



Contribution of liquid-phase and gas-phase ionization in extractive electrospray ionization mass spectrometry of primary amines

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In this study, we investigated how binary mixtures of compounds influence each other's signal intensity in electrospray ionization (ESI), extractive electrospray ionization (EESI) and secondary electrospray ionization (SESI) experiments. The experiments were conducted using a series of homologous primary amines (from 1-butyl to 1-decylamine). In every experiment, two of the amines were present, and all 21 possible combinations were measured with EESI, ESI and SESI as ionization sources. Except for the volatility, which decreases with increasing molecular weight, the physico-chemical properties of the amines are very similar, so that the intensity ratio obtained in each experiment provides information about discrimination effects occurring during the ionization process. The results show that for the relatively volatile compounds investigated, the EESI ionization mechanism resembles the SESI-like gas-phase charge transfer more than ESI-like analyte ionization in solution. In addition, almost no discrimination effects were observed in the spectra obtained in EESI experiments. Quantitative EESI experiments with nonylamine as internal standard showed that EESI is capable of providing both more accurate and more precise results than SESI and ESI.

Keywords: electrospray ionization (ESI), secondary electrospray ionization (SESI), extractive electrospray ionization (EESI), ionization mechanism

Introduction

Extractive electrospray ionization (EESI) mass spectrometry (MS), first introduced by Chen *et al.*,¹ is a soft ionization technique that was developed to investigate samples with complex liquid matrices such as undiluted milk^{1,2} or urine¹ at ambient pressure. In EESI experiments, the sample is nebulized via a capillary pointing towards the MS inlet. In most cases, plume formation is assisted with a nebulizing gas (nitrogen). A second high-voltage spray is oriented in such a way that the plumes cross each other in front of the MS inlet. In this way, the charges necessary for ionization of the analyte are provided. In a typical EESI experiment, both the sample and charging spray solvents are composed of a 1 : 1 (v/v) water/methanol mixture, with the

charging spray solvent being additionally acidified with acetic acid.¹ EESI tolerates complex matrices, allowing, for example, mass spectral analysis of components in olive oil,^{3,4} honey,³ milk^{1,2} and raw urine.¹ Despite the fact that EESI has been used in many publications, not much work has been done to elucidate its mechanism. It is commonly assumed that during the collision of droplets from the charging and the sample sprays, liquid-liquid extraction followed by solvent evaporation accounts for the formation of analyte ions. Recent work by Law *et al.*⁴ showed that by modifying the solvent composition of the charging spray, the mass spectral intensity pattern obtained from compounds of varying polarity contained in

extra virgin olive oil could be influenced, supporting the liquid-liquid extraction hypothesis. We have also shown that, at high solvent flow rates (charging spray $200\ \mu\text{L}\ \text{min}^{-1}$, sample spray $100\ \mu\text{L}\ \text{min}^{-1}$), charged ESI droplets and neutral analyte molecules interact predominantly in the liquid phase.⁵ It would be interesting to investigate whether the situation is similar for typically used EESI flow rates (around $3\text{--}5\ \mu\text{L}\ \text{min}^{-1}$), as well as for a broad range of compounds, for example, more volatile substances, as these might be expected to undergo a gas-phase charge transfer rather than being ionized in the liquid phase.

EESI is similar to three other ionization techniques, secondary electrospray ionization (SESI)^{6–9} desorption electrospray ionization (DESI)^{10,11} and fused droplet–electrospray ionization (FD-ESI).^{12,13} For all three techniques, either liquid-phase interactions between droplets of the charging spray and the sample, gas-phase charge transfer, or a combination of both can contribute to ion formation. In SESI experiments, analyte molecules are evaporated, transported through a tube and then crossed with a charging spray. Therefore, liquid-liquid extraction followed by ionization is not possible. Not surprisingly, the mechanism was found to be essentially a gas-phase charge transfer.⁶ All of the above-mentioned ionization techniques are based on electrospray ionization (ESI) first described by Fenn and co-workers.¹⁴ As opposed to EESI and FD-ESI, there is only one spray involved in ESI experiments. A high voltage is applied to the capillary containing the analyte to provide the charges necessary for ionization. Depending on the size of the analyte molecules, two different theories explaining the ionization mechanism are described in literature; the ion evaporation model¹⁵ (IEM) for small molecules and the charged residue model¹⁶ (CRM) for macromolecules. The CRM suggests that electrospray droplets undergo evaporation and fission cycles until only one ion is left within the droplet. After complete evaporation of the solvent, the remaining ion typically carries multiple charges. In the IEM, evaporation of the solvent causes shrinking droplets to reach a point where the field strength on the surface eventually becomes so high that ions are spontaneously released from the droplet surface.

The IEM usually results in analyte molecules being detected as singly charged $[M+H]^+$ ions (which may be related to the low molecular weight of analytes ionized by this mechanism), which is also the case in EESI experiments. For non-volatile compounds, the spectra of both EESI and ESI are very similar, suggesting a similar mechanism.

In this study, we compared EESI with ESI and SESI using a homologous series of primary amines (butylamine to decylamine) as examples. Except for the changing volatility, these compounds all have very similar physico-chemical properties. We could show that, for these volatile compounds, ionization via gas-phase charge transfer accounts for a large share of the analyte ion current. The lighter the amine, the more pronounced the effect, which means that the proposed liquid-liquid extraction mechanism is not globally valid, but compound dependent.

Materials and methods

Chemicals

1-Butylamine, 1-pentylamine, 1-hexylamine, 1-octylamine, 1-nonylamine and 1-decylamine were obtained from Sigma-Aldrich (Buchs, Switzerland). 1-Heptylamine, methanol and acetic acid were obtained from Acros Organics (Geel, Belgium). Nanopure water with a resistivity of $>18.1\ \text{M}\Omega\ \text{cm}$ was obtained from a NANOpure water purification system (Barnstead, IA, USA).

Ionization sources

All spectra of the amines were obtained in positive ion detection mode on a commercial three-dimensional ion trap (LCQ-Deca; Thermo Finnigan, San Jose, USA). The set-ups for the ESI, EESI and SESI sources are shown in Figure 1. For all experiments, self-made ionization sources were used consisting of 1/16 inch Swagelok Tees (Swagelok Corp., Solon, OH, USA), PEEK tubing (i.d. $250\ \mu\text{m}$, o.d. $1/16\ \text{inch}$; BGB Analytik, Boeckten, Switzerland) for the delivery of nitrogen as nebulizing gas ($270\ \text{mL}\ \text{min}^{-1}$) and polyimide-coated fused silica capillaries (i.d. $75\ \mu\text{m}$, o.d. $150\ \mu\text{m}$;

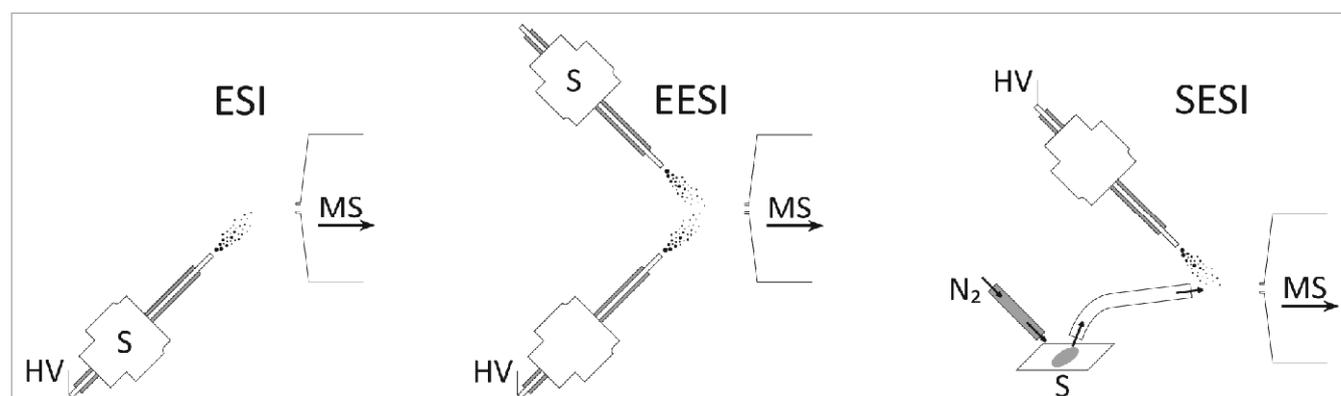


Figure 1. Schematic diagram of the set-ups. Electrospray ionization (ESI; left), extractive electrospray ionization (EESI; middle), and secondary electrospray ionization (SESI; right).

BGB Analytik, Boeckten, Switzerland) for the sprays. For the EESI experiments, the angle between the charging and the sample spray was optimized for highest ion yield and set to 120°, with a distance of 5 mm from each other. The angles between the charging spray (below) and the MS inlet and the sample spray (above) and the MS inlet were set to 120°, with a distance of 10 mm from the spray tips to the MS inlet. These parameters are similar to those used in previous work.^{5,12,13} For ESI experiments, the amines were electrosprayed with the bottom spray while the upper spray was removed. For the SESI experiments, amine vapors were delivered from above into the electrospray plume via a separate heated (80°C) polytetrafluoroethylene (PTFE) tube at a flow of ~20 $\mu\text{mol s}^{-1}$. Fourteen microliters of an amine solution (MeOH/H₂O in a ratio of 1 : 1) were deposited into the PTFE tube where the amines were evaporated with a N₂ flow of 4.5 mL s⁻¹. A single experiment lasted 2 min, during which about half of the spotted solution evaporated. The flow of amines delivered to the electrospray was considered constant as no change in signal intensity could be detected. The charging spray (MeOH/H₂O in a ratio of 1 : 1 acidified with 1% acetic acid) and the sample spray in the EESI experiment were infused at 3 $\mu\text{L min}^{-1}$. Other parameters were optimized for maximum ion yield in each experiment with typical conditions as follows: capillary temperature 250°C, charging spray and MS cone voltages were set to +3.5 kV and +26 V, respectively. MS data acquisition was controlled by the Xcalibur 2.0 software (Thermo Fisher Scientific, Waltham, MA, USA). Mass spectra were collected in scanning mode for 120 s, in the m/z range of 60–200 Th.

Results and discussion

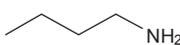
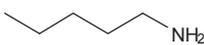
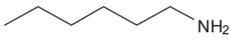
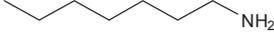
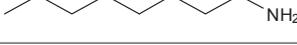
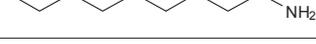
As the name suggests, the main mechanism of EESI is thought to be an extraction of neutral analyte molecules

from sample spray droplets into the charging spray as the droplets collide, to generate analyte ions that are subsequently released in a normal ESI process. Such a mechanism results in spectra that are very similar to ESI spectra.^{1,17} However, compared to ESI experiments, signals from water, water–methanol and methanol clusters are more intense than those of the analyte in EESI. If extraction processes really dominate the EESI mechanism, the intensity distribution of different analyte ions is expected to resemble that obtained by ESI. On the other hand, a SESI spectrum differs significantly from an ESI spectrum. Depending on the volatility of a compound as well as on the distance gaseous analyte molecules have to travel prior to ionization by the charging spray, one expects fewer volatile molecules to be discriminated against more volatile ones in SESI, where a gas-phase charge transfer mechanism is thought to dominate. For volatile analytes, it is easily possible that gas-phase charge transfer contributes to the ionization in EESI experiments. Hence, both extraction followed by ionization in liquid droplets as well as gas-phase charge transfer are possible ionization mechanisms in EESI experiments with volatile analytes. Comparing EESI mass spectra with ESI and SESI spectra should thus allow one to estimate which ionization mechanism is more important.

To study the influence of these two possible ionization mechanisms, a series of homologous amines (Table 1) was chosen as analyte molecules, for several reasons: they have a readily ionizable primary amine functional group, they have very similar gas-phase basicities, and are all in the same molecular weight range, with a mass difference of only 14.02 Da between homologous members.

In every experiment conducted, only two of the seven amines were present, always at the same concentration (1 $\mu\text{mol L}^{-1}$ corresponding to 500 fmol s^{-1} for ESI, 100 $\mu\text{mol L}^{-1}$ corre-

Table 1. Some properties of the amines used in this study.

Chemical	Molecular formula	Structure	Gas-phase basicity (kJ mol ⁻¹) ¹⁸	m/z [M + H] ⁺
1-Butylamine	CH ₃ (CH ₂) ₃ NH ₂		886.6	74.14
1-Pentylamine	CH ₃ (CH ₂) ₄ NH ₂		889.5	88.17
1-Hexylamine	CH ₃ (CH ₂) ₅ NH ₂		893.5	102.20
1-Heptylamine	CH ₃ (CH ₂) ₆ NH ₂		889.3	116.22
1-Octylamine	CH ₃ (CH ₂) ₇ NH ₂		895.0	130.25
1-Nonylamine	CH ₃ (CH ₂) ₈ NH ₂		—	144.28
1-Decylamine	CH ₃ (CH ₂) ₉ NH ₂		896.5	158.30

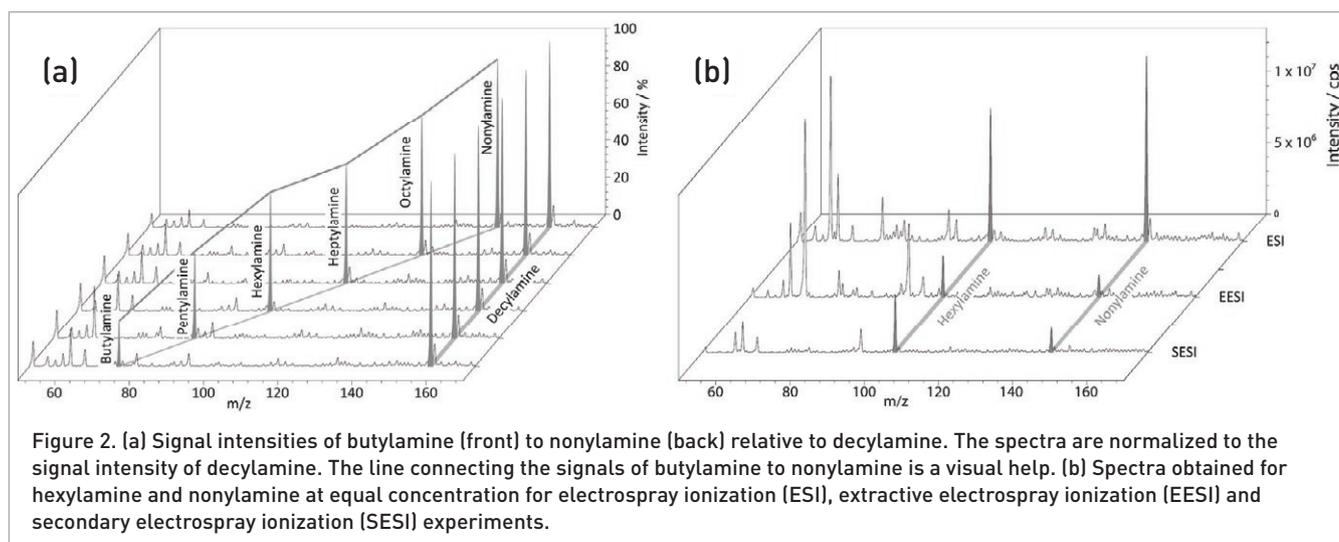


Figure 2. (a) Signal intensities of butylamine (front) to nonylamine (back) relative to decylamine. The spectra are normalized to the signal intensity of decylamine. The line connecting the signals of butylamine to nonylamine is a visual help. (b) Spectra obtained for hexylamine and nonylamine at equal concentration for electrospray ionization (ESI), extractive electrospray ionization (EESI) and secondary electrospray ionization (SESI) experiments.

sponding to 50 pmols^{-1} for both EESI and SESI experiments). Every one of 21 possible combination was analyzed and the intensities detected together with the total ion current (TIC) were recorded. To allow a comparison of different spectra, all intensities were normalized by the corresponding TIC. Figure 2(a) shows all six ESI experiments with decylamine. In this figure, all spectra are normalized by the intensity of decylamine, for comparison. The line connecting the tips of the amine signals is a visual help. Analogous lines are plotted for all compounds in Figure 3, to show the trend of the relative intensities of all amines relative to each other. The intensity ratios are calculated by dividing the intensity of the amines on the x-axis by the intensity of the amine plotted in the graph. Therefore, the increase in intensity ratio for butylamine towards heavier amines in the case of ESI (Figure 3, top) means that heavier amines are detected with higher intensity. This can be explained by the fact that our LCQ system generally shows a better sensitivity for higher molecular weight compounds measured at the same concentration in this mass range. Not surprisingly, the plot in the case of the SESI experiments (Figure 3, bottom) shows the opposite behavior. Here, the heavier ions are strongly discriminated against compared to the lighter ions despite the previously mentioned characteristic of the LCQ. Note that the scale on the y-axis is logarithmic. The plot for the EESI experiments (Figure 3, middle panel) shows only slight discrimination of heavier ions. It resembles the SESI experiment more than the ESI experiment. Gas-phase charge transfer seems to contribute significantly to the ionization of the primary amines, even if they are delivered via a typical EESI configuration.

We next asked the question whether it is possible in EESI experiments to use one amine as an internal standard to quantify other amines present. This is not trivial, because signal intensities vary greatly both between experiments and between consecutively collected spectra. If this were possible, other classes of compounds such as alcohols,

ketones or aldehydes could be quantified as well, quite likely with an internal standard of the corresponding class and possibly even across classes. Our experiments show that an external calibration was not sufficient for generating a calibration curve with sufficiently small errors due to the high signal intensity variation between experiments (data not shown). Table 2 lists the signal variation for hexylamine and nonylamine obtained for the three different ionization techniques. The signal stability decreases markedly with increasing contribution of a gas-phase charge transfer to the total ion current: the signal is most stable for ESI, but varies more in the case of EESI and SESI. This might be due to small air drafts that influence the fragile EESI and SESI set-up to a higher extent than ESI. It is interesting to note that upon normalizing of the signal intensity with the TIC, the stability of the ESI signals for hexylamine and nonylamine is not improving, on the contrary, the normalized signal shows even greater variability. However, the variation for the normalized SESI signals is smaller, whereas in the case of EESI the standard deviation hardly changes at all. In the ESI experiments, the TIC is subject to small variations that are unrelated to the amine signals. In both EESI and especially SESI experiments, however, variations in the TIC correlate more strongly with the signal intensity of the amines.

When an external calibration is not satisfactory, internal standards are often used for quantitative analysis. Table 3 shows the results for the quantitative analysis of hexylamine with nonylamine as internal standard for all three ionization techniques. The quantification was carried out for three different concentration ratios of hexylamine to nonylamine, 7:3, 1:1 and 3:7. The intensity ratio of hexylamine to nonylamine was calculated using 200–600 spectra, which corresponds to a scan time of 2–6 min. Note that for every single amine, the intensity ratio (signal intensity divided by the TIC) is more constant than the signal intensity itself and that the deviation of the intensity ratio is highest for ESI and smallest for EESI. Except for the last three ESI quantifica-

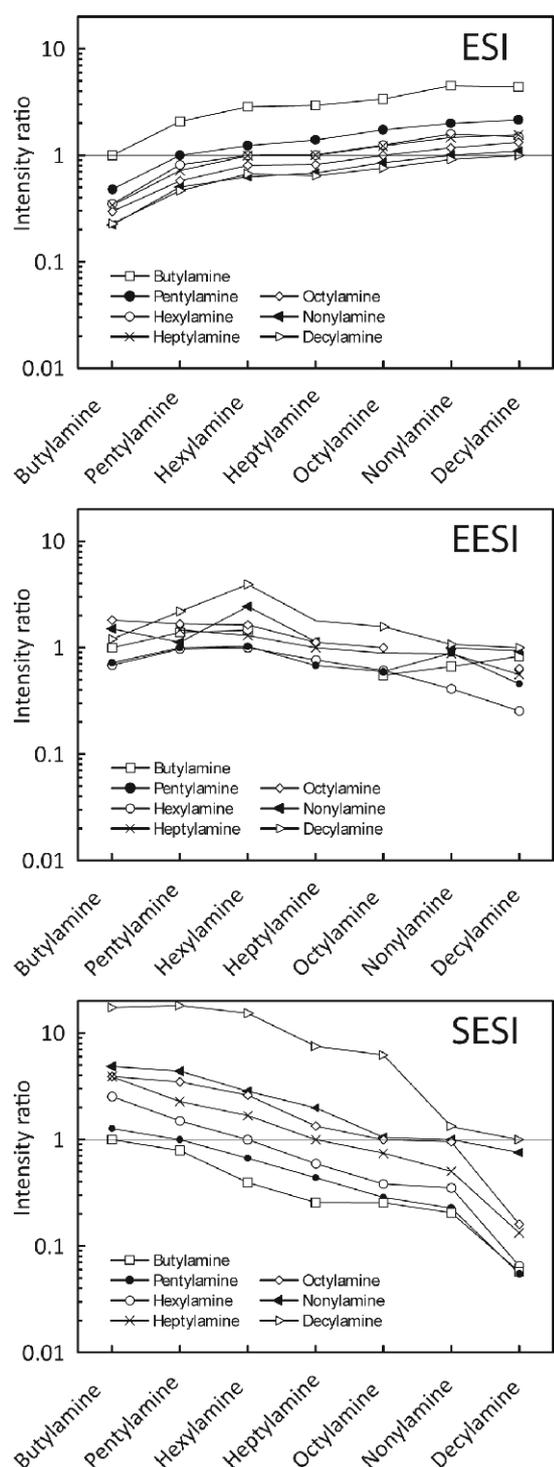


Figure 3. The signal intensity ratios of the amines for the three different ionization techniques used. From top to bottom: electrospray ionization (ESI), extractive electrospray ionization (EESI), and secondary electrospray ionization (SESI). Note that the y-axis is logarithmic. The intensity ratios are calculated by dividing the intensity of the amines on the x-axis by the intensity of the amine plotted in the graph. Therefore, the increase in intensity ratio for butylamine towards heavier amines in the case of ESI (top) means that heavier amines are detected with higher intensity.

tion experiments, the concentrations used were chosen in such a way that the signal/noise (S/N) ratio of nonylamine to the background signal were in the same range (ESI $S/N=29$, EESI $S/N=23$, SESI $S/N=43$) for better comparison. Note that the ESI experiments are roughly a factor of 100 more sensitive than both EESI and SESI experiments. In order to be able to calculate the concentration of hexylamine in the sample based on the known concentration of the internal standard nonylamine, the signal intensity ratio obtained in Figure 2(a) was used; we call this ratio the response factor. The calculated amount of hexylamine is expressed as a flow in units of picomoles per second and is compared to the true concentration present in the analyte solution. The accuracy of the quantification is expressed as a deviation (expressed in %) of the calculated amount from the known amount. EESI experiments yield the most accurate results. In two of three experiments, the calculated concentrations are within the standard deviation of the signal variation. The ESI experiment with the same S/N ratio as the EESI and SESI experiments yields inaccurate results. Separating the high voltage supply needed for ionization from the solution containing the analyte (EESI/SESI set-up) obviously ameliorates the accuracy of the results. While the precision increases for ESI experiments that have higher S/N ratio, surprisingly, the accuracies obtained are worse than for experiments with lower S/N ratios.

Taking both accuracy and precision into account, EESI yields more reliable results than ESI and SESI for this class of compounds for the quantification via internal calibration. However, the concentration difference between internal standard and analyte must not be more than one order of magnitude. As expected, the accuracy decreases with larger concentration differences between the two amines, because the absolute signal variation has a greater influence on the signal intensity. We see this effect confirmed in both ESI and EESI experiments but not in the SESI experiment.

Conclusions

A series of homologous amines was used to estimate which of the two possible ionization mechanisms for the generation of ions in EESI experiments dominates for these volatile compounds: extraction followed by ionization in droplets or gas-phase charge transfer. Comparing the results obtained in EESI experiments with pure liquid-phase ionization (ESI) experiments and pure gas-phase charge transfer ionization (SESI), our experiments showed that the mechanism of EESI for volatile amines is dominated by gas-phase charge transfer ionization. Additionally, in comparison to both ESI and SESI experiments, despite the mass discrimination of the instrument, hardly any discrimination effects occur in the spectra.

In a second set of experiments it was examined whether, in EESI experiments, quantitative statements about compounds can be made. A comparison of the three different ionization techniques showed that EESI experiments yield results that

Table 2. Variation (standard deviation) of the signal intensities for hexylamine and nonylamine, and for hexylamine and nonylamine normalized with the total ion current (TIC) for the three different ionization techniques, expressed as a percentage.

Technique	Hexylamine (%)	Hexylamine/TIC (%)	Nonylamine (%)	Nonylamine/TIC (%)
SESI	44	37	51	43
EESI	28	27	25	24
ESI	12	21	13	20

ESI, electrospray ionization; EESI, extractive electrospray ionization; SESI, secondary electrospray ionization.

Table 3. Quantification results for hexylamine with nonylamine as internal standard for the three different ionization techniques.

Ionization technique	N	Hexylamine:nonylamine		Hexylamine		
		Concentration ratio	Measured intensity ratio	True concentration flow (pmols ⁻¹)	Calculated concentration flow (pmols ⁻¹)	Deviation from true value (%)
SESI	590	0.43	0.30±0.03	32.6	35±4	+7
	580	1	0.64±0.08	23.3	13±2	-44
	510	2.33	1.4±0.2	14	4.8±0.7	-66
EESI	600	0.43	0.44±0.05	15	17±2	+13
	600	1	0.9±0.2	25	25±5	0
	600	2.33	1.3±0.3	35	23±6	-34
ESI	200	1	0.44±0.05	0.125	0.05±0.007	-60
	530	0.43	0.193±0.008	15	4.0±0.2	-73
	600	1	0.82±0.04	25	12.1±0.6	-52
	600	2.33	0.47±0.02	35	4.2±0.2	-89

ESI, electrospray ionization; EESI, extractive electrospray ionization; SESI, secondary electrospray ionization.

are less precise but more accurate than in ESI experiments and results that are both more precise and more accurate than in SESI experiments. This could be explained by the stability and robustness of the EESI set-up.

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