Inlet Ionization: A New Highly Sensitive Approach for Liquid Chromatography/Mass Spectrometry of Small and Large Molecules

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Inlet Ionization: A New Highly Sensitive Approach for Liquid Chromatography/Mass Spectrometry of Small and Large Molecules

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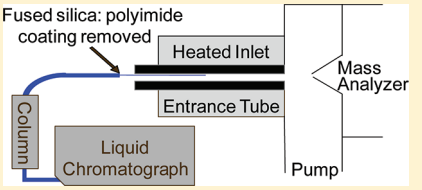
Supporting Information

ABSTRACT: Inlet ionization is a new approach for ionizing both small and large molecules in solids or liquid solvents with high sensitivity. The utility of solvent based inlet ionization mass spectrometry (MS) as a method for analysis of volatile and nonvolatile compounds eluting from a liquid chromatography (LC) column is demonstrated. This new LC/MS approach uses reverse phase solvent systems common to electrospray ionization MS. The first LC/MS analyses using this novel approach produced sharp chromatographic peaks and good quality full mass range mass spectra for over 25 peptides from injection of only 1 pmol of a tryptic digest of bovine serum albumin using an eluent flow rate of 55 μL min^{-1}. Similarly, full acquisition LC/MS/MS of the MH$^+$ ion of the drug clozapine, using the same solvent flow rate, produced a signal-to-noise ratio of 54 for the major fragment ion with injection of only 1 μL of a 2 ppb solution. LC/MS results were acquired on two different manufacturer's mass spectrometers using a Waters Corporation NanoAcquity liquid chromatograph.



Ionization methods used in liquid chromatography/mass spectrometry (LC/MS) are currently dominated by electrospray ionization (ESI) for polar and large molecules[1−3] and atmospheric pressure chemical ionization (APCI) for small molecules of low polarity.[4,5] These methods have been developed over several decades and have become rugged and sensitive. Thus, new ionization approaches have a high bar to overcome. ESI is by far the most utilized ionization method for LC/MS, and the highest sensitivity with this method is achieved using low solvent flow because the method is concentration rather than flow rate sensitive. However, the low-flow conditions of micro- and nano-ESI[6] make these methods technically difficult and greatly increases the time needed to complete a separation.[7,8] Attempts at further increasing the sensitivity of nanoflow ESI have involved subambient ESI in combination with ion funnel technology to avoid ion losses associated with transferring ions from atmospheric pressure (AP) through an aperture leading to the vacuum of the mass analyzer.[9] It is estimated that even with nano-ESI, 80−90% of the produced ions are lost in the transfer from AP to the first vacuum region.[10,11]

A new approach to ionization was first introduced as an AP matrix assisted laser desorption/ionization (MALDI) method[12,13] but was later called laserspray ionization (LSI)[14] to reflect the very different ionization mechanism of the new method versus MALDI.[15] Because the highly charged ions can be generated from laser ablation of a matrix/analyte mixture at AP[13,14] and vacuum,[16,17] these methods are distinguished as LSI *inlet* (LSII) and LSI *vacuum* (LSIV). In LSII, the laser is not directly involved in the ionization process and, unlike MALDI, the ions observed are not generated close to the matrix/analyte surface.[15,18] Identical mass spectra to those obtained using LSII can also be produced from a variety of methods for introducing matrix/analyte particles into a heated transfer tube linking AP with the first vacuum region of the mass analyzer and has been termed matrix assisted inlet ionization (MAII) because a laser is not required.[19] It was recently shown that the matrix may be solvents such as water, acetonitrile, methanol, and mixtures thereof. This approach called solvent assisted inlet ionization (SAII)[20] was shown to be a highly sensitive method for ionization of small molecules as well as peptides and proteins. The ability to use solvents identical to those employed with ESI suggests that SAII may be a useful method for LC/MS. Instead of applying a high voltage to initiate ESI at AP and then sampling a portion of the ions through the vacuum inlet for mass analysis,[10] SAII introduces the solvent/analyte solution directly into the heated inlet tube without application of a voltage, a laser, or other external means of ionization. Ionization is initiated in the confined sub-AP environment of the inlet tube so that ion losses associated with transfer of ions from AP through the entrance orifice of the mass analyzer are eliminated.

SAII is similar in some respects to thermospray ionization (TSI) introduced by Vestal in the early 1980s[21] but is also significantly different in both the physical method and the results obtained. A brief discussion of SAII fundamentals and the similarities and differences relative to TSI are provided in the Supporting Information. SAII produces highly charged ions similar to ESI[22] even for proteins and with sensitivity orders of magnitude higher than those reported for TSI.[19,21,23] Here, we demonstrate first LC/MS

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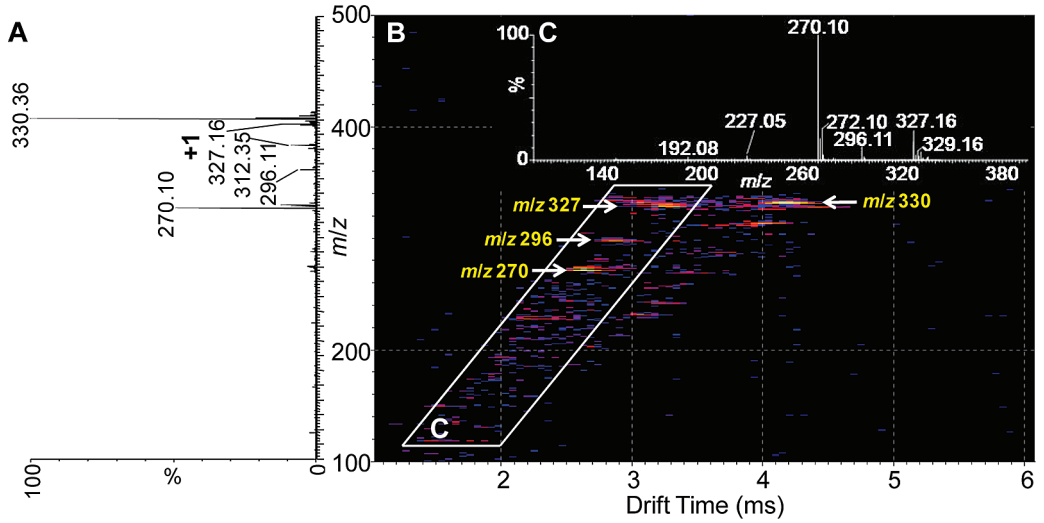

Figure 1. Acquisition of a 1 ppb solution of the drug clozapine at a solvent flow of 16 μL min^{-1} using SAII IMS/MS/MS analysis on the SYNAPT G2. (A) The full mass spectrum, (B) the DriftScope representation of drift time vs m/z of the 1 ppb solution of clozapine, and (C) the region for extracting the mass spectrum which is shown at the top of (B).

experiments using SAII achieved on mass spectrometers of two different manufacturers. LC/MS results using the drug clozapine as well as a tryptic digest of bovine serum albumin (BSA) are reported.

EXPERIMENTAL SECTION

Materials. HPLC grade water and methanol were from EMD Chemicals (Gibbstown, NJ). Acetonitrile (ACN) and formic acid (FA) were from Fisher Scientific (Pittsburgh, PA). Clozapine was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and the BSA tryptic digest was from Proteo Biosciences (Morgantown, WV). Bovine insulin was obtained from Sigma Aldrich (St. Louis, MO). All chemicals were used as received.

LC/MS. A Waters Corporation NanoAcquity liquid chromatograph was used in this work and was interfaced to either a Waters Corporation SYNAPT G2 with a home-built heated inlet capillary or to a Thermo Fisher Scientific LTQ Velos with a commercial AP to vacuum heated inlet tube. A Waters 1 × 50 mm BEH C18 column with 1.7 μm particles was used for the analysis of clozapine by employing a 3 min gradient using ACN/H_2O/0.1% FA from 40% to 95% ACN at a flow rate of 55 mL min^{-1}. For separation of the peptides of the BSA digest, a Waters 1 × 100 mm BEH300 C18 column with 1.7 μm particles was used with a 30 min gradient of 3 to 60% ACN at 55 mL min^{-1}. The LC column was interfaced to the mass spectrometers through a 15 cm length, 0.050 ID × 0.220 OD mm fused silica tubing (Eksigent, Dublin, CA) with ca. 3 cm of the polyimide coating burned from the exit end. Approximately 1 to 3 cm of the tubing was inserted into the heated capillary inlet of the MS as demonstrated in the Graphical Abstract. The fused silica tubing was attached with tape to an x,y,z-stage that was scavenged from a discarded optical microscope.

SYNAPT G2 Mass Spectrometer. Adjusting the fused silica from the LC column to achieve maximum ion current on the SYNAPT G2 was accomplished as follows. A low volume tee fitting was inserted between the LC column and the fused silica tubing described above. The LC flow rate was set at 55 μL min^{-1} at 70% ACN/H_2O/0.1% FA, and the drug clozapine was blended into the solution from the LC through the tee connection by infusing 5 μL min^{-1} of a 1 μM solution. With the exit-end of the fused silica inserted into a constructed heated inlet tube (see below), the x,y,z-stage was used to adjust the end of the fused silica capillary within the home-built heated MS inlet tube for maximum ion current of the MH^+ ion of clozapine. Once the position was set, the heat applied to the inlet capillary was adjusted for maximum ion current but was no hotter than a faint glow from the nichrome wire. The temperature of the home-built inlet for the SYNAPT G2 was unknown but, because of the large heat sink, was likely below 250 °C. Construction of the home-built inlet capillary for the SYNAPT G2 is described in the Supporting Information.

Velos LTQ Mass Spectrometer. A Velos LTQ mass spectrometer was used for LC/MS/MS analysis of clozapine and for LC/MS analysis of a BSA tryptic digest. The commercial heated ion transfer tube allowed SAII operation similar to previously reported SAII analysis on an Orbitrap Exactive.[20] Determining the optimum position for the exit-end of the same fused silica tubing used with the SYNAPT G2 followed a similar procedure to that described above, except a 1 μM solution of bovine insulin in water infused at 5 μL min^{-1} was blended into the 55 μL min^{-1} flow from the LC column. The ion abundance of the +4 charge state of insulin optimized at about 350 °C at 50% ACN in water with 0.1% FA, but because good abundance with no appreciable background was achieved at 270 °C, this lower inlet temperature was used in the studies reported here. The depth of the fused silica in the instrument inlet tube and its position was then adjusted using an x,y,z-stage to obtain a constant ion current for the +4 charge state of insulin. Once the position was adjusted, no other manipulation was required. The tee connector was replaced with a straight low volume connector for the LC/MS runs.

RESULTS AND DISCUSSION

A SYNAPT G2 mass spectrometer was previously modified by inclusion of a desolvation device attached at the inlet skimmer to provide the ability to produce highly charged ions by LSII.[24] This modification was not satisfactory for SAII, and a new inlet design was necessary (description in the Supporting Information). The drug clozapine (MW 326) was used to determine the sensitivity

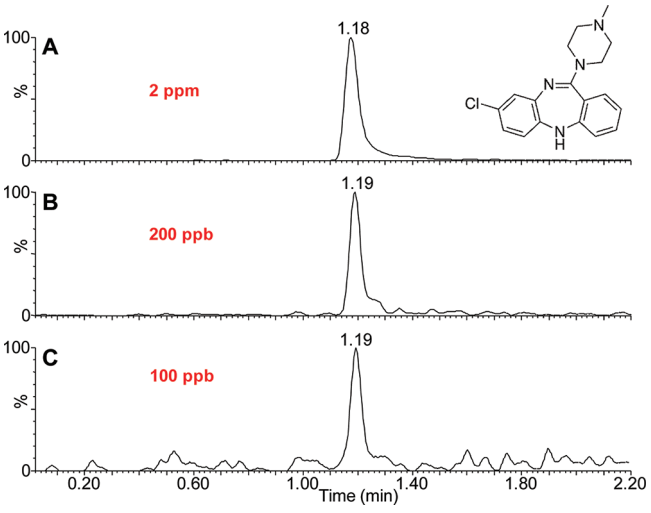

Figure 2. Three consecutive 3 min acquisitions injecting 3 μL of clozapine at a concentration of (A) 2 ppm, (B) 200 ppb, and (C) 100 ppb on the SYNAPT G2 using a NanoAcquity liquid chromatography with a Waters C18 BEH 1 × 50 mm column and a solvent flow rate of 55 μL min^{-1}.

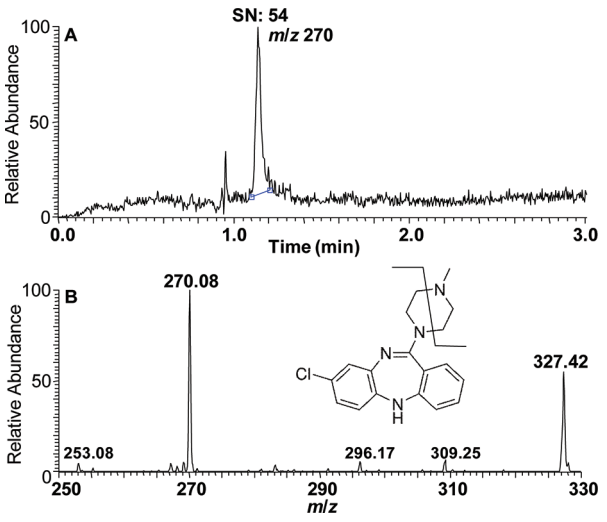

Figure 3. Injection of 1 μL of a 2 ppb solution of clozapine onto a Waters C18 BEH 1 × 50 mm column using a solvent flow rate of 55 μL min^{-1} on the Velos LTQ. The m/z 327 (MH^+) ion was selected and fragmented, and the selected ion current chromatogram of the m/z 270 fragment ion was plotted in (A). The MS/MS spectrum obtained at the apex of the m/z 270 selected ion current chromatogram in (A) is shown in (B).

and reproducibility using the constructed modification of the Z-Spray AP to vacuum interface. The capability of the inlet design was demonstrated by acquiring IMS-MS/MS data for a 1 ppb solution of clozapine by inserting the exit-end of a fused silica tube into the constructed heated MS inlet tube and the entrance-end into the clozapine methanol solution. The flow rate was measured to be ca. 16 μL min^{-1}. The MS/MS fragment ion mass spectrum obtained by selecting the MH^+ (m/z 327) ion and fragmenting it in the trap using a collision energy setting of 30 V prior to the IMS separation with data summed for 1 min is shown in Figure 1A. At this concentration, the chemical background produces the most abundant ions (e.g., m/z 330) in the

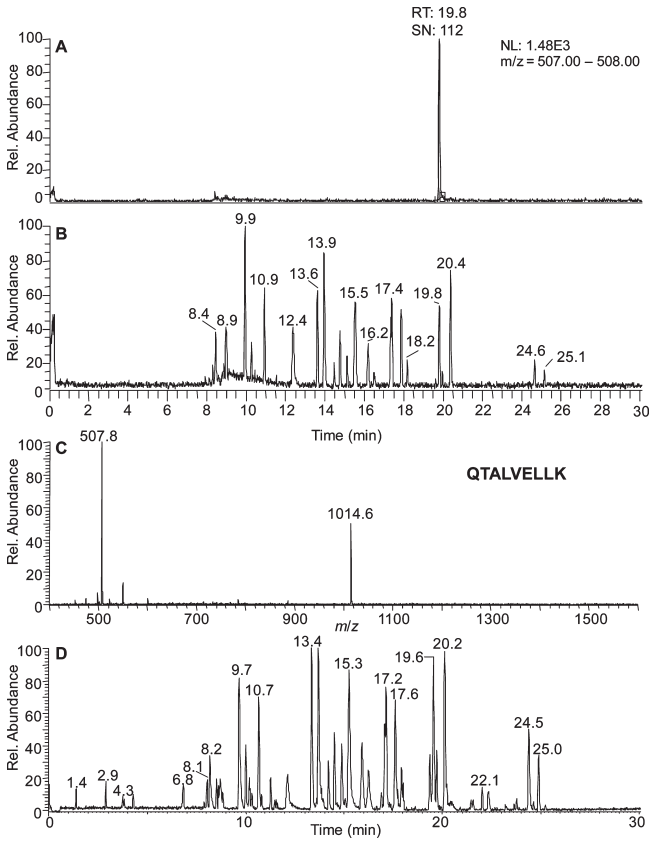

Figure 4. BSA tryptic digest obtained using a Waters Corporation C18 BEH300 1 × 50 mm column with an ACN/H_2O/0.1%FA gradient from 3 to 60% ACN in 30 min using a NanoAcquity liquid chromatography interfaced using the SAII method to a Velos LTQ mass spectrometer. (A) Selected ion chromatogram of the m/z 507.8 ion for 1 pmol injected, (B) base peak chromatogram of 1 pmol injected, (C) mass spectrum of the chromatographic peak at 19.8 min with 1 pmol injected, and (D) the base peak chromatogram for 3 pmol injected.

mass spectrum. However, by utilizing the ion mobility dimension, only ions belonging to clozapine are extracted from the two-dimensional plot using DriftScope (Figure 1B) to produce a clean fragment ion mass spectrum (Figure 1C). These results suggested that SAII is well suited for interfacing to liquid separation methods, provided a heated inlet tube is available.

The entrance-end of the fused silica was then connected to a liquid chromatography column as shown in the TOC. The reproducibility and change in signal-to-noise of LC-IMS/MS/MS is demonstrated by the full scan acquisitions of a 3 μL injection of 2 ppm (Figure 2A), 200 ppb (Figure 2B), and 100 ppb (Figure 2C) solutions of clozapine in water using a Water's NanoAcquity LC at a flow rate of 55 μL min^{-1}. The high organic gradient (40−90% ACN, 0.1% FA) was necessary because insufficient thermal energy could be supplied to the constructed inlet tube to allow analysis of higher water content solvents. Consequently, the constructed inlet did not allow gradient elution LC/MS to operate efficiently for peptides and proteins under optimum gradient conditions (3−60% ACN/0.1% FA).

The ability to obtain ions using the SAII method from solutions of water/0.1% FA to water/90% ACN/0.1% FA was achieved on the Velos LTQ mass spectrometer by setting the instrument AP to vacuum inlet tube temperature as low as 270 °C and inserting the exit-end of the fused silica tubing into

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the instrument heated AP to vacuum inlet tube. Tuning the fused silica position within the inlet required only a few minutes to accomplish. Injecting 1 μL of a 2 ppb solution of clozapine using an LC flow rate of 55 μL min^{-1}, as described above for the clozapine analysis on the SYNAPT G2, produced ample ion current to obtain an MS/MS spectrum with a signal-to-noise of 54 (Figure 3A). Data were acquired by selecting the m/z 327 MH$^+$ ion using a mass tolerance of $\pm$1 Da and obtaining the MS/MS spectrum from m/z 100−300 using a collision energy setting of 32 V. The MS/MS spectrum for the acquisition at the apex of the ion current chromatogram is shown in Figure 3B. These results demonstrate that by tuning on a small protein, in this case insulin, without need for further adjustment, the SAII LC/MS method is highly sensitive for small molecules.

Excellent results were also obtained for a BSA digest using the same fused silica position applied to the clozapine analysis. This suggests applicability for a wide range of compounds in a single acquisition. The selected ion current chromatogram of the peak eluting at 19.8 min from injection of 1 μL of a 1 μM solution (1 pmol) of a tryptic digest of BSA at a flow rate of 55 μL min^{-1}, which allows excellent peptide separation in a 30 min gradient, is shown in Figure 4A. As can be seen from the width of the eluted peak, SAII does not degrade the chromatographic resolution or cause peak tailing under these conditions, indicating that the ionization occurs sharply in a confined time and space. The base peak chromatogram (BPC) obtained from this injection is shown in Figure 4B. The BPC displays over 25 peptide peaks, and additional peptides can be found that do not trigger a peak in the BPC. Figure 4C is the mass spectrum obtained from the peak shown in Figure 4A and shows both the doubly charged ion at m/z 507.8 and the singly charge ion at m/z 1014.6. The base peak chromatogram obtained with 3 μL (3 pmol) injected of the BSA digest is shown in Figure 3D. As expected, more peaks are observed with the higher column loading.

CONCLUSION

Experiments demonstrating LC/MS and LC/MS/MS using SAII on two different mass spectrometers suggest that this new ionization method is highly sensitive for small molecules and a wide range of peptides without compromising the chromatographic separation. Using equivalent solvent flow rates, SAII was reported to be more sensitive than ESI for bradykinin[20] and is shown here to produce excellent results for peptides from a 1 pmol injection at a flow rate of 55 μL min^{-1}. Considering the early stage of development of SAII, it seems likely that sensitivity improvements will occur which may allow this ionization method to compete in sensitivity with nano-ESI but using higher flow rates. Future work is aimed at determining the long-term ruggedness of the SAII method and its potential to ionize a wide range of ionic, polar, and low polarity compounds of high and low mass with high sensitivity. Mechanistic aspects to ionization will be addressed in a forthcoming paper.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supplemental Section

Mechanistic Considerations

SAII[1] is similar in some respects to thermospray ionization (**TSI**) introduced by Vestal in the early 1980's,[2] but is also significantly different in both the physical method and the results obtained. Briefly, TSI involves flowing a liquid stream, typically from a liquid chromatograph at flow rates of 1 - 2 mL min^{-1}, through a heated vaporizer tube into a vacuum which is closed to air. Ionization is believed to be a combination of statistical droplet charging with ion evaporation, and chemical ionization processes.[3] TSI has also been reportedly conducted at AP.[4] Ions produced under vacuum conditions are sampled through an orifice into the mass analyzer. Some multiply charged ions are observed in TSI, but typically singly charged ions are dominant.[3-4] A report of observation of proteins up to 30,000 Da was reported using TSI but required >100 pmol of sample.[5] These results were obtained at a solvent flow >1 mL min^{-1} and only 78 ºC vaporizer temperature which is much lower than typically used in TSI where optimum results generally require 90 to 95% of the solvent be vaporized inside the vaporizer tube.[6] In SAII, comparable protein mass spectra are obtained with ca. 1000X less sample, but requires significantly higher inlet temperature. Another attribute of TSI is the small temperature range (as little as 10 ºC) of the heated vaporizer tube in which optimum

results are observed. In SAII ions are often observed over several hundred degrees with a broad maximum. [3,5]

In SAII, the liquid is introduced directly to the heated walls of the inlet in a region of high gas flow from AP to the first vacuum region because the AP end of the inlet is open to the atmosphere.[1] Centering the solvent inlet capillary within the inlet, as is done in TSI produces significantly poorer results. Liquid flow rates from 1 to 200 µL min^{-} have thus far been shown to be applicable. High charge states similar to ESI allow proteins at least as large as BSA to be observed with sensitivity similar to ESI.[7] Small molecules and peptides for which TSI is applicable are observed with several orders of magnitude greater sensitivity in SAII than has been reported with TSI.[1]

Inlet ionization is shown to be a general method for solid and liquid samples with and without matrix[1,8-10] while TSI is only applicable to analytes in solvents.[6] Nevertheless, it seems reasonable that commonalities exist in the ionization mechanisms of TSI,[6] sonicspray ionization,[11] ESI,[12] and inlet ionization[1,8-9] methods. Most notably, all of these methods probably involve ion formation from charged droplets.[6] It seems highly probable that even methods such as fast atom bombardment[6,13] and matrix assisted laser desorption/ionization (MALDI)[14] also derive some portion of ions from a charged droplet mechanism. TSI, inlet ionization, and MALDI probably also have a chemical ionization component.[3,14-15]

In inlet ionization, just as with TSI and MALDI,[3,14] there is evidence for multiple ionization mechanisms. In any confined space where there are multiple collisions occurring and gas phase ions present, ion-molecule reactions will occur to transfer charge in exothermic processes. **Figure S1** is a plot of ion abundance vs. temperature for bradykinin (**BK**) and shows that the doubly charged ions maximize at an inlet

temperature of ca. 300 ºC but that singly charged ions begin to become important at ca. 275 ºC and continue to increase to the maximum inlet temperature of 450 ºC. This is interpreted as probably the result of different mechanisms of ion formation, a lower energy mechanism with formation of multiply charged ions and a higher energy mechanism producing singly charged ions. It is possibly that the higher energy process involves vaporization of neutral BK with ionization by a chemical ionization process. The efficiency of droplet charging, droplet size, and desolvation may be major differences that distinguish the ion formation mechanisms of the various methods for observation of nonvolatile compounds.

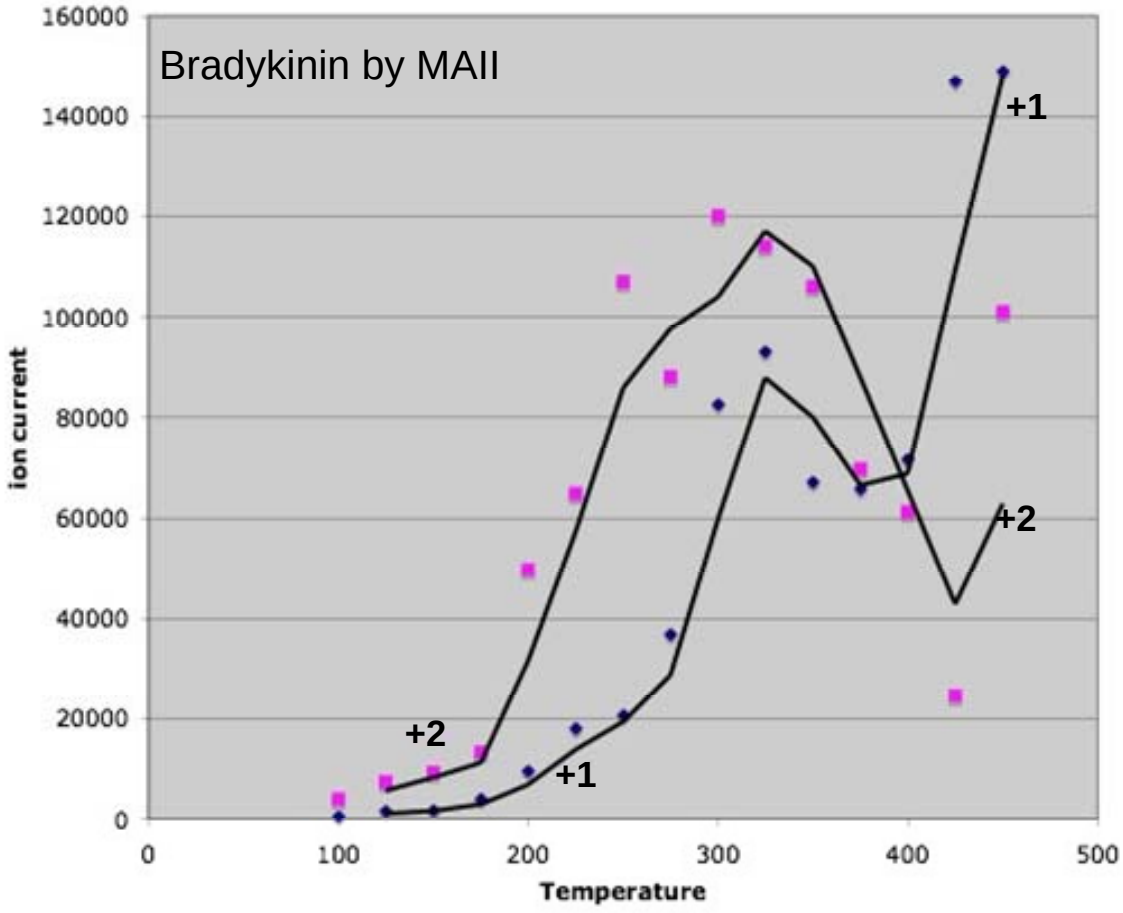

Figure S1

Inlet Construction for SYNAPT G2

The homebuilt inlet capillary for the SYNAPT G2 was constructed using a 5 cm long section of 0.530 ID X 680 OD mm fused silica tubing in which about 0.5 cm was inserted through the inlet skimmer orifice and held in place with Sauereisen Insa-lute No.1 cement (Pittsburgh, PA). Sanding the tip of a Waters Z-Spray ion source skimmer using emery cloth was sufficient to open the skimmer inlet aperture to just allow insertion of the 0.680 mm OD fused silica. The heater was constructed by placing a wound section of nichrome wire over the fused silica and holding it in place with additional Sauereisen cement. A laboratory Variac Powerstat (Bristol, CT) was used to place a voltage through the wire (<9 volts) which was sufficient to cause a faint glow of the heater wire.

Cation Adduction

SAII has been shown to be applicable for ionization of small volatile compounds such as drugs and large nonvolatile compounds such as proteins,[1] but with extensive metal cation adduction.[1] The addition of acid such as 0.1% formic decreases the ultimate sensitivity of SAII but has the advantage of reducing metal cation adduction and lowering the inlet temperature necessary to achieve good results. The LC separation further reduces cation adduction so that almost exclusively MH^+ ions are observed. The inlet temperature required for effective ionization depends on both the solvent and acid. Water requires a higher inlet temperature than high organic solvents, especially those containing 0.1% formic acid. Solvent mixtures containing from zero to 90% ACN:0.1% FA in H_2O:0.1% FA demonstrate excellent sensitivity using SAII on Orbitrap Exactive and LTQ Velos mass spectrometers which have commercial heated inlet tubes linking AP to the first vacuum stage of the mass analyzer.

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