV) collision energies to generate both molecular ion and fragment ion data).

The extracted ion chromatograms of *m/z* 397.1499, corresponding to the hydroxylated glucuronide metabolites of ibuprofen, are shown in Figure 4. For the most intense peak eluting with a retention time of 3.7 min, the signal increased by over 50% when IPA was used (bottom chromatogram).

The advantage of this approach was further applied towards the detection of other urine metabolites. Hippuric acid (*m/z*=178.0504), an endogenous polar biomarker produced an intense signal in the presence of isopropanol, but was completely undetected under standard conditions (Figure 3).

2. ENHANCED SENSITIVITY OF PROTEOMIC EXPERIMENTS BY USING DMSO AS A MOBILE PHASE ADDITIVE

Increasing the electrospray responses of peptides by adding a low percentage of dimethylsulfoxide (DMSO) to the LC solvent has been investigated in several studies.[9,10] DMSO is a polar aprotic solvent with an elution strength similar to acetonitrile. Therefore, addition of DMSO to the LC solvents requires adaptation of the elution gradient to avoid the loss of hydrophilic peptides. The post-column addition iKey, with its dual-channel configuration, enables the introduction of DMSO through a side channel without influencing chromatographic performance.

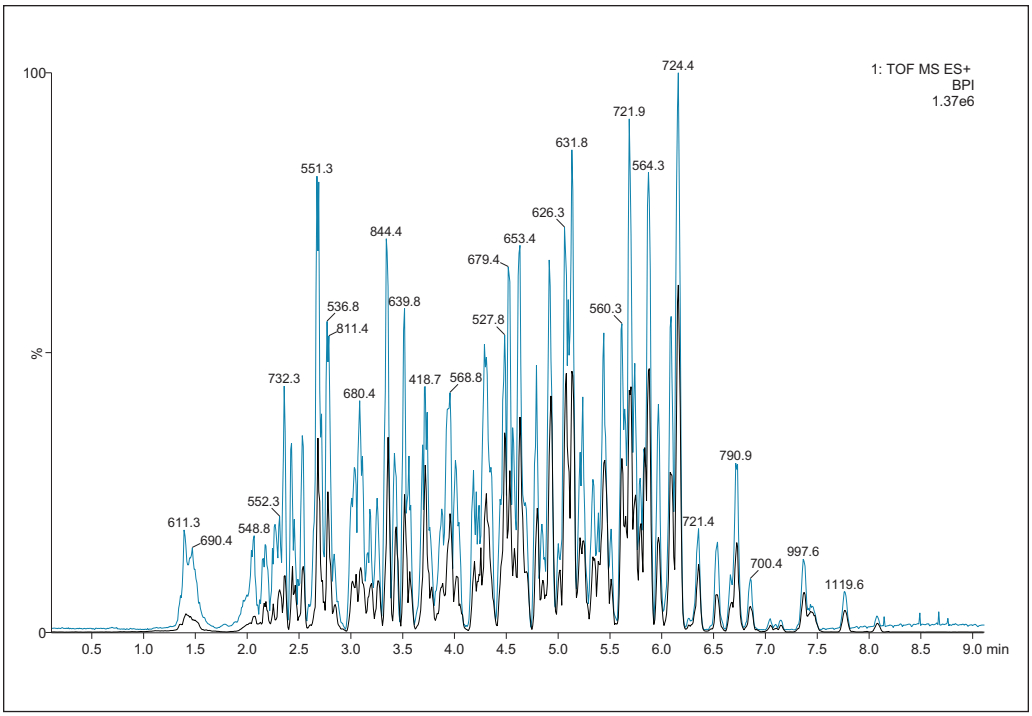

Figure 5. ***Improved LC-MS/MS performance using DMSO of a four protein digest.*** *Base peak intensity chromatograms of a four protein digest (MassPREP protein digestion standard mixture 1) in the presence (blue) and absence (black) of 5% DMSO.*

In order to maximize the sensitivity enhancement, it is necessary to optimize the amount of DMSO added to the solvent. The extra channel of the PCA iKey enables the introduction of reagents post LC separation, which simplifies and speeds up the method development process.

Alternately, DMSO can be added to the LC solvents directly. However, the addition of DMSO in the mobile phases without gradient adjustment impacts the chromatography as illustrated in Figure 6. The peak shape of the hydrophilic peptides is significantly deteriorated. The change in retention times depends on the amount of DMSO added in the mobile phases, whereas with the post-column addition the retention times are the same regardless of the DMSO concentration.

As previously observed,[9] the addition of DMSO may result in lowering the charge states of certain peptides, and therefore, proper identification and updating the MRM transitions while performing targeted analysis is critical for the post column DMSO addition. In the example presented in Figure 7., the dominant species of the peptide TIAQYAR is the doubly charged ion *m/z* 411.7. However, with the post-column addition of DMSO, the singly charge ion *m/z* 822.4 became the most abundant species.

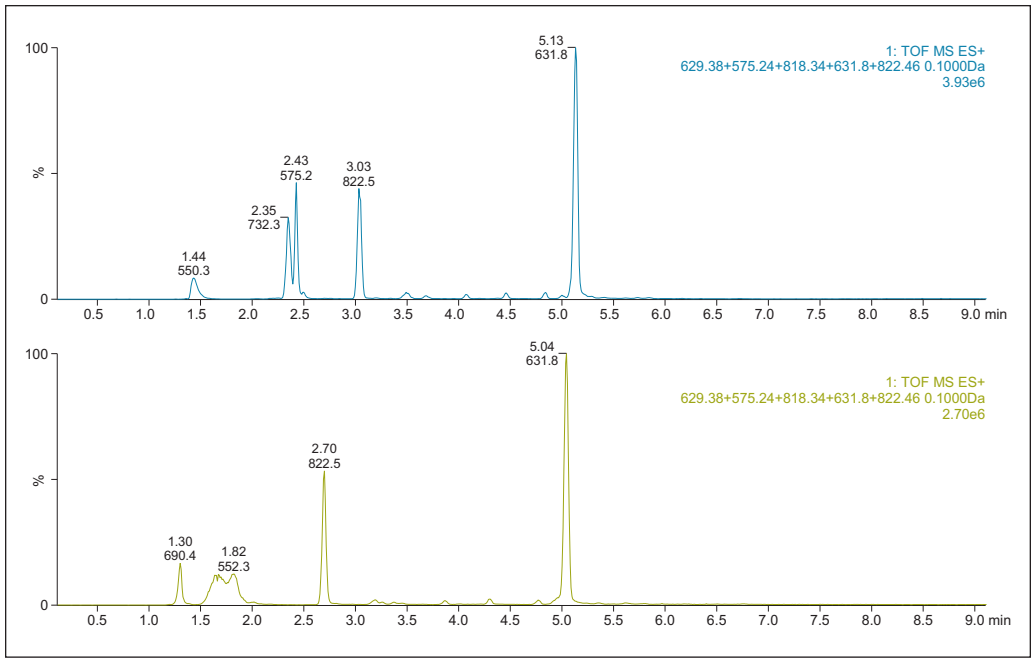

Figure 6. ***DMSO effect on chromatography*** *Post-column addition of DMSO (blue) vs. in-solution addition of DMSO (green).*

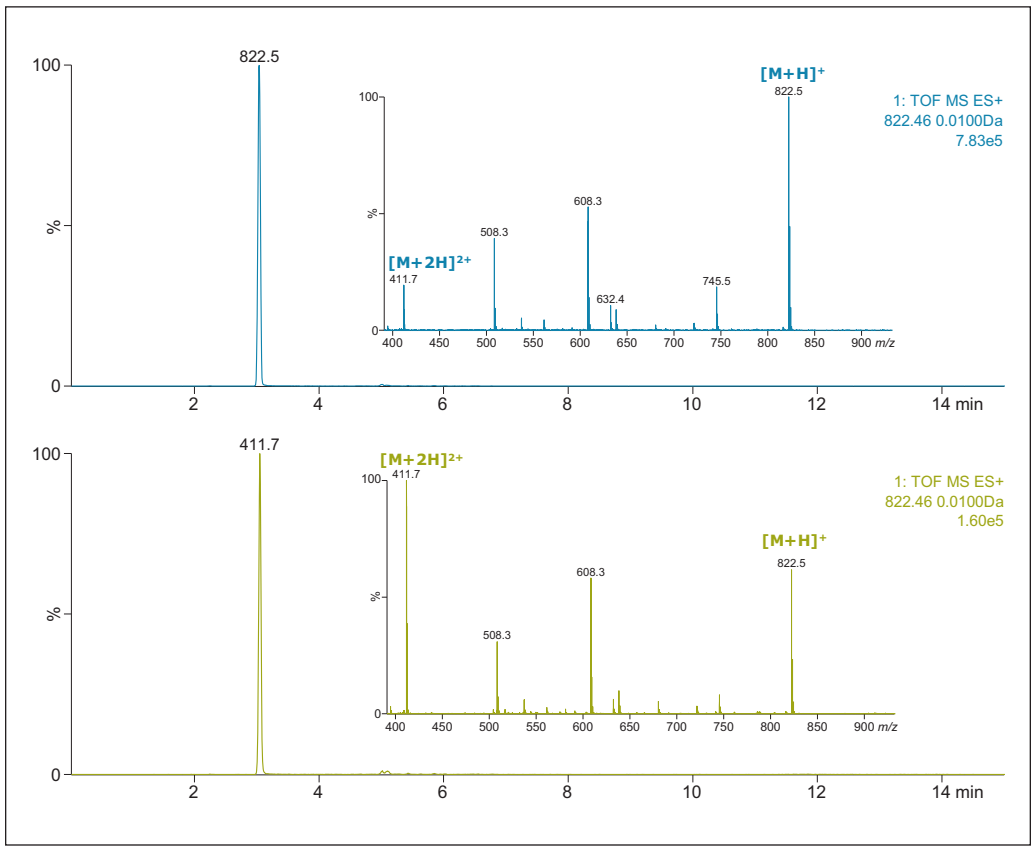

Figure 7. Extracted Ion Chromatograms and Mass Spectra of TIAQYAR peptide in the presence (blue) and absence (green) of 5% DMSO.

CONCLUSIONS

By applying post-column addition, optimization of the ESI MS condition can be achieved without compromising the optimal LC conditions, thus maximizing system performance.

There are numerous ways that post-column additions can be employed to improve electrospray sensitivity, as listed below:

1. Post-column addition of solvents, such as isopropanol, facilitates the electrospray process by reducing the surface tension.
2. Post-column addition of DMSO can be used to enhance sensitivity in shotgun proteomics.
3. The mobile phase pH can be adjusted to improve ESI sensitivity. The addition of acetic acid or formic acid can lower the pH to improve positive-ion detection (e.g. LC-MS detection of amines can be improved by post-column acidification of the mobile phase). Similarly, the negative mode ESI sensitivity can be improved by increasing the pH through adding ammonium hydroxide post-column (e.g. bile acids analysis).
4. Derivatization of a sample to improve electrospray sensitivity can be performed post column.
5. Post-column addition can be used to displace an additive (e.g. TFA, HFBA) forming stronger ion pairs with the analytes with an additive forming weaker ion pairs (propionic acid). This methodology is known as the "TFA Fix".[11]

References

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