Reversed phase liquid chromatography electrospray ionization mass-spectrometry (LC-ESI-MS) is a method of choice to analyze complex mixtures. In addition to low limits of detection, it enables to confirm the identity of analytes and identify unknown compounds.

Limit of detection of LC-MS analysis depends on the efficiency of generating gas-phase ions of particular analyte and on the influence of other compounds present in ESI—the matrix effect. Ionization efficiency of an analyte can be improved by adding a property-modifying group with derivatization. Derivatization also alters the retention of analytes, which may reduce matrix effect, but the reagents introduced for derivatization can cause additional matrix effect.

The matrix effect (%ME) can be quantitatively expressed by Equation [1], where \( A_{\text{matrix}} \) and \( A_{\text{standard}} \) are peak areas of analyte, respectively, in the presence and in absence of possibly interfering compounds. The %ME value of 100% indicates that the compound in question does not affect the ionization of an analyte. %ME values below and over 100% indicate ionization suppression and enhancement, respectively.

\[
\%\text{ME} = \frac{A_{\text{matrix}}}{A_{\text{standard}}} \times 100\%
\]

The present work was inspired by observations made by LC-ESI-MS analysis of amino acids derivatized with 9-fluorenylmethylmethoxy-carbonyl chloride (Fmoc-Cl) and diethyl ethoxymethylenemalonate (Deemm) derivatives of three amino acids and five other compounds. Influence of boric acid on their ionization was investigated and dramatic impact on the signal was observed. The strongest signal suppression (6% of signal remains) was observed for the Deemm derivative of \( \beta \)-Alanine (with ammonium acetate in eluent). With only formic acid as the eluent pH modifier, signal enhancement was observed, being largest for Fmoc-Cl derivative of Phenylalanine, 267%. Investigation of the influence of boric acid shows that it is a possible signal enhancer for LC-ESI-MS analysis.

**Keywords:** electrospray ionization; boric acid; ionization suppression/enhancement; ionization efficiency

**Introduction**

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The present work was inspired by observations made by LC-ESI-MS analysis of amino acids derivatized with 9-fluorenylmethylmethoxy-carbonyl chloride (Fmoc-Cl) and diethyl ethoxymethylenemalonate (Deemm) in borate buffer. Influence of low concentrations of boric acid on the ionization of Fmoc-Cl and Deemm derivatives of three amino acids and five compounds with comparable ionization efficiencies was studied.

**Experimental**

**Chemicals**

HPLC-grade acetonitrile (J.T. Baker). Tetraethylammonium perchlorate, Fmoc-\( \beta \)-Alanine and Fmoc-Glycine were
purchased from Fluka and Fmoc-Phenylalanine from Sigma-Aldrich. Deemm-Serine, Deemm-β-Alanine, Deemm-Leucine were synthesized in the laboratory. Diphenylamine and 2-nitroaniline were from Reakhim. Dimethyl glutarate was from Merck and diphenyl phthalate from Riedel de-Haën.

Other chemicals: sodium hydroxide (Sigma–Aldrich); ammonium acetate and formic acid (Fluka); hydrochloric acid, orthophosphoric acid, boric acid, sodiumtetraborate, sodium bicarbonate and ammonium hydroxide were from Reakhim. All reagents were of analytical grade or equivalent.

All aqueous solutions were prepared with ultrapure water purified by Millipore Milli-Q Advantage A10 (Millipore, USA). Stock solutions (0.26–3.89 mg g⁻¹) were prepared once and dilutions (0.002–0.39 mg g⁻¹) were freshly prepared before each measurement.

Mobile phase components: A1—0.1% formic acid, A2—1 mM ammonium acetate in 0.1% formic acid (pH = 3.2), B—acetonitrile.

Equipment

LC-MS system Agilent Series 1100 LC/MSD Trap XCT (Agilent Technologies, Santa-Clara, USA) was equipped with an in-line degasser, a binary pump, an autosampler and a column thermostat. For detection, a photodiode array detector and ESI-MS were used. The system was controlled with Chemstation (Rev.A.10.02) and LCMS Trap Control (Version 5.2) software. Chemstation and DataAnalysis (Version 3.2) were used for data analysis.

Most of the chromatographic separations were carried out using Eclipse XDB-C18 (4.6 × 250 mm, 5 μm; Agilent) analytical column with guard column (4.6 × 12.5 mm, 5 μm). Only for Deemm derivatives with A2 as the buffer, Synergi Hydro-RP 80A (4.60 mm × 250 mm, 4 μm; polar endcapped stationary phase) [Phenomenex] with a guard cartridge 4.0 mm × 2.0 mm, polar endcapped C18 (Phenomenex) was used as in Reference 6.

LC-MS analysis

- HPLC conditions for Deemm derivatives: mobile phase components A1 and B with linear gradient of B from 10% to 100% in 45 minutes.
- HPLC conditions for Fmoc-Cl derivatives: mobile phase components A1 and B with linear gradient of B from 30% to 100% in 45 minutes.
- HPLC conditions for analysis of mixture of other compounds: mobile phase components A1 and B with linear gradient of B from 10% to 100% in 30 minutes.
  For all the above, the eluent flow rate was 0.8 mL min⁻¹, column temperature 30°C and injection size 10 μL. The UV detection wavelength was 280 nm.
  HPLC conditions for Deemm with A2 were from Reference 6.
  Common ion source parameters for all compounds: nebulizer gas (nitrogen) 50 psi (345 kPa), drying gas (nitrogen) 12 L min⁻¹ and drying gas temperature 350°C. ESI voltage and MS parameters were optimized separately for each compound.

**Experiment setup**

To test the influence of boric acid on chromatographic peaks in ESI source and mass spectra of the analytes, boric acid solution or ultrapure water was added to the chromatographic effluent before the ESI inlet via a tee-piece. The addition was carried out using a syringe pump at 0.5 mL h⁻¹ (8.3 μL min⁻¹) flow rate. Concentration of boric acid in the ESI source was 1.8 mM for Deemm derivatives and 2 mM for other compounds. For each analyte, %ME was calculated from peak areas with boric acid (A_{intra}) and water (A_{standard}) infused [Equation (1)].

**Results and discussion**

Deemm and Fmoc-Cl amino acid derivatives were obtained as pure substances, i.e. no borate was present in the solutions. Other compounds [diphenylamine (DPA), 2-nitroaniline (2-NA), tetraethylammonium perchlorate (TEA), dimethyl glutarate (DMG) and diphenyl phthalate (DPhP)] were chosen according to the ESI efficiency scale of organic compounds in order to include compounds with higher and lower logRIE values than Deemm and Fmoc-Cl derivatives of amino acids (Table 1). Experiments were repeated five times over a period of one month.

In order to evaluate the influence of boric acid, an experiment without boron acid was carried out and ultrapure water was added to the ESI source in order to keep the concentration of the analytes identical. For the boric acid experiments, 0.2 M boric acid in eluent (1 mM ammonium acetate in 0.1% formic acid) was infused. Identical results were obtained if the boric acid was dissolved in ultrapure water or 0.1% formic acid. At lower boric acid concentrations, no effect on signal intensity was observed.

Compared to the eluent flow rate (0.8 mL min⁻¹), the infusion rate of either boric acid or water was low (8.3 μL min⁻¹). pH of the original eluent (A1 and A2) and pH of the eluent after addition of boric acid or water was measured. Identical pH values were recorded for the eluent before and after addition of boric acid or water. Consequently, the observed effects cannot be explained by eluent pH change and must be attributed to the presence of boric acid in the ESI source.

The influence of boric acid on the mass spectra was also studied. In positive ion ESI mode, boric acid has no influence [Figure 1(a)]. In negative ESI mode, addition of boric acid gives rise to a multitude of new peaks. These can be related to various anionic complexes of boric acid. It is interesting to note that despite of the presence of these ions, the signal of an analyte can be enhanced, such as in the case of Fmoc-Cl derivatives [Figure 2]. However, additional peaks due to the presence of boric acid complicate the interpretation of mass spectra.

**Positive ion mode**

For positive ion mode ESI, %ME values [Figure 2] show that the effect of boric acid varies strongly. For most compounds (except 2-nitroaniline), the signal was enhanced by the boric...
acid and for Fmoc-Cl derivatives the enhancement was most substantial. Enhancement can be up to 267%, as in the case of Fmoc-Phenylalanine. In case of Deemm derivatives, the influence of boric acid depends on the composition of the elution buffer. With eluent A1, signal enhancement was also observed for Deemm derivatives, but if the eluent contains ammonium acetate (eluent A2), ionization suppression (as low as 6% for Deemm-β-Alanine) is observed (Figure 2).

It can be concluded that the ionization enhancement is related to the use of boric acid and formic acid but if ammonium acetate is present, suppression occurs. This is confirmed by preliminary results of boric acid influence on Fmoc–Cl derivatives with ammonium acetate present.

Two other tendencies should be mentioned. Signal enhancement is stronger when analytes have longer retention times, meaning that the percentage of organic component in the solvent is higher (Fmoc–Phenylalanine, Deemm–Leucine and diphenyl phthalate) and compounds with higher logRIE values are more likely to observe signal enhancement in positive ion mode if borate buffer is present. However, considering the limited volume of experimental data, these results are inconclusive.

### Negative ion mode

Negative ion mode was used for amino acid derivatives and diphenyl phthalate (Figure 2). The remaining compounds did not ionize in negative mode ESI. It is distinct that for Deemm derivatives strong signal suppression (40–55%) is observed with ammonium acetate in eluent. With only formic acid present in the eluent, similarly to the positive ion mode, enhancement was observed (116–160%). For Fmoc-Cl derivatives, there is also signal enhancement of up to 181% as in the positive mode. For diphenyl phthalate, the signal is also strongly enhanced, as it is more than twice the signal when no boric acid is present.

### Mechanism of action of boric acid

Results demonstrate that boric acid strongly influences the ESI ionization. Surface activity of matrix compounds is most often regarded as the cause of suppressing %ME. As boric acid caused both enhancement and suppression, then other mechanisms must be sought.

Borates are known to form complexes. One may assume that borate ion forms complexes with analyte molecules and those complexes facilitate evaporation of the analytes into the gas phase. This could be the case with all the experiments where ammonium acetate was not present (Figure 2). If ammonium acetate simply prevents formation of the complex, then there should be no matrix effect (%ME = 100%). However, this is not the case, since severe ionization suppression is observed when ammonium acetate is present (Figure 2). Consequently, borate complexation with the analyte can hardly be the reason for the observed effects.

As other mechanisms of action, one should consider Lewis acidity and electrochemical properties of boric acid. Influence of borate on solvent structure may also play a role.

The mechanism of action of borate remains unclear, but it certainly deserves further investigation.

### Practical considerations

For compounds for which boric acid has substantial enhancement effect, it could be used to enhance sensitivity of ESI-MS determinations and if reproducibility is adequate, quantitative analysis is possible.

As for amino acid derivatization, where borate buffers are widely used, these should be given another look at and
made sure that they do not suppress the signal or cause poor reproducibility especially when buffers containing ammonium acetate are used. To solve the problem, buffers other than borate buffer could be considered for amino acid derivatization. Another solution would be to divert effluent from the initial part of the chromatographic run into waste.

Figure 1. Comparison of chromatograms (EIC) of Fmoc-β-Alanine with boric acid and with ultrapure (MilliQ) water: a) positive ion mode ($m/z$ 312); b) negative ion mode ($m/z$ 310). Insets are the respective mass spectra.
Conclusions

Boric acid can severely influence ionization of analytes in LC-ESI-MS. For Deemm amino acid derivatives, strong signal suppression in both positive and negative modes was observed when ammonium acetate was present in the eluent. On the other hand, ionization of Fmoc-Cl and Deemm amino acid derivatives was enhanced if boric acid was present and only formic acid was used to control the pH of the eluent.

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References


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**Figure 2.** Calculated %ME values for analytes in positive and negative ion mode. (a): with 0.1% formic acid as an eluent component and (b): with 1 mM ammonium acetate in 0.1% formic acid as an eluent component (n = 5).