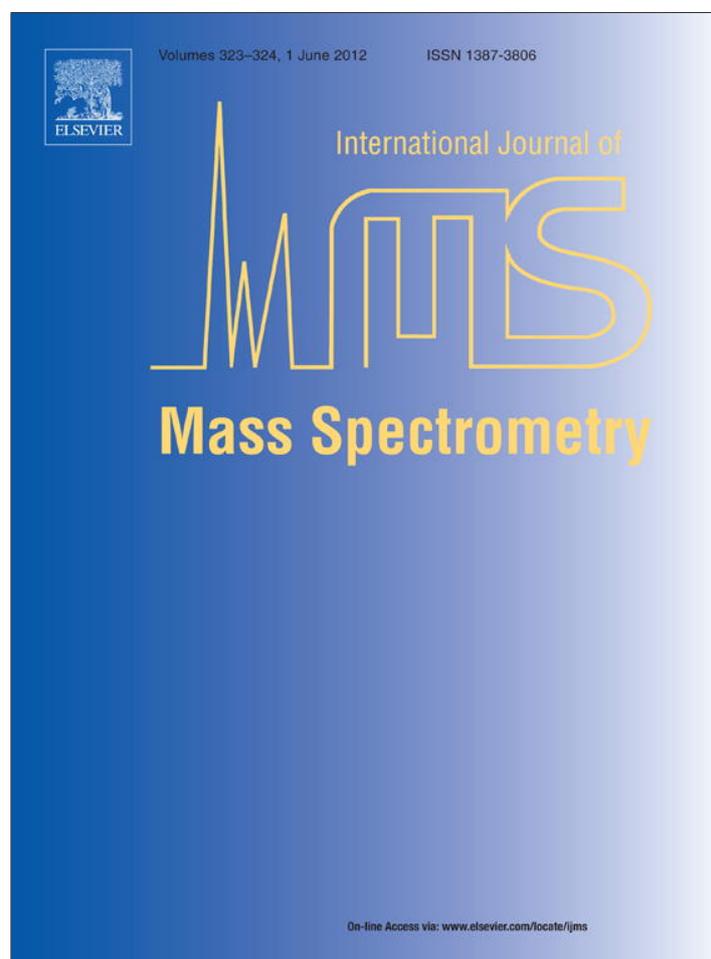


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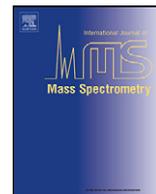


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Classroom Article

Pressurized sample infusion: An easily calibrated, low volume pumping system for ESI-MS analysis of reactions

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ABSTRACT

Pressurized sample infusion (PSI) continuously delivers solution from a flask through capillary tubing at a flow rate that makes it suitable as a low internal volume pumping method for electrospray ionization mass spectrometry (ESI-MS). The flow rate can be predicted from the applied pressure by using the Hagen–Poiseuille equation, but variability in internal diameter of the PEEK tubing used is such that each individual length should be calibrated if accurate results are sought. Once calibrated, the values hold well for different solvents across a range of pressures. The technique has been exemplified in two reactions: the deprotection of a protected amino acid using trifluoroacetic acid, and in the platinum-catalyzed redistribution of two silanes. In both cases, the abundances of starting material, product(s) and intermediates were tracked in real time as the reaction proceeded.

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1. Introduction

There are numerous methods of introducing sample solutions to an electrospray ionization mass spectrometer (ESI-MS) [1]. Most commonly, an HPLC pump delivers the analyte through a column in a flow of mobile phase, and some of the eluant from the column passes into the source of the spectrometer, hence LC-MS [2]. Syringe pumps expel solutions directly from a syringe containing the sample by means of a worm-drive [3], and these are robust and tolerant to a wide range of solvents, but cannot be easily adapted to operation at different temperatures. Peristaltic pumps produce a pulsating flow and are therefore not suitable for ESI-MS, in which a steady flow is desirable for spray consistency (though these are used in, for example, inductively coupled plasmas mass spectrometry, for sample introduction at a rate of $\sim 1 \text{ ml min}^{-1}$) [4]. Microreactors can be coupled to ESI-MS instruments, but the temporal element can only be controlled by flow rate or tubing length, so collection of each data point involves a new experiment [5]. Solutions can also be pressurized with an inert gas and delivered into the source of the mass spectrometer. Agilent's calibrant delivery system pushes calibrant solution into the solvent selection valve, whereupon it is pumped into the

mass spectrometer source conventionally through means of the LC pump [6]. Pressurized chambers have been used for capillary electrophoresis/electrospray ionization, in which the solution is driven through the separation capillary and electrospray capillary by an electropneumatically regulated supply of nitrogen. [7] A sample infusion bomb apparatus using 5–20 psi of nitrogen to drive a sample through 50 μm i.d. fused silica tubing into an ESI-MS, capable of on-line sample concentration and clean-up, has also been demonstrated [8].

Pressurized sample infusion (PSI) is a simple method for infusing a dilute solution directly into a mass spectrometer without further treatment [9]. PSI involves no mechanical pumping; only what is essentially a cannula transfer from a flask into the instrument through a length of chromatography tubing, and we developed it with the intention of applying to the online monitoring of catalytic reactions. It works well in this application, and with simple normalization of the data to total ion current or an internal standard it provides very high data density along with good point-to-point reproducibility of quantifiable data [10]. The total solvent volume of the pumping system is simply the volume of the tubing. While the experimental set-up inherently allows the analysis of compounds under anaerobic conditions, it works equally well for air- and moisture-stable materials, so its application is much broader than just organometallic catalysis, and can be used in all situations where a simple, low internal volume pumping system is desirable.

All required materials for a PSI system are easily accessible: a flask with two ground glass joints (or one joint and an arm), rubber septum, rubber hose, a short length of PEEK tubing ($\sim 0.5 \text{ m}$), a PEEK chromatography fitting (nut and ferrule) and a source of

Abbreviations: PSI, pressurized sample infusion; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Pmf, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TFA, trifluoroacetic acid.

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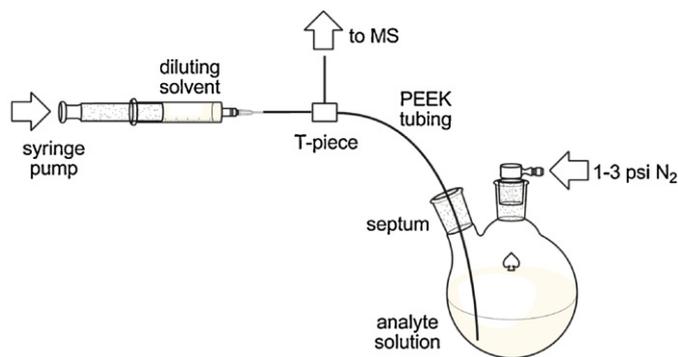


Fig. 1. Pressurized sample infusion (PSI), set-up with on-line sample dilution via a syringe pump. This step is not required if the sample in the flask is sufficiently dilute.

pressurized and regulated gas (e.g. N_2). The flask is positioned as close as is practical to the ESI-MS source and connected to a source of pressurized, carefully regulated inert gas via a short length of rubber tubing. One end of a piece of PEEK capillary tubing is then inserted through a punctured rubber septum (which is wired to the flask to ensure it does not pop out) into the flask, while the other end is connected to the electrospray ionization inlet (Fig. 1). Standard glassware with ground glass joints are shown; we generally use Schlenk flasks, including those with integrated condensers, to minimize the chances of leakage. A slight overpressure (0.5–5 psi) is applied to the contents of the flask to facilitate continuous introduction of the sample into the mass spectrometer. It is important that a regulator capable of accurately measuring these small pressures is used as over-pressurizing the flask is dangerous. Any ground glass joints should be secured to prevent fittings from popping out under pressure. For monitoring reactions the flask may be equipped with a stir bar and placed in a temperature controlled bath to allow for stirring and temperature control. The sample solution must be homogeneous to ensure the PEEK tubing stays free of blockage. If dilution of the sample solution is required, this may be accomplished on-line by incorporating a tee in the PEEK tubing immediately outside the flask as shown in Fig. 1. Up to a 100:1 dilution can be obtained in this fashion with careful adjustment of pressure and flow rate. Dilution with large volumes of room temperature or cold solvent also acts to effectively quench any reaction that may be occurring, which may be advantageous in some scenarios. PEEK tubing with a larger inner diameter should be used after the T-piece to ensure backflow into the flask does not occur.

The PSI method has no mechanism for measuring flow rate, so we were interested in using the pressure – the most easily altered experimental parameter – to control flow rate in a predictable way, without having to actually measure it for a given combination of solvent, pressure and tube diameter and length. Drawing on well-established relationships from fluid mechanics, the Hagen–Poiseuille equation [11] predicts the change in pressure to be related to the volumetric flow rate as follows:

$$\Delta P = \frac{128\pi LQ}{\pi d^4} \quad (1)$$

where ΔP is the pressure drop, μ is the dynamic viscosity, L is length of tube, Q is the volumetric flow rate and d is the inner diameter of the tube. Rearranging to predict a flow rate from a given overpressure, for water ($\mu = 0.001$ Pa.s), 1 psi (6900 Pa) of pressure applied to a tube of length 0.5 m and diameter 127 μm should generate a flow rate of $(6900 \times 3.14 \times \{1.27 \times 10^{-4}\}^4) / (128 \times 0.001 \times 0.5) = 8.8 \times 10^{-11} \text{ m}^3 \text{ s}^{-1} = 8.8 \times 10^{-2} \mu\text{L s}^{-1} = 5.3 \mu\text{L min}^{-1}$. This flow rate is entirely compatible with ESI-MS experiments.

Scholarly implementation. The practical part of this work was done within an undergraduate lab course, CHEM 361, at the University of Victoria under the supervision of NT in the spring of 2010 and the spring of 2011. CHEM 361 is a required laboratory course for chemistry majors students and focuses on practical analytical chemistry lab skills and learning instrumental techniques of analysis. This course has been involved in several collaborative projects within the chemistry department as well as with other departments at the University of Victoria. For this project, students individually performed flow rate measurements for a particular solvent and tubing length with varying pressure of nitrogen in 1 psi steps from 1 to 5 psi. From this data collection they were to calculate an optimal pressure to achieve a certain flow rate. The students used the ESI-MS to do some measurements using the same system (solvent, tubing length, and pressure). They were introduced to the ESI-MS instrument with hands-on instruction. Overall there were 71 undergraduates involved in collecting the data. KLV developed the initial technique as part of her research, and was also a teaching assistant for CHEM 361. She was therefore involved in all aspects of development, implementation, as well as checking all of the student data submitted.

2. Materials and methods

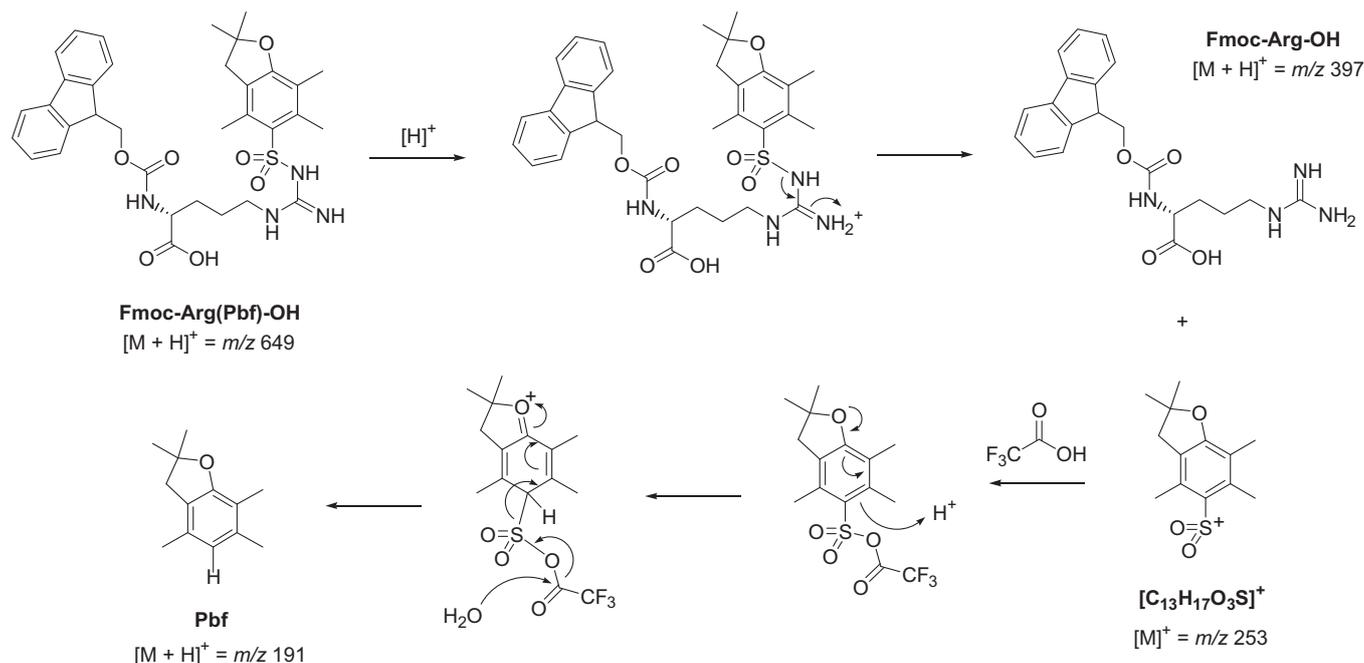
Deprotection: 2.1 mg Fmoc-Arg(Pbf)-OH (Merck Schuardt OHG) was dissolved in 1 ml acetonitrile (99.5%, CALEDON) in a 50 ml flask attached to a well-regulated supply of nitrogen (at 5 psi) and equipped with a septum and PEEK tubing leading to the mass spectrometer. 4 ml of trifluoroacetic acid (99.9%, CALEDON) was injected in the reaction flask.

Silane redistribution: 6 mg of (3-(methylsilyl)propyl)triphenylphosphonium hexafluorophosphate(V) (0.0121 mmol, 1 equivalent), 93 mg of Karstedt's catalyst (Pt 3.91% 0.000954 mmol, 0.8 equivalent), 6 mg of triphenylphosphine (0.0229 mmol, 1.9 equivalent) were dissolved in 20 mL of freshly distilled fluorobenzene in a 50 mL Schlenk flask in an inert atmosphere glovebox. The flask was equipped with a septum, removed from the glovebox and installed with PEEK tubing leading to the mass spectrometer. The flask was pressurized with N_2 and monitored by ESI-MS. Once a steady signal was obtained, 0.15 g of freshly distilled phenylsilane (1.39 mmol, 115 equivalents) was injected into the reaction flask. The reaction was initiated at room temperature and then was heated to reflux after 20 min to drive it to completion.

Nitrogen gas (5 psi) was used as the driving force for sample infusion in both experiments. Mass spectra were collected on a Micromass Q-ToF *micro* mass spectrometer in positive-ion mode using pneumatically-assisted electrospray ionization. Capillary voltage: 2900 V; cone voltage: 10 V; extraction voltage: 0.5 V; source temperature: 80 °C; desolvation temperature: 150 °C; cone gas flow: 100 L/h; desolvation gas flow: 200 L/h; scan time was 3 s and the inter scan time was 0.1 s. The reaction was continued until the amount of starting material dropped to <5% of the original value.

3. Results

To demonstrate the utility of PSI, we monitored the acid-catalyzed hydrolysis of a protected amino acid, Fmoc-Arg(Pbf)-OH (Scheme 1), where trifluoroacetic acid cleaves off the Pbf group selectively and under mild conditions. The 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) protecting group was developed as a TFA-sensitive arginine side-chain protecting group for use in Fmoc-based peptide synthesis [12], because when compared to the 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmf) protecting group, Pbf removal is faster when monitored by HPLC



Scheme 1.

[13]. The mechanism has been adapted from that proposed by Ramage and co-workers for the cleavage of Pmc [14]. Similar reactions have been studied using FAB-MS via periodic sampling of the reaction mixture [15].

Here we observe Pbf cleavage from Fmoc-protected arginine under typical conditions, which proceeds as a (pseudo) first order process. Mass spectra from $t=0$ and $t=10$ min appear in Fig. 2. The reaction took ~ 20 min to go to completion.

PSI allows the constant monitoring of all charged species at a time resolution equal to the scan rate, and the abundances of the various species of interest can be plotted against time. Reaction times are those the reactants spend in the flask plus the time they spend in the tube. The additional reaction time can be calculated (vide supra) but fast reactions may be somewhat difficult to follow. Here, making the tube as short as possible is beneficial. In the case of the hydrolysis reaction, we see the protonated starting material, [Fmoc-Arg(Pbf)-OH + H]⁺ at m/z 649, disappear to be replaced by protonated forms of both fragments, [Fmoc-Arg-OH + H]⁺ at m/z 397 and [Pbf + H]⁺ at m/z 191. We present the traces in 2 forms: (a) raw intensity data and (b) normalized to the total intensity of all ions of interest (i.e. those at m/z 649, 397 and 191).

Note that the traces are somewhat ragged (individual spectra have different intensities due to fluctuations in spray quality). Addition of trifluoroacetic acid caused a huge boost in ion current at ~ 30 s, due to its ability to protonate neutrals to form [M + H]⁺ ions (as well as catalyze the hydrolysis)—the ion count per data point jumped from 3000 to a momentary value of 48,000 (Fig. 3 shows only 0–15,000 counts, to keep the rest of the data on scale). To smooth out these fluctuations and to focus in on the relative abundances of the ions of interest, we normalized the data to the total intensity of the three key ions (Fig. 4); the product traces disregard the other product, to take account of the different ionization efficiencies of these two ions.

The disappearance of m/z 649 is approximately first order, with a slope of -0.21 and $R^2 = 0.96$ for a plot of $\ln[\text{intensity}]$ vs. time, with $t_{1/2} = 3.3$ min. This number is consistent with the assumption that the hydrolysis reaction is essentially complete within 20 min. It is clear from the traces that the two hydrolysis products are generated at different rates; m/z 397 is produced rapidly (cleavage of the S–N

bond), whereas the production of the ion at m/z 191 (cleavage of the S–C bond) through several additional steps is somewhat slower. Trace amounts of a key intermediate, the cationic [C₁₃H₁₇O₃S]⁺ at m/z 253 (see Scheme 1), were detected, and while the abundance was low (a maximum of $\sim 0.3\%$), it was observed to be roughly proportional to the overall rate of reaction. Overall, the data is con-

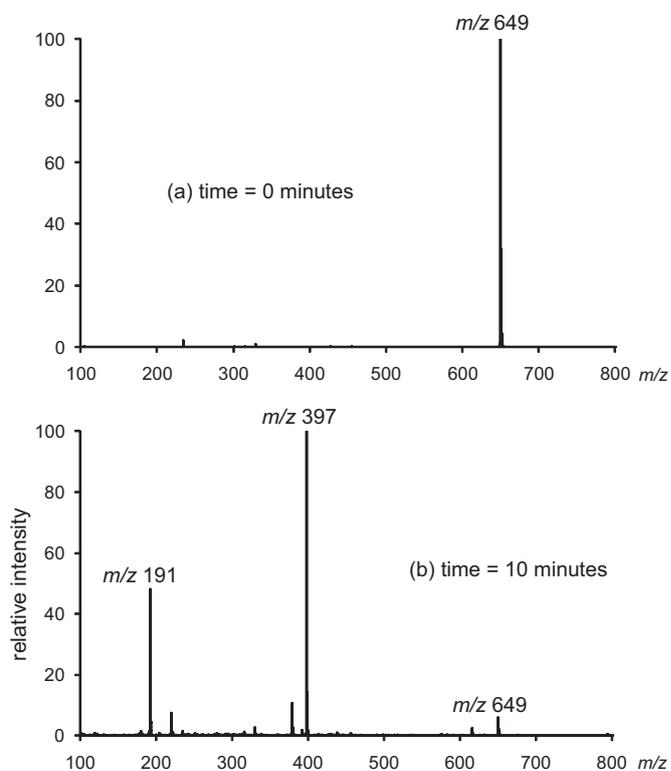


Fig. 2. Individual positive ion electrospray ionization mass spectra taken at time (a) 0 and (b) 10 min into the reaction. The disappearance of the peak at m/z 649 and the growth of the products at m/z 191 and 397 are clearly evident.

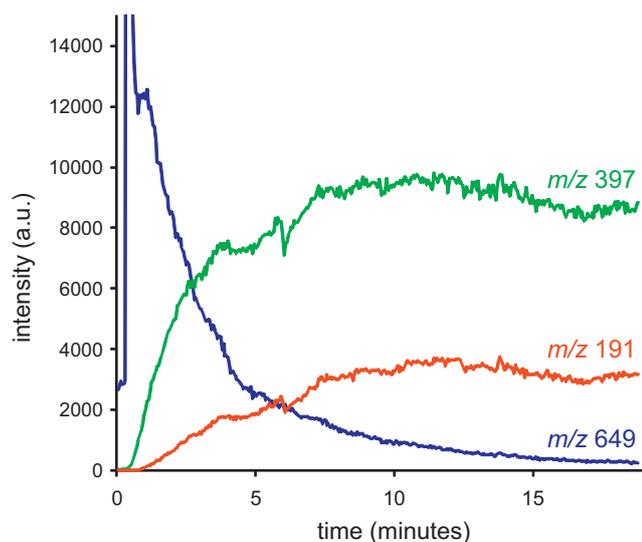


Fig. 3. Raw intensity vs. time traces for the protected amino acid (m/z 649) and its hydrolyzed products (m/z 397 and 191). ESI-MS spectra were accumulated for 3 s to generate each set of data, so each trace consists of ~ 380 points.

sistent with Ramage's postulated mechanism for TFA deprotection [14]. This experiment demonstrates the capability of PSI-ESI-MS to extract the type of data necessary for a mechanistic analysis, especially the high dynamic range that allows it to simultaneously monitor species present in high (starting material, products) and very low concentrations (intermediates).

Another example of the data provided by our approach is shown in Fig. 4, which tracks the platinum-catalyzed redistribution of two silanes [16], one charge-tagged with a phosphonium group. This demonstrates the ability of the technique to monitor air-sensitive reactions in non-routine solvents; in this case, fluorobenzene. The temperature of the reaction was increased from room temperature to reflux at 20 min; the catalyst used was Karstedt's catalyst, $\text{Pt}[(\text{H}_2\text{C}=\text{CHMe}_2\text{Si})_2\text{O}]_2$ (Fig. 5).

We were also interested in how the parameter space (tube dimensions, overpressure, solvent) would affect the flow rate. For ESI-MS, flow rates of $1\text{--}50\ \mu\text{L}\text{min}^{-1}$ cover the normal range, with $5\ \mu\text{L}\text{min}^{-1}$ being typical. We used $0.005''$ i.d. PEEK tubing,

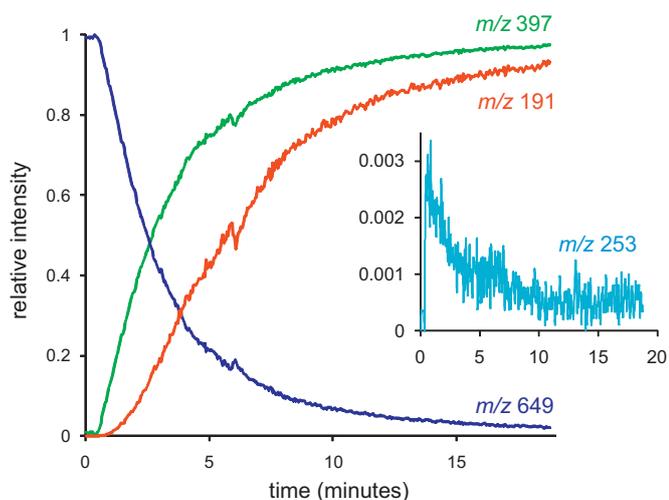


Fig. 4. Normalized intensity vs. time trace for the protected amino acid (m/z 649) and its hydrolyzed products (m/z 397 and 191). The inset shows the abundance of the $[\text{C}_{13}\text{H}_{17}\text{O}_3\text{S}]^+$ intermediate on a different intensity scale; note that its abundance approximates the instantaneous rate of reaction.

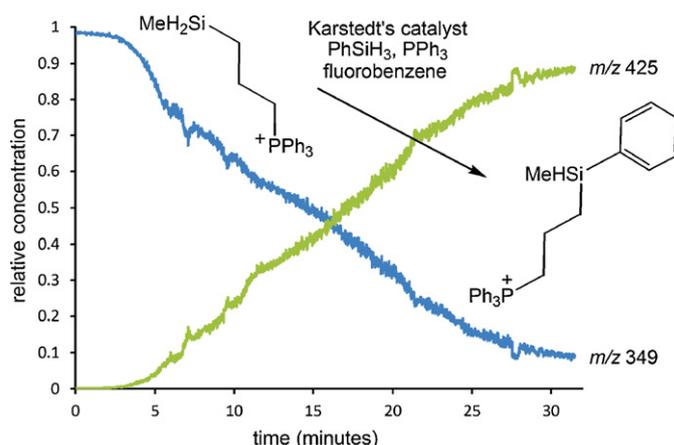


Fig. 5. Normalized intensity vs. time trace for charged silane (m/z 349) and appearance of the product of redistribution (m/z 425). The phosphonium silane has hexafluorophosphate as counterion; the catalyst was added at $t=2$ min and the solution heated to reflux at ~ 20 min.

as this diameter strikes a good balance between low volume and appropriate flow rates. It is also an LCMS standard, and is reasonably resistant to blocking. Its internal volume is approximately $13\ \mu\text{L}$ per meter (the tubing volume can be easily estimated by using the equation for the volume of a cylinder, $\pi r^2 h$, so for PEEK tubing of inner diameter $127\ \mu\text{m}$ and length 1 m, the volume is $3.14 \times (0.5 \times 127 \times 10^{-6})^2 \times 1 = 5.06 \times 10^{-8}\ \text{m}^3 = 12.65\ \mu\text{L}$). To determine the pressure required to obtain an appropriate flow rate, a series of measurements at different pressures were taken for a variety of solvents and using a range of PEEK tubing lengths. PEEK tubing for LCMS applications has an outer diameter of 1/16 of an inch ($1.5875\ \text{mm}$) and an inner diameter (typically) of 0.005 inches ($0.0127\ \text{mm}$). The solvents investigated were those commonly used for ESI-MS experiments: water, acetonitrile, a 50:50 water:acetonitrile mixture, methanol and dichloromethane. 0.45 m is the minimum required PEEK tubing length for our PSI apparatus, and PEEK tube lengths of 0.45, 0.50, 0.55 and 0.60 m were examined. Pressures between 0.5 psi and 3 psi were examined at increments of 0.5 psi. The mass of solvent forced through a length of PEEK tubing over time was recorded and each flow rate was measured a minimum of 10 times.

The intercepts in plots of flow rate vs. pressure were at slightly positive values of overpressure, and as expected, the least viscous solvents showed the highest flow rates at a given pressure (Fig. 6). Vertical error bars are included, but they are almost too small to

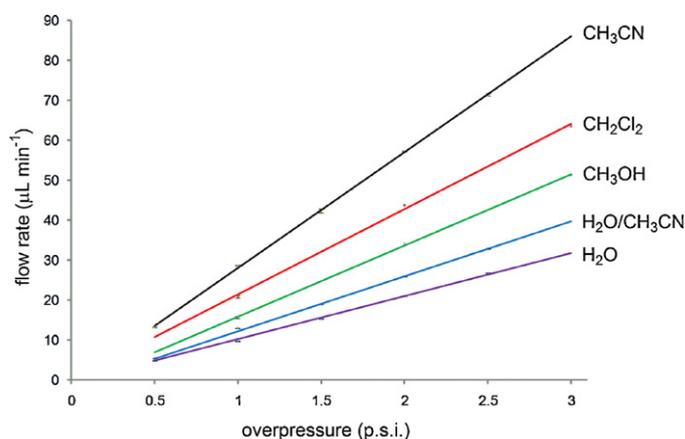


Fig. 6. Data from flow rate vs. overpressure experiments with different solvents and using PEEK tubing of length 0.6 m.

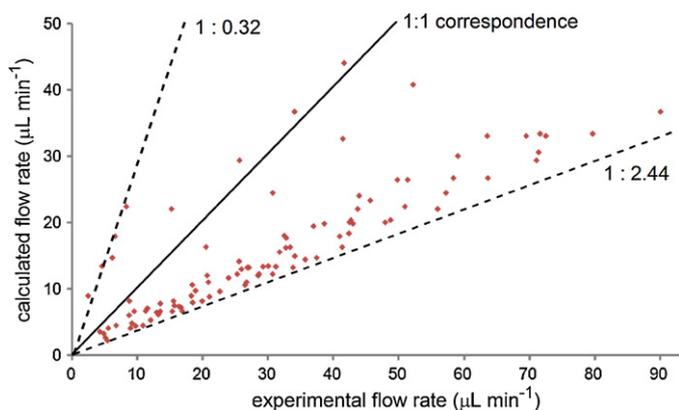


Fig. 7. Experimental flow rates plotted vs. theoretical flow rates calculated using the Hagen–Poiseuille equation. The 100 data points include flow rates calculated using five different solvents, four different tube lengths and five different values of overpressure (see Supporting information for data used to generate this plot). The dashed lines indicate the variation expected if the inner diameter was 25% less (1:0.32) or 25% more (1:2.44) than the claimed value.

be seen indicating high precision in the flow rate measurements. Some slight deviations from linearity are observed and this can be attributed to the precision with which the pressure was set for each experiment—the errors in the pressure reading were estimated to be of the order of ± 0.1 psi (gauge markings are at 0.2 psi increments). The relative (to water) viscosities of the solvents at 20 °C are 0.36 for acetonitrile, 0.44 for dichloromethane, 0.59 for methanol, 0.68 for 50:50 acetonitrile:water (approximated from data collected at 25 °C) [17], and 1 for water [18].

The large amount of data collected is best summarized by plotting the actual flow rate at a given combination of solvent, overpressure and tubing length and plot it against the calculated value using the Hagen–Poiseuille equation (Fig. 7). In all cases, the flow rates vs. overpressure graphs were almost perfectly linear ($R^2 > 0.99$).

What is immediately clear from the plot is that different pieces of tubing produce markedly different results. Because the overpressure, tubing length and solvent viscosity can be measured accurately, the likely source of error is in the inner diameter of the tube, for which any discrepancy in the listed value is magnified to the fourth power. A $\pm 25\%$ error in diameter is enough to account for most of the variation ($1.25^4 = 2.44$, $0.75^4 = 0.32$). The manufacturer's listed tolerance for 1/16" o.d. 0.005" i.d. PEEK tubing is ± 0.001 ", i.e. $\pm 20\%$ error, and this tolerance matches well with our observed experimental variability of $\pm 25\%$. Nonetheless, it is very easy to calibrate a given length of tubing by following the procedure we describe here (i.e. using a stopwatch and a balance) and apply the appropriate correction to the Hagen–Poiseuille equation for all samples run using the PSI method. Once this correction is obtained, the equation simplifies to $Q = k\Delta P/\mu$, where k combines the experimental correction with $\pi d^4/128L$, which is a constant for a given piece of tubing. We regard this approach to be more reliable than direct measurement of the diameter, because not only is it difficult to measure inner diameters of PEEK tubing accurately on the sub-millimeter scale, there is no guarantee that that diameter at the end(s) is representative of the entire length of tubing. Knowing the flow rate at a given pressure allows for quick optimization of the experimental set-up, helping ensure that good spray conditions are obtained and that consumption of the solution is appropriate for the length of the experiment. Reproducibility of conditions is a key consideration when attempting to extract kinetic information from an online monitoring experiment.

4. Conclusions

The Hagen–Poiseuille equation can be used to reliably estimate flow rates using pressurized sample infusion, but calibration of flow rate for a given piece of tubing is recommended in order to estimate the internal diameter accurately. Once this simple, one-off experiment is performed, the analyst has a reliable pumping system for ESI-MS with a low internal volume and robustness towards a wide variety of solvents. The high dynamic range of mass spectrometer in conjunction with continuous monitoring allows the simultaneous measurement of all charged species in solution, including low abundance intermediates, as exemplified by the acid-catalyzed deprotection of an amino acid. Air-sensitive systems in unconventional solvents at temperatures up to reflux are also readily accessible to this method, as shown by the silane redistribution experiment. We anticipate our approach to be useful for all chemists interested in reproducible, continuous monitoring of reactions in which the relative abundance of ions is dynamic and varies across several orders of magnitude.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijms.2012.03.007>.

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