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Sensitivity improvement in hydrophilic interaction chromatography negative mode electrospray ionization mass spectrometry using 2-(2-methoxyethoxy)ethanol as a post-column modifier for non-targeted metabolomics☆

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ABSTRACT

The application of ammonia acetate buffered liquid chromatography (LC) eluents is known to concomitantly lead to ion suppression when electrospray ionization mass spectrometry (ESI–MS) detection is used. In negative ESI mode, post column infusion of 2-(2-methoxyethoxy)ethanol (2-MEE) was shown in the literature to help to compensate this adverse effect occurring in reversed phase liquid chromatography mass spectrometry (RP-LC–MS) analyses. Here a setup of direct infusion and hydrophilic interaction chromatography (HILIC) post-column infusion experiments was established in order to investigate systematically the beneficial effects of 2-MEE. We demonstrate that, 2-MEE can help to improve ESI–MS sensitivity in HILIC too and reveal analyte structure specific behaviors. Our study indicates that 2-MEE especially improves ESI response for small and polar molecules. The ESI response of stable isotope labeled amino acids spiked into biological matrices increases up to 50-fold (i.e. D_5-L-glutamic acid) when post column infusion of 2-MEE is applied. A non-targeted analysis of a pooled urine sample via HILIC–ESI–QTOF–MS supports this hypothesis. In direct infusion, the combined application of an ammonia acetate buffered solution together with 2-MEE results in an improved ESI response compared to a non-buffered solution. We observed up to 60-fold increased ESI response of L-lysine. We propose this effect is putatively caused by the formation of smaller ESI droplets and stripping of positive charge from ESI droplets due to evaporation of acetic acid anions. In summary, post-column infusion of 2-MEE especially enhances ESI response of small and polar molecules. Therefore it can be regarded as a valuable add-on in targeted or non-targeted metabolomic HILIC–MS studies since this method sets a focus on this molecule category.

1. Introduction

Liquid chromatography (LC) coupled to mass spectrometry (MS) via electrospray ionization (ESI) is one of the key technologies in analytical science. It is widely used in chemical synthesis, pharmaceutical, clinical, environmental and –omics sciences [1]. It is a generally accepted fact that pH-control of liquid chromatography eluents is one of the crucial points to provide robust and valid LC separations. The use of volatile buffers and mobile phase additives (such as formic acid (FA), acetic acid (AcOH) and

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its corresponding ammonia salts and trifluoracetic acid (TFA)) is particularly necessary when analytes of interest contain ionizable moieties such as amino or carboxylic groups [2]. The effects of these additives on electrospray ionization (ESI) are various and are concentration dependent. The formation of charged adducts such as $[M+NH_4^+]^+$, $[M+FA^-]^-$, $[M+AcO^-]^-$ reduce the peak height of the (de)protonated mass signal, decreasing sensitivity and increasing spectral complexity [3].

The application of strong acids (e.g. TFA) or bases (e.g. triethylamine (TEA)) lead to neutral salts formation such as $[M+H]^+ + [TFA]^-$ (in positive mode ionization) and $[M-H]^- + [TEA]^+$ (in negative mode ionization) due to their inherent ion paring properties. It is not possible to detect these ion pairs via MS; this effect drastically decreases MS sensitivity [4].

The complete or partial suppression of protonated and deprotonated molecules in positive and negative ionization modes, respectively, can be observed. This is a consequence of the excessive ion formation derived from basic or acidic modifiers in mobile phase (e.g. pos mode: TEA, neg mode: TFA, acetic acid and formic acid) competing for the charge during ESI droplets formation [5,6]. It was reported that TEA is accumulating on the inner surface of the mass spectrometer vacuum chamber [5].

On the other side, some favorable effects such as an increase of sensitivity can be observed during the application of weak acids and their corresponding ammonia salts (in concentration lower than 1 mM) [7] [8] or ammonia fluoride in [9]. Yamaguchi et al. [10] introduced 2-(2-methoxyethoxy)ethanol (2-MEE) as an ESI dopant able to compensate the ion suppression effect of ammonium acetate (NH_4OAc) buffer in negative ESI. Investigations on ibuprofen and its metabolites via ESI–MS, reported an increase in ESI response (up to 100 fold) using ammonium acetate buffer, as mobile phase and 2-MEE as post-column modifier. These results contrast the ones obtained in absence of 2-MEE.

Nowadays the introduction of silanol deactivated silica-based and hybrid materials in reversed phase (RP) LC–MS separations almost superseded the necessity of NH_4OAc buffer as mobile phase [11,12]. Many separations are mainly performed by TFA or FA acidified mobile phases. In hydrophilic interaction chromatography (HILIC) ammonium acetate and formate buffers still remain quite popular mobile phases [13]. HILIC stationary phases generally need to exhibit a highly polar surface in order to be able to establish an adsorptive molecular layer of water which actually represents or acts as stationary phase [14]. Usually used column packing materials exhibit ionizable groups such as, silanol groups (bare silica), sulfobetaine groups (ZIC HILIC) or aminopropyl groups (amino HILIC). In order to suppress, minimize or adjust secondary interaction s such as ion exchange, a sufficient cation and anion concentration in the mobile phase has to be provided [14]. Of course there are also virtually non-ionizable HILIC stationary phases available (amide, diol, pentafluorophenyl ...) however also here very often ammonia buffers need to be applied, probably due to incomplete silanol capping. Due to the high organic content used in HILIC eluents, ESI response is significantly higher compared to ESI response during RP separations. However the use of 2-MEE as post-column dopant in HILIC–MS applications could provide an additional increase of sensitivity.

The aim of this study is the investigation of the beneficial effect of post-column application of 2-MEE during HILIC–MS separations. Metabolite standards where analyzed to elucidate the 2-MEE dopant mechanism. An extended/enhanced mechanism, compared to the initially introduced one, is proposed which is responsible for the dopant effect.

To evaluate matrix effects of biological samples on the proposed HILIC–MS 2–MEE post-column infusion system, stable isotope labeled amino acids are spiked into biological samples (human urine and plasma) and analyzed with and in absence of post-column infusion of 2-MEE.

2. Experimental

2.1. Chemicals

All standards were obtained from (Sigma-Aldrich, Taufkirchen, Germany). Each standard was dissolved in water/acetonitrile/methanol (4:3:1) at a final concentration of 500 mg/L. Each solution was acidified with 0.2% formic acid. 2-(2-Methoxyethoxy)ethanole (methyl carbitol) was purchased from Sigma Aldrich. LC–MS grade acetonitrile and methanol were obtained from Sigma Aldrich. LC–MS grade water was obtained from a MilliQ water purification system (Millipore, Molsheim, France).

2.2. Biological samples

Pooled samples of human urine and plasma from healthy volunteers were used. Ice cooled acetonitrile (400 μL) was added to an aliquot of sample (100 μL). The resulting mixture was vortexed for 10 s and centrifuged at 4 °C 20,817 × *g* for 5 min. The obtained supernatants were dried in a vacuum centrifuge.

The evaporated residues were reconstituted in a solution of acetonitrile/methanol (3:1) and 0.2% formic acid (reconstitution solvent). The reconstituted samples were subjected to supersonic bath (5 s) vortexed (10 s) and centrifuged at 4 °C 20,817 × *g* for 5 min. The supernatants were transferred to ultra-pressure liquid chromatography (UPLC)-vials for the analysis. All plasma and urine samples were prepared according to this procedure.

2.3. U(H)PLC–QTOF–MS measurements

LC–MS experiments were performed using a Acquity-UPLC system (Waters Milford, MA, USA) coupled to a SYNAPT–G1–QTOF HD mass spectrometer (Waters Micromass, Manchester, UK). In post column infusion experiment 2-MEE flow was facilitated via Ultimate Plus separation system (Dionex/LC Packings). The following ESI parameters were applied: capillary voltage 2.3 kV, sample cone 24 V, extraction cone 4.0 V, source temperature 120 °C, desolvation temperature, 350 °C, cone gas Flow 20.0 L/h, desolvation gas flow 800 L/h. Scan time was set to 300 ms in and *m/z* range from 50 to 1000 in V-mode (average 9000 resolution).

HILIC separations were performed on an Acquity UPLC BEH HILIC (150 × 2.1 mm 1.7 μm, Waters) column in isocratic and gradient mode. The column temperature was set to 40 °C. Mobile phase A consists of 10 mM ammonium acetate and 0.2 v/v% acetic acid in water, mobile phase B consists of acetonitrile. The flow rate was set to 300 μL/min, the injection volume was set to 5.0 μL. Isocratic elution was performed with 33% mobile phase A and 67% mobile phase B for 10 min. Gradient elution was performed with a linear gradient: 0–2 min: 5% A, 15–20 min 50% A, 20.5–25.5 min 5% A. For spiking experiments stable isotope labeled standards were pooled and diluted in reconstitution solvent to a final concentration of 20 mg/L each. For all other HILIC–MS experiments standards were diluted in reconstitution solvent to a final concentration of 20 mg/L each. 2-MEE was post-column infused with a flow rate of 150 μL/min. A source:waste (1:1) split was applied in non-post column infusion experiments. In post-column infusion experiments a source:waste (1:2) split was applied in order to maintain constant flow rate of ~100 μL/min to the ESI-source.

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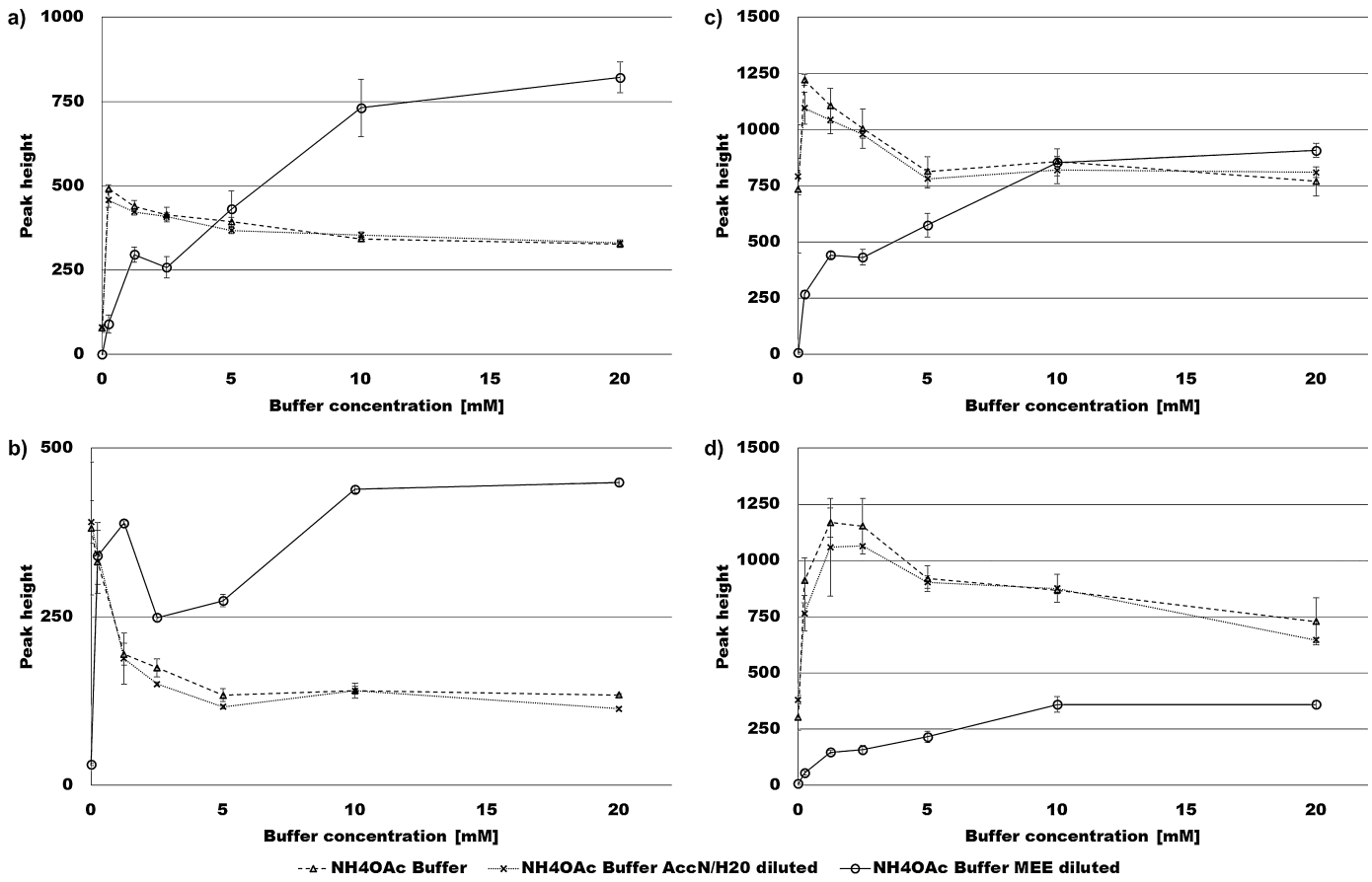

Fig. 1. ESI-responses (peak height in counts) to various buffer types and concentrations of different standards, (a) L-leucine, (b) D10-adipic acid, (c) D6-sulfadimethoxine, (d) D4-cholic acid.

2.4. Direct infusion experiments

2.4.1. Experiment A

Standard mixture A (see Supplementary material Fig. 1) was diluted by factor 100 in acetonitrile/water (4:1 v/v). The resulting solution was split into 7 aliquots and spiked with increasing ammonia acetate concentrations. Each aliquot was again split into 3 sub-aliquots. The first sub-aliquots were not further modified. The second sub-aliquots were diluted 1.5 factors in acetonitrile/water (4:1 v/v). The third sub-aliquots were diluted by factor 1.5 in 2-MEE. The aliquots where analyzed in triplicates in direct infusion mode (200 μL/min for undiluted samples, 300 μL/min for diluted samples). Additionally, a dilution row of standard mixture A was prepared and analyzed (triplicates) to ensure applied concentrations are within linear or dynamic range of the instrument data not shown (applied dilution factors: 50, 75, 100, 200 and 500).

2.4.2. Experiment B

Standard mixture B containing aspartic acid (133.3 mg/L), lysine (166.7 mg/L) and phenylalanine (166.7 mg/L) were diluted by factor 100 in acetonitrile/water (2:1 v/v). The resulting solution was split into 7 aliquots. The aliquots were spiked with different ammonia acetate and 2-MEE concentrations. All samples were analyzed in triplicates at a flow rate of 100 μL/min.

2.5. Software and data processing

Physiochemical parameters of applied standards were predicted using Jchem for excel 6.1.0.688 2013 ChemAxon (http://www.chemaxon.com).

Chromatogram alignment and peak picking for non-targeted HILIC–U(H)PLC–QTOF analysis was performed using Genedata MS Refiner 8.0. Mass annotation of detected peaks was facilitated via Genedata in combination with HMDB human metaboloite database (HMDB) version 3.5 [15–17] with a mass annotation error of ±7 ppm.

Quantification of targeted HILIC–U(H)PLC–QTOF analysis was performed using Targetlynx software (included in Masslynx 4.1 (SCN639)).

UPLC–MS system was controlled via MassLynx 4.1 (SCN 639) software.

3. Results & discussion

3.1. Impact of 2-MEE on ESI response in HILIC mobile phase condition

The beneficial effects of post-column infusion of 2-MEE on ESI efficiency during RP separations carried out in buffered (ammonia acetate) solvents has been shown previously [10]. ESI response of ibuprofen and related metabolites was significantly enhanced when post-column infusion of 2-MEE was applied [10]. In RP separations polar analytes elute at relatively low concentrations of organic solvents, such as acetonitrile or methanol. In contrast to RP, HILIC necessitate the use of high percentage of organic solvent (e.g. acetonitrile content 95–50%) which can also be buffered (i.e. NH_4OAc). A set of direct infusion experiments (experiment A see material and method) was performed to estimate the dopant effect of 2-MEE on ESI efficiency in HILIC solvent conditions. Standard mixtures containing different NH_4AcO concentrations were

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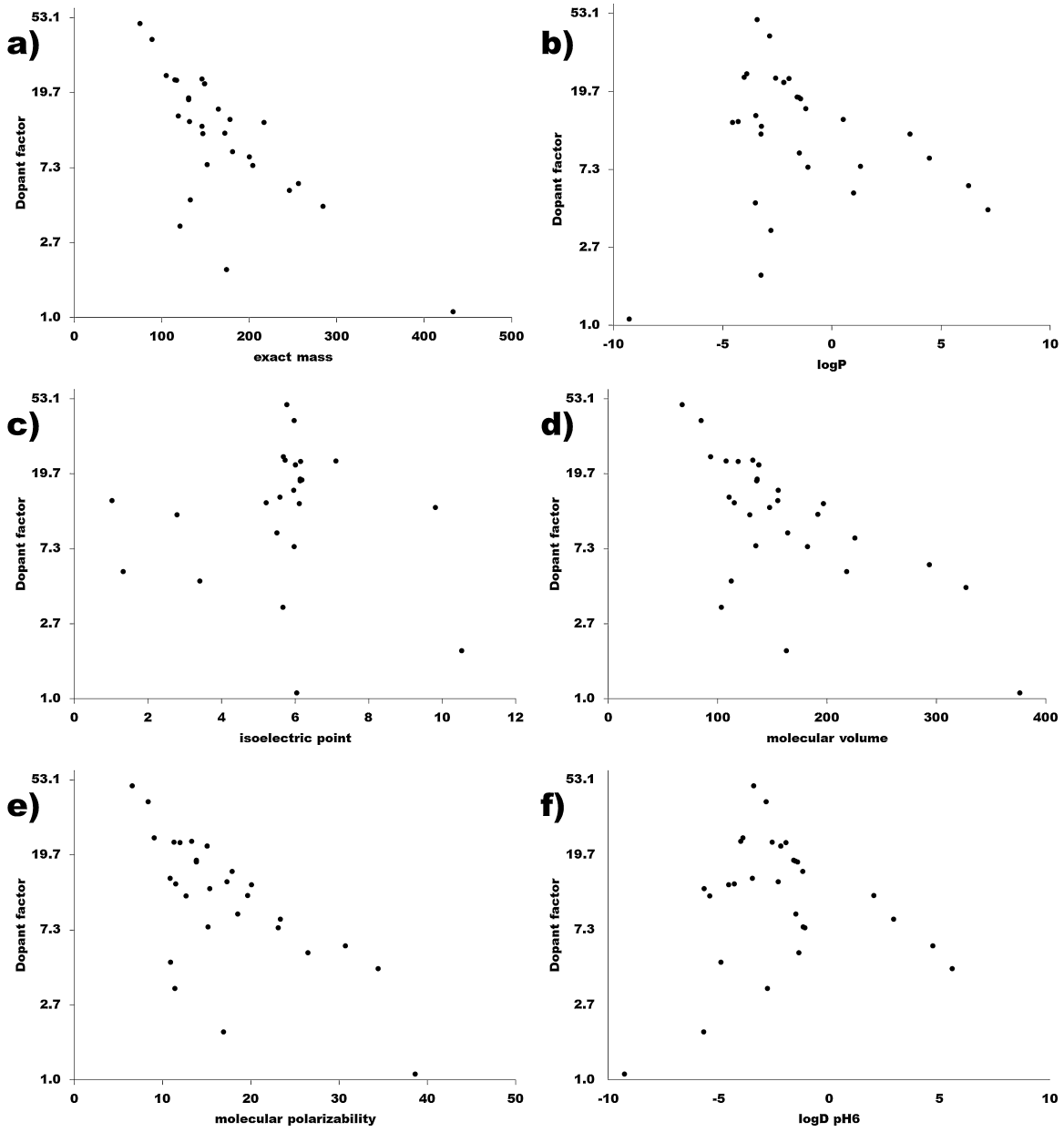

Fig. 2. Dopant factor over different physiochemical parameters (a) molecular weight, (b) logarithmic water–octanol partition coefficient of unionized solutes (log *P*), (c) isoelectrical point, (d) molecular volume, (e) molecular polarizability, and (f) logarithmic water–octanol partition coefficient at pH 6. *y*-Axes are ln-scaled.

analyzed with and without the addition of 2-MEE. Each experiment was performed in triplicates.

Fig. 1 exemplary shows the dopant effect of 2-MEE in direct infusion analysis (figures of all analyzed standards can be found in Supplementary material Fig. 2). Especially highly polar analytes ($m/z < 200$) seem to benefit. As expected a suppressing effect of the buffer arises. Besides a trend can be observed, when 2-MEE is applied increasing buffer concentrations lead to better ionization efficiencies. Similar effects could be observed when standards were analyzed via HILIC–LC–MS (see Supplementary material Fig. 3).

3.2. Impact of physicochemical parameters on 2-MEE dopant effect

In order to understand better the dopant mechanism, an extended array of standards (mostly amino acids) were analyzed with and without 2-MEE (for complete list see Supplementary material). Each experiment was performed in triplicates (isocratic HILIC separation method). The resulting peak areas were analyzed and a dopant factor was generated. The dopant factor is the ratio of the peak area under the influence of 2-MEE to the peak area in absence of 2-MEE. Plots of the dopant factor over different physiochemical parameters (via Jchem predicted) are depicted in Fig. 2. In addition a plot of the dopant factor based on signal to noise ratios over molecular weight is provided in Supplementary Fig. 3. For detailed information see Supplementary data Table 2.

It is possible to observe a logarithmic correlation between dopant factor and: molecular weight, molecular volume, molecular polarizability and log *P*. These 4 physiochemical properties are confounding factors within the set of standards. The influence of strong acidic or basic moieties such as guanidino-, *tert*-, amino-, sulfo- or phosphorous-groups cannot be examined with the applied standards

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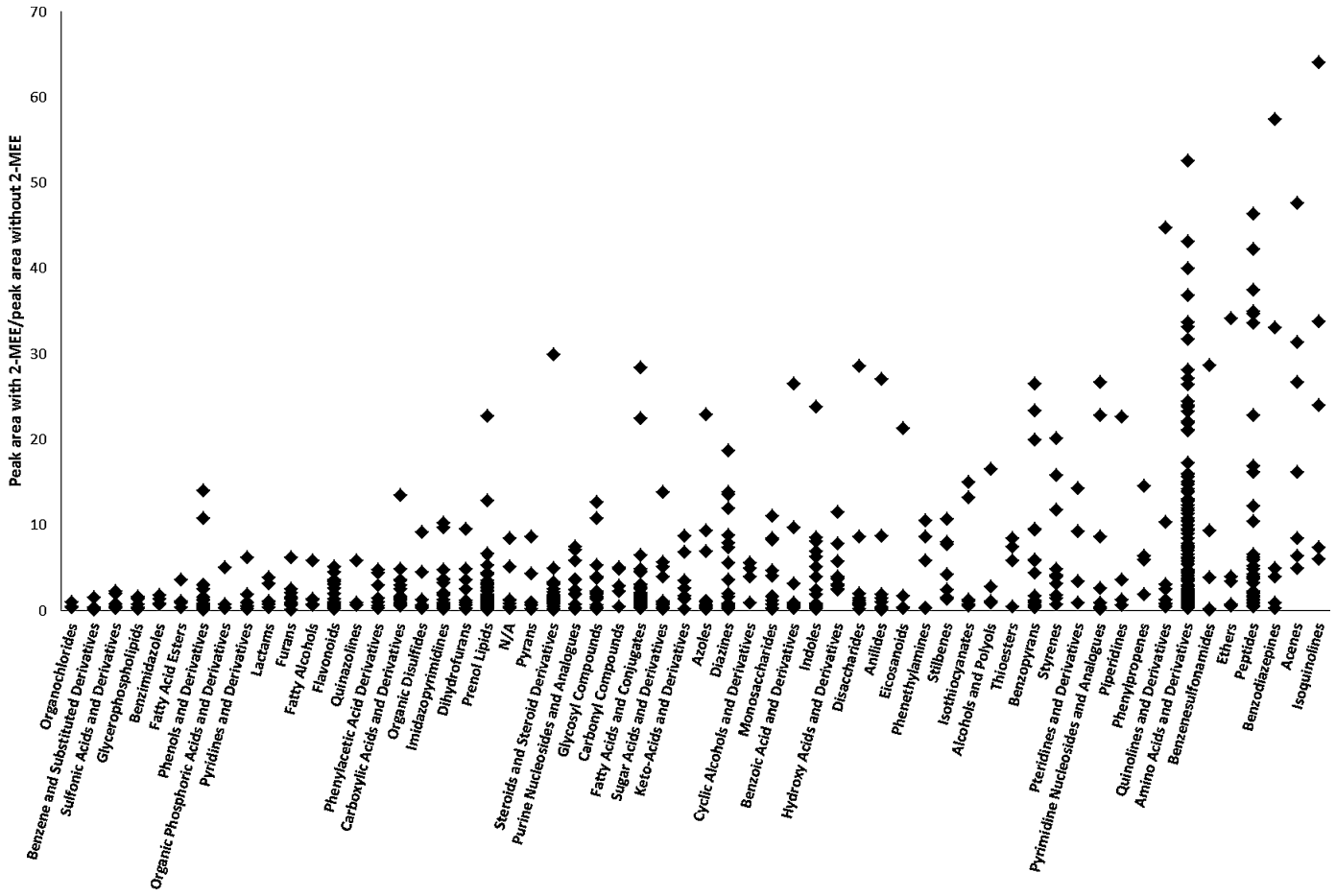

Fig. 3. Dopant factor over HMDB metabolite taxonomy class. Individual dots depict the dopant factor of mass annotated peaks and their corresponding HMDB metabolite taxonomy class resulting from non-targeted HILIC–MS analysis of human urine samples.

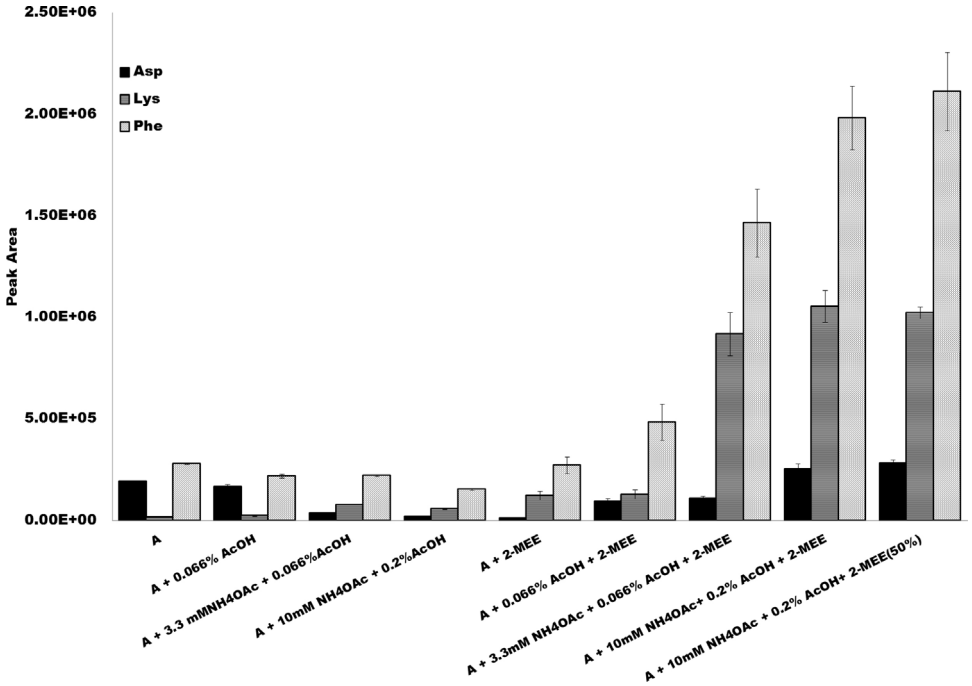

Fig. 4. Influence of buffer additives and 2-MEE on signal abundances (with A = acetonitrile:water (2:1 v/v)).

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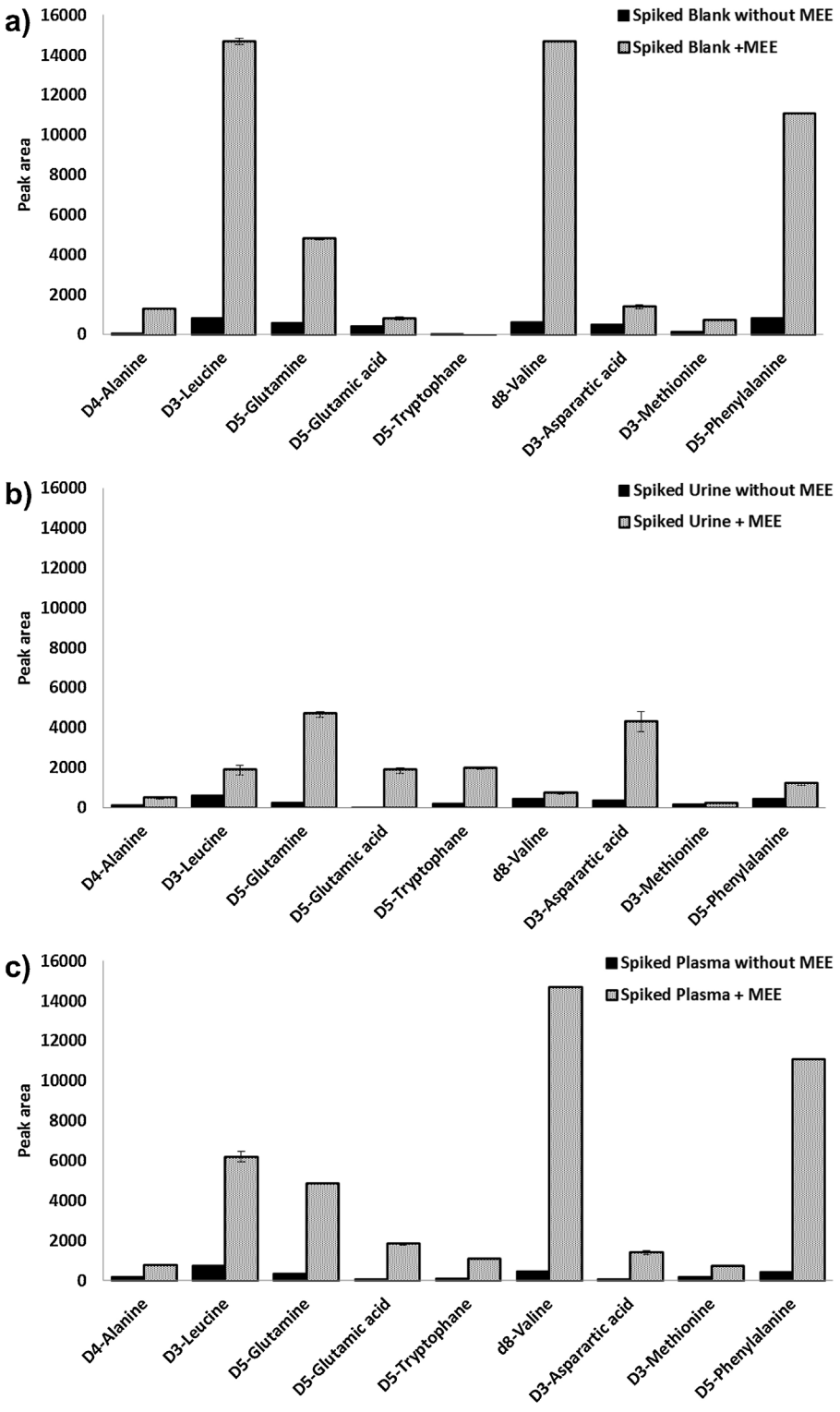

Fig. 5. Comparison of stable isotope labeled amino acids peak areas in (a) blank and (b) and (c) biofluids with and without post column infusion of 2-MEE.

3.3. 2-MEE dopant effect on metabolite classes

In order to study the effect of 2-MEE in a more holistic way, a non-targeted investigation of a pooled urine sample from healthy volunteers was conducted via HILIC–U(H)PLC–QTOF–MS with and without post-column infusion of 2-MEE. Every experiment was performed in quadruplicates. Only peaks detected under both conditions (with and without post column infusion) and putatively assigned via Genedata were considered. In Fig. 3 the dopant factor is plotted over the HMDB metabolite taxonomy class. Among the different metabolite groups a trend is observed. The molecules that benefit most of the dopant effect are of small molecular weight, highly polar and of amphoteric character or combinations of these properties. ESI-response of metabolites containing strong acidic

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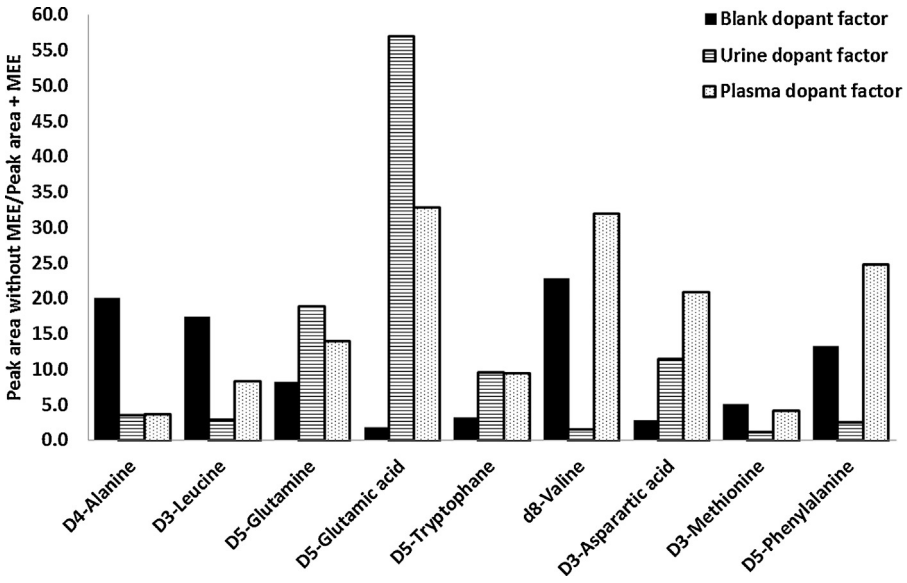

Fig. 6. Dopant factor in spiked blank, urine and plasma showing differences in the matrice effect but systematic increase in sensitivity.

functional groups such as sulfone- or phosphate groups is more suppressed than enhanced.

3.4. Impact of buffer concentration on 2-MEE dopant effect

Yamaguchi [10] proposed a mechanism that explains how 2-MEE can increase ESI efficiency. A buffer additive (i.e. ammonia acetate) has to be used in high performance liquid chromatography (HPLC) to achieve a reasonable chromatographic separation. Subsequently, the detection of the analytes via ESI–MS can be suppressed due to an excessive formation of acetate anions $(AcO)^-$ originated from the buffered HPLC eluents. Analyte molecules and acetic acid are in direct competition for negative charges in the ESI chambers. The addition of a solvent with a high boiling point (2-$MEE_{bp} \sim 194\,^{\circ}C$) results in the selective evaporation of the neutral acetic acid molecules (acetic $acid_{bp} \sim 118\,^{\circ}C$) from the ESI droplets. Therefore, this phenomenon leads to less charge competition.

In order to verify and validate this hypothesis a further direct infusion experiment was conducted (direct infusion experiment B). A mixture containing 3 amino acids (i.e. Asp, Lys and Phe) was spiked with different ammonia acetate and 2-MEE concentrations respectively. The obtained combinations were analyzed via direct infusion ESI–QTOF–MS.

It is expected that, the analysis without any modifiers would yield to highest signal intensity and this ESI is the reference experiment. Following the hypothesis of Yamaguchi [10], the addition of ammonia acetate would significantly reduce mass signal abundances; the addition of 2-MEE (without addition of ammonia acetate) would result in equally or slightly reduced signal abundances; the addition of NH_4AcO + 2-MEE would also result in equally or slightly reduced signal abundances due to incomplete acetic acid evaporation.

As expected each of the additives on its own showed a suppressing effect (Fig. 4) on signal intensities (except for lysine). However, the analyses of the amino acid mixture in combination with the addition of ammonia acetate and 2-MEE outperform the analysis without any modifier. The analyses also showed that the ratio of the applied solvent to 2-MEE can be reduced from 2:1 to 4:1, without decreasing the beneficial effects of 2-MEE.

The obtained results indicate not only a compensating effect of 2-MEE for acetic acid suppression but also a multiple step mechanism to increase electrospray ionization efficiency.

Acetic acid evaporates from ESI droplets due to its lower boiling point compared to 2-MEE. This phenomenon may lead to a reduced acetic acid concentration in the droplets. Due to the lower acetic acid concentration less competition for negative charges takes place in the ESI droplet resulting in a more efficient ionization of the analyte and in an increase of signal heights. During the transfer from liquid to gaseous phase, deprotonated acetate anions draw protons from ESI droplets. Stripping protons from ESI droplets may in addition facilitate the deprotonation of analyte molecules and improves ionization efficiency.

The removal of acetate anions decreases the surface charge of ESI droplets before they reach Rayleigh limit, with a consequent smaller ESI droplets formation and an improved ionization efficiency [18,19]. To verify this hypothesis ESI droplet size could be measured via phase Doppler anemometry (PDA) [20,21]. Additionally there is possibility to indirectly follow the droplet size via concentration determination of a fluorescence resonance energy transfer (FRET) dye pair. Reducing ESI droplet size due to solvent evaporation leads to an increase of the dissolved dye pair concentration until FRET occurs [21].

Two additional dopant effects can thus be proposed to the already identified mechanism. The reduced size of ESI droplets and the substraction of positive charge from analytes in ESI droplets converge to the finding that especially small, less acidic and very polar compounds benefit from the 2-MEE dopant effect. The proposed mechanism also complies with the ESI charge residue process theory [22]. Small and polar molecules are predicted to stay in the inner side of ESI droplets especially when lipophilic, or generally surface active molecules are also present in the droplets. Due to this effect, the probability of getting ionized is lower for small and polar compounds. When the ESI droplet size is reduced, the relative surface area of the droplets increases. At the same time, the probability of getting ionized during the ESI process is increased for small and polar compounds.

3.5. HILIC–MS negative ESI mode. 2-MEE post column infusion in practice

A spiking experiment was conducted to test the signal dopant potential of 2-MEE under HILIC conditions in biological samples. Stable isotopes labeled amino acids were spiked into pooled urine and plasma samples of healthy volunteers. The types of samples

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were chosen to evaluate the 2-MEE dopant effect in presence of matrix effects originating from diverse biological sources. Urine is considered a biological matrix containing highly polar and small molecules and a high inorganic salt content; protein precipitated plasma on the other hand exhibits a high complexity of various chemical compounds, e.g. a high content of lipids [23] and low amounts of peptides and small proteins remaining from incomplete protein precipitation [24].

HILIC–LC–MS analyses were performed with and without post column infusion of 2-MEE. The obtained peak areas are depicted in Fig. 5.

Comparing the retrieved peak areas leads to the conclusion that, in HILIC–LC–MS, urine exhibits a stronger matrix effect compared to plasma. This observation complies with HILIC theory since polar analytes are retained on HILIC materials whereas non-polar molecules elute in the void volume of the chromatogram. The application of post-column infusion of 2-MEE improved signal intensities of the analyzed standards even in presence of matrix effect (dopant factors are depicted in Fig. 6).

In order to restore initial ESI performance/characteristics/response peek tubing and ESI capillary need to be flushed with water:acetonitrile (1:1 v/v) for about 20 min and ESI chamber needs to be cleaned according to vendors specification (tested with Bruker and Waters mass spectrometers, data not shown). Even after several days of 2-MEE infusion no persistent negative side effects could be observed when following this cleaning procedure.

4. Conclusion

HILIC separation techniques offer an orthogonal retention behavior and improved sensitivity in MS detection for many polar compounds of biological interest compared to RP separations. However in most HILIC separation volatile buffers such as ammonia acetate need to be applied in order to obtain reasonable chromatographic separation. As a side effect these buffers suppress electrospray ionization efficiency leading to lower signal intensity. Here the application of post-column infusion of 2-(2-methoxyethoxy)ethanol gives a new opportunity to increase in sensitivity in targeted or non-targeted metabolomics applications. Our findings show that 2-MEE improves ESI sensitivity especially for small and polar compounds which can be considered as the general target in HILIC separations. The application of 2-MEE appears to be a convenient add-on especially to HILIC–MS in negative ESI mode.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.07.104.

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