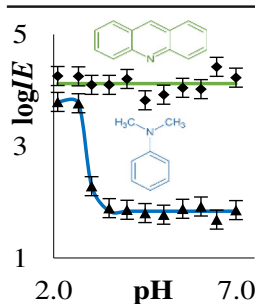


RESEARCH ARTICLE

pH Effects on Electrospray Ionization Efficiency

Jaanus Liigand,  Asko Laaniste, Anneli Kruve

Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Ravila 14A, 50411, Tartu, Estonia



Abstract. Electrospray ionization efficiency is known to be affected by mobile phase composition. In this paper, a detailed study of analyte ionization efficiency dependence on mobile phase pH is presented. The pH effect was studied on 28 compounds with different chemical properties. Neither pK_a nor solution phase ionization degree by itself was observed to be sufficient at describing how aqueous phase pH affects the ionization efficiency of the analyte. Therefore, the analyte behavior was related to various physicochemical properties via linear discriminant analyses. Distinction between pH-dependent and pH-independent compounds was achieved using two parameters: number of potential charge centers and hydrogen bonding acceptor capacity (in the case of 80% acetonitrile) or polarity of neutral form of analyte and pK_a (in the case of 20% acetonitrile). It was also observed that decreasing pH may increase ionization efficiency of a compound by more than two orders of magnitude.

Keywords: pH, ESI, Ionization efficiency, Ionization degree, Solvent effects, Electrospray

Received: 20 May 2016/Revised: 18 November 2016/Accepted: 20 November 2016/Published Online: 13 December 2016

Introduction

Although numerous studies of ESI have been performed [1–3], the mechanisms of ESI are still not fully understood [4]. One aspect of great interest is the ionization efficiency of analyte in the ESI source. Different groups have proposed models to predict ESI ionization efficiency based on the physicochemical properties of various analytes [5–9]. However, these models have been developed in a single solvent and, therefore, the obtained models do not fully account for the effects arising from mobile phase composition when paired to liquid chromatographic separation.

Kostiainen and Kauppila [1] have reviewed the effect of eluent on the ionization process. It has been observed that both organic solvent content [3, 10, 11] and the pH of aqueous phase [3, 12] have significant impact on ESI/MS sensitivity. The best ESI sensitivities have been observed in cases where the analyte is already ionized in the liquid phase [1, 3]. Marwah et al. [13] showed that adding volatile organic acids in low concentrations (1–500 ppm) to the mobile phase enhances ESI ionization efficiency of steroids by several orders of magnitude. Choi et al. [14]

showed that formic acid, ammonia, and ammonium formate can decrease signal suppression in complex matrices. They found that the highest sensitivity to methoxyfenozide in matrix (wheat forage) was obtained with 5 mM ammonium formate and 0.2% ammonia and formic acid, whereas the highest sensitivity in the analysis of standard solutions was observed with 1 mM concentration of ammonium formate, ammonia, and formic acid [15].

There are some studies showing that increasing the degree of ionization in solution could result in a decrease in sensitivity. Kamel et al. [15] studied the effect of trifluoroacetic acid, acetic acid, ammonia, and sodium acetate on the sensitivity of antiviral agents using ESI (positive mode). They showed that trifluoroacetic acid suppresses and acetic acid enhances the signal in ESI. Additionally, they showed that addition of ammonia to the aqueous phase significantly enhances analyte response. A significant conclusion drawn [16] from that work was that the major processes for the formation of ions from pyrimidines occurred via gas phase ion/molecule reactions and not through solution phase reactions. The effects of trifluoroacetic acid and acetic acid on the sensitivity of basic drugs in ESI positive mode were also studied by Mallet et al. [12]. Dams et al. showed [16], like Kamel et al. [15], that trifluoroacetic acid has a suppressive effect and added that all other acids with higher concentrations, too, have a suppressive effect. They also reported the suppressive effect of volatile acids (formic and acetic acid) on morphine analysis, with the most suppression observed at acid concentrations 0.03–

Electronic supplementary material The online version of this article (doi:10.1007/s13361-016-1563-1) contains supplementary material, which is available to authorized users.

Correspondence to: Jaanus Liigand; e-mail: jaanus.liigand@ut.ee

0.04 vol%; higher concentration did not yield further suppression [16].

Silvester [17] observed the highest responses in positive mode ESI for various analytes (propanolol, rosuvastatin, and drug AZ-X) in basic solutions (ammonium acetate buffer, pH = 9.89), for which the analytes were expected to be in a neutral form. Mansoori et al. [18] first observed, and Zhou and Cook [19] later studied in detail, “the wrong-way-round ionization” this means that analytes give high response in conditions where the analyte is not expected to ionize according to solution phase chemistry (pK_a and pH). They explained the phenomenon by gas-phase reactions occurring with precursors either present in solution or induced by corona discharge. Hua and Jenke [20] showed that some compounds form ammonium adducts in the droplet that may lead to increased protonated analyte signals. Peng and Farkas [21] also showed that a high mobile phase pH is suitable for analysis of basic analytes.

The pH effect on ESI efficiency becomes even more complex if changes in solution pH during the ESI process [22–24] are considered. Van Berkel et al. [22] showed that electrochemistry occurring on the needle tip changes the pH of the solution remarkably. In unbuffered solutions, the pH decreases in positive ion mode by as much as 1.8 pH units in the case of low flow rates ($0.008 \text{ mL min}^{-1}$) and 0.6 pH units using typical chromatographic flow rates (0.2 mL min^{-1}). Additionally, Zhou et al. [24] and Girod et al. [23] have shown that the pH of droplets decreases along the ESI plume by approximately 0.6 pH units.

Previously, analytes were divided into two groups based on their ionization efficiency dependence on solvent pH: (1) analytes for which ionization degree changes with pH, and (2) analytes for which ionization degree is independent of pH [25]. However, contradictory results have also been observed by Henriksen et al. [26].

None of the previous observations distinguish if the ESI sensitivity dependence is caused by particular additive or whether the overall pH of the solvent affects the sensitivity. Additionally, existing observations are limited in the number of compounds and mobile phases studied. Therefore, a detailed study is needed to relate the chemical properties and pH-dependent/pH-independent nature of analytes. By studying more analytes of different chemical origins that display pH-dependent ionization efficiency, insight into the ESI mechanism may be achieved.

The aim of this work is to study the dependency of ESI ionization efficiency on solvent pH and to relate these effects to solvent and analyte properties. In order to study the effect of pH on electrospray ionization efficiency in a detailed manner, the ionization efficiencies of 28 analytes in 22 different solvents (all containing acetonitrile and buffer) with aqueous phase pH ranging from 2.1 to 7.0 and acetonitrile percentages of 20% and 80% were used. The studied analytes covered a wide range of $pK_a(\text{aq})$ values from -15.2 to 9.5 , and a wide hydrophobicity range: $\log P$ (octanol-water partition coefficient) values from -2 to 5 . The ESI results are compared with the ionization degree in solution, estimated by UV-Vis spectrophotometric

measurements or from chromatographic data. This is the most comprehensive study of ionization efficiencies of analytes in various solvents to our knowledge.

Experimental

Chemicals

For the study of aqueous phase pH effects, the following compounds were purchased from Sigma-Aldrich (St. Louis, USA): 3-methoxy-*N,N*-dimethylaniline, 2,4-dinitroaniline, 3-nitroaniline, 4-nitroaniline, 4-aminobenzoic acid, quinoline, 2-aminophenol, 2,6-diaminopyridine, 2-aminobenzimidazole, 2,4,6-trinitroaniline, 2-aminopyridine, Trizma base (further referred to in text as tris), 4-amino-*N,N*-dimethylaniline, 3-hydroxypyridine, 3-(dimethylamino)benzoic acid, 2-nitroaniline, and aniline. The following were purchased from Fluka (Buchs, Switzerland): 3-aminobenzoic acid, tetramethylammonium chloride, tetraethylammonium perchlorate, pyridine, and acridine. From Reakhim, 8-aminoquinoline, 3-aminophenol, 2,6-dimethylpyridine, 1-naphthylamine, and *N,N*-dimethylaniline were purchased. 4-(Dimethylamino)-*N,N*-dimethylaniline was purchased from Merck (Darmstadt, Germany). *N,N*-diphenylbispidine and (dinitrophenyl)imino)tris(pyrrolidino)phosphorane (further referred to in text as 2,6-(NO₂)₂-C₆H₃-P1(pyr)phosphazene) were synthesized in our group according to [27] and [28], respectively. The structures and physicochemical parameters calculated with the COSMO-RS method [29, 30] are listed in Supplementary Information (see Supplementary Table S1).

The concentrations of standard solutions of analytes were between 5 and 50 μM . Owing to the low ionization efficiencies in more basic media, concentrations of 100 μM were needed for 2,4-dinitroaniline and 3-nitroaniline. The concentrations of analyte in the spray varied from 0.125 to 2.5 μM depending on the analyte and mobile phase studied.

Acetonitrile (Chromasolv Plus for HPLC, $\geq 99.9\%$; Sigma-Aldrich, USA), ultra-pure water (purified with Millipore Advantage A10 MILLIPORE GmbH, Molsheim, France), formic acid (98%, Fluka, USA), trifluoroacetic acid (99 + %, Aldrich, USA), ammonia solution (25%, Lach:NER, Czech Republic), and ammonium acetate ($\geq 99.0\%$, Fluka, USA) were used as solvent components.

Twenty-two different mobile phase compositions were studied: two different acetonitrile percentages (20% and 80% v/v) in combination with different aqueous phases: 0.1% trifluoroacetic acid (pH = 2.1), 0.1% formic acid (pH = 2.7), and buffers with pH = 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0. Buffer solutions were obtained by adjusting the pH of 5 mM ammonium acetate solution with formic acid or ammonia.

Equipment

The ionization efficiency measurements were carried out in the positive ion mode on an Agilent XCT ion trap mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). For flow

injection analyses, an Agilent 1100 series HPLC (Agilent Technologies) was used. For instrument control, an Agilent ChemStation for LC (rev. A. 10.02) and MSD Trap Control (ver. 5.2) were used. The following MS and ESI parameters were used: nebulizer gas pressure, 15 psi; drying gas flow rate, 7 L min⁻¹; drying gas temperature, 300 °C; needle voltage, 3500 V. Additionally, only the target mass (*TM*) was optimized [31].

For degree of ionization measurements, UV-Vis spectra were recorded with a PerkinElmer Lambda 2S UV/Vis spectrometer (PerkinElmer, Cambridge, MA, USA). Spectra were recorded from 190 to 750 nm using 1 cm cuvettes.

For chromatographic experiments, the same equipment was used as for ionization efficiency measurements with a 250 mm long Zorbax Eclipse XDB-C18 column (internal diameter of 4.6 mm and a particle size of 5 µm). Isocratic elution with 80% acetonitrile was used for chromatographic ionization degree determination in corresponding solvent. The aqueous phase pH was measured with pH-meter (Evikon pH Meter E6115, Tartu, Estonia) using a glass electrode (Evikon pH631).

Ionization Efficiency Measurement

In this work, ionization degree is defined in solution and described with Equation (3), and ionization efficiency describes the entire process occurring during electrospray ionization.

Since it is complicated to measure absolute ionization efficiency values [32], we measured the relative ionization efficiency (*RIE*) of the analytes studied herein, a methodology that has been successfully used in our group [5]. For ionization efficiency measurements, flow injection analysis was performed and a calibration graph was constructed for each analyte using standard solutions constructed by a series of dilutions (5×, 2.5×, 2×, 1.67×, 1.25×, and 1×) of stock solutions with the solvent by the autosampler and injected to MS. The injection volume was 10 µL and the mobile phase flow rate was 0.2 mL min⁻¹. For each measurement set, the linearity of calibration graph was assessed and was found to be acceptable for all compounds for which numerical ionization efficiency values are presented in Supplementary Table S3. The *RIE* value is obtained by dividing the slopes of analytical signals [6] according to Equation 1:

$$RIE(M_1) = \frac{\text{slope}([M_1 + H]^+)}{\text{slope}(Et_4N^+)} \quad (1)$$

where *M*₁ denotes the analyte and all analytes were measured relative to tetraethylammonium (Et₄N⁺). The tetraethylammonium log*IE* values were determined with infusion measurements at different pH values. It was observed that tetraethylammonium has a nearly constant log*IE* value at all studied pH values and, therefore, was taken to be log*IE* = 4.16 as assigned previously [25].

The reproducibility of measurements was calculated as a pooled standard deviation (Equation 2):

$$s_{\text{pooled}} = \sqrt{\frac{\sum_i^k (n_i - 1) \cdot s_i^2}{\sum_i^k n_i - k}} \quad (2)$$

where *s*_{*i*} is the standard deviation of an analyte in one solvent system, *n* is the number of measurements performed on an analyte in one solvent system, and *k* is the number of different solvent systems multiplied by the number of analytes.

Ionization Degree Measurement

Ionization degree of an analyte is calculated according to Equation 3:

$$\alpha = \frac{[AH^+]}{[AH^+] + [A]} = \frac{1}{1 + \frac{K}{[H^+]}} \quad (3)$$

where [AH⁺] denotes concentration of protonated analyte, [A] denotes concentration of analyte, and *K* the constant of protonation of analyte.

For measurements of the degree of ionization, solutions of analyte with different aqueous phase pH were made. The pH ranged from pH 1 (0.1 M hydrochloric acid) to pH 13 (0.1 M sodium hydroxide). The intermediate pH values were obtained by titrating 5 mM ammonium acetate with formic acid or ammonia.

The absorbances at the corresponding wavelengths were recorded, and the ionization degree, *α*, was calculated using Equation 4:

$$\alpha = \frac{A_{pH(i)} - A_{\text{cation}}}{A_{\text{neutral}} - A_{\text{cation}}} \quad (4)$$

where *A*_{pH(*i*)} is absorbance at particular pH and *A*_{neutral} and *A*_{cation} are the absorbances corresponding to the purely neutral and purely cationic form, respectively. The absorbance maxima with the largest difference in absorbance between neutral and cationic forms of an analyte were used for calculations (see Supplementary Table S2).

Compounds without chromophores (tris) could not be studied with UV-Vis spectroscopy; for these compounds, HPLC measurements were performed [33]. For three analytes, the HPLC measurements were used to confirm the results obtained with UV-VIS spectroscopy. For chromatographic ionization degree measurements, the analytes were analyzed after isocratic elution with two different aqueous phase pH values: 0.1% trifluoroacetic acid (pH 2.1) and 0.1% ammonia (pH 10.7). The mobile phase consisted of 80% acetonitrile and 20% buffer (v/v). The change in retention times at different pH values was an indicator of change in ionization degree.

Discriminant Analysis

Ions are known to escape droplets as solvent clusters, or as associates with solvent modifiers, or combined [34]. However, there is no certainty to which extent the escaping ions are clustered under specific conditions used in experiments. Therefore, we use molecular parameters calculated for ions without solvent shell. The eligibility of this approach has also been demonstrated before by successfully modeling the ionization efficiencies of various compounds by parameters calculated for analyte ion [5, 6, 35].

Various physicochemical properties of the analytes in the solvent phase were calculated with COSMO-RS method to determine the appropriate analyte form (neutral or ionic) for linear discriminant analysis (LDA) and quadratic discriminant analysis (QDA) (see [Supplemental Information](#)). The calculated parameters were: aqueous pK_a , $\log P$, the charge delocalization weighted average negative sigma ($WANS$) parameter, and the Klamt parameters (molecular area, molecular volume, σ_{sig2} and σ_{sig3} , which describe polarity and polarizability, respectively, and H_{bdon3} and H_{bacc3} , which are quantitative measures of hydrogen bonding donor and acceptor capacities, respectively). These parameters were calculated for both neutral and cationic forms of an analyte. Additionally, parameters such as change in the degree of ionization (see Ionization Degree Measurements), and number of potential charge centers were used.

To conduct linear discriminant analysis, the analytes were randomly divided into training and validation sets. In the validation set, there were nine compounds: *N,N*-diphenylbispidine, 2,4-dinitroaniline, 2,6-dimethylpyridine, *N,N*-dimethylaniline, acridine, 2,4,6-trinitroaniline, 3-hydroxypyridine, quinoline, and 3-aminobenzoic acid. The other 19 compounds were used for developing a linear discriminant function. The modeling was first performed using a one-parameter-at-a-time approach and the subsequent steps were performed using up to five parameters. All possible combinations were considered in discriminant function development. First, the models were generated with either one, two, three, four, or five parameters for training set (including leave-one-out cross-validation step). For validation, the best models (for each number of parameters) were also tested on a validation set (Supplementary Figures S2 and S3). In case of small number of compounds (size of training set and validation set) it is possible that overfitting of the LDA model occurs. Using training set results, a model with smallest number of parameters having sufficient prediction precision was chosen (see Supplementary Figures S2 and S3). It was observed that more than two parameters in the LDA model do not significantly improve the prediction precision, neither for training nor for validation set. Therefore, it is possible that models with higher number of parameters give small improvement by chance. The two parameter model was considered the best composing of two parameters that alone gave the best prediction precisions compared to others. For quadratic discriminant analysis, all 28 compounds were used to study the relationship. The quadratic

discrimination analysis was not used to make any prediction but to describe the observed phenomena.

Results

The results of the $\log IE$ measurements for different solvents are presented in Supplementary Table S3. Altogether, 22 solvent compositions were measured. The pooled standard deviation of the results (Equation 2) in the case of 80:20 acetonitrile:buffer was 0.14 $\log IE$ units and in the case of 20:80 acetonitrile:buffer, 0.23 $\log IE$ units. All 28 analytes were measured in both mobile phase compositions with pH 2.1 and pH 7.0 and if the difference between $\log IE$ values was larger than 0.5 (the threshold for statistical significance, calculated based on the reproducibility of the results), the compound was placed in a pH-dependent group. Analytes with statistically insignificant $\log IE$ change were grouped into the pH-independent group. The $\log IE$ values determined for the analytes in both mobile phase compositions and both pH values studied are listed in Table 1. All measured $\log IE$ values are listed in Supplementary Table S3 (Supplemental Information).

The largest decrease in $\log IE$ with a pH change from 2.1 to 7.0 was observed for 3-nitroaniline (2.3 $\log IE$ units) and smallest statistically significant $\log IE$ decrease was observed for 2-nitroaniline (0.6 $\log IE$ units) in case of 80:20 acetonitrile:buffer. The average decrease of $\log IE$ in the pH-dependent group was 1.2 $\log IE$ units. Similarly, in the case of 20:80 acetonitrile:buffer, the largest decrease was observed for 3-nitroaniline (2.5 $\log IE$ units) and the smallest for 2-nitroaniline (0.6 $\log IE$ units) with a pH change from 2.1 to 7.0. Of the 28 studied analytes, 13 were pH-dependent and 15 were pH-independent.

The pH-dependent group was studied in detail at all solvent compositions (pH from 2.1 to 7). The flow rate effect on pH dependency was determined for three compounds: *N,N*-dimethylaniline, pyridine, 1-naphthylamine. The pH dependence of the $\log IE$ values of these compounds was evident and numerically consistent at flow rates of 0.008 mL min⁻¹ and 0.2 mL min⁻¹. The $\log IE$ change has the same profile and change occurs at the same aqueous phase pH (see Supplementary Figure S1). The obtained $\log IE$ values with both flow rates at corresponding mobile-phase compositions were in a good correlation ($0.84 < R^2 < 0.99$). Therefore, it can be assumed that ionization efficiency change with pH is a flow rate-independent effect. Typical behavior of a pH-dependent analyte is presented in Figure 1 for the case of *N,N*-dimethylaniline.

In addition to the change in the ionization efficiency, the degree of ionization (α) in solution phase was determined for all analytes. The results of corresponding changes in α in solution are indicated in Table 2. Based on the results of degree of ionization measurements in solution and the results of ionization efficiency measurements, analytes were divided into four groups: (I) compounds for which both the $\log IE$ and α changes (10 compounds), (II) compounds for which α changes but the $\log IE$ does not change (13 compounds), (III)

Table 1. The Ionization Efficiency Values of Analytes in Both Solvent Compositions (80% and 20% (v/v) Acetonitrile) with Two Different Aqueous Phases: pH = 2.1 and pH = 7.0. The Asterisk Indicates that Analyte Ionization Efficiency is Dependent on Aqueous Phase pH

Analyte	Acetonitrile:buffer			
	80:20		20:80	
	pH = 2.1	pH = 7.0	pH = 2.1	pH = 7.0
2,6-(NO ₂) ₂ -C ₆ H ₃ -P1 (pyrr) phosphazene	5.27	5.22	4.62	4.84
<i>N,N</i> -diphenylbispidine	4.63	4.84	4.13	4.28
acridine	4.20	4.17	3.90*	2.88*
3-methoxy- <i>N,N</i> -dimethylaniline	3.93*	2.70*	3.62*	2.06*
4-(<i>N,N</i> -dimethylamino)- <i>N,N</i> -dimethylaniline	3.82	3.68	3.56	3.19
8-Aminoquinoline	3.74	3.46	3.55*	1.52*
<i>N,N</i> -dimethylaniline	3.74*	1.81*	3.31*	2.46*
Quinoline	3.65*	2.61*	3.29*	1.98*
2,6-Dimethylpyridine	3.60*	2.34*	3.19*	1.84*
2-Aminobenzimidazole	3.60	3.42	3.14	3.30
1-Naphthylamine	3.49*	2.85*	3.01*	1.95*
4-Amino- <i>N,N</i> -dimethylaniline	3.44	3.17	2.73	2.75
2,6-Diaminopyridine	3.32	3.06	3.10	2.63
2-Aminopyridine	3.20	2.85	2.32	2.36
Aniline	3.12*	0.92*	2.92*	0.78*
3-Hydroxypyridine	2.92	2.64	2.27	1.67
2-Aminophenol	2.92*	2.32*	2.82*	2.18*
3-Nitroaniline	2.85*	0.57*	2.23*	-0.31*
Pyridine	2.76*	1.03*	2.48*	1.00*
3-Aminobenzoic acid	2.70*	1.74*	2.08*	1.23*
4-Aminobenzoic acid	2.68*	1.73*	1.94*	1.29*
3-Aminophenol	2.66	2.34	2.94	2.56
3-(<i>N,N</i> -dimethylamino)benzoic acid	2.64	2.67	3.16	2.41
4-Nitroaniline	2.59	2.56	2.15	2.08
Tris	2.53	2.16	2.66*	1.64*
2-Nitroaniline	2.48*	1.92*	1.89*	0.86*
2,4-Dinitroaniline	1.39*	-0.53*	0.41*	NA ^a
2,4,6-Trinitroaniline	1.27	0.96	NA ^a	NA ^a

^aNot possible to measure.

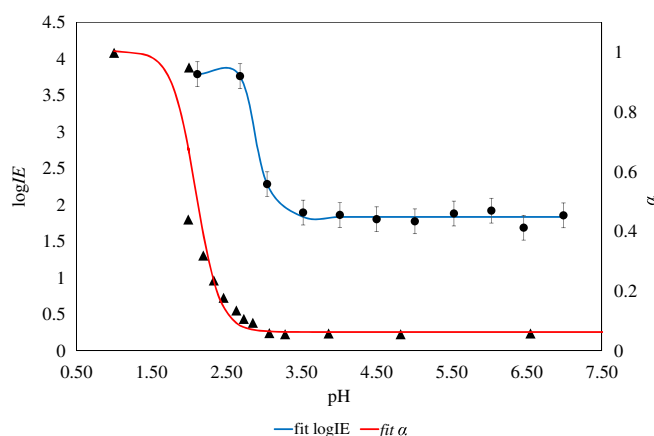
compounds for which α does not change but $\log IE$ changes (3 compounds), and (IV) compounds for which neither α nor $\log IE$ changes (2 compounds) (Table 2). However, it is possible that for some analytes, the $\log IE$ change occurs but is too small to be statistically significant.

Three analytes – acridine, 8-aminoquinoline, and tris – had ionization efficiencies independent of pH in a mobile phase containing 80% acetonitrile but in a mobile phase containing

20% acetonitrile, their ionization efficiency depended significantly on pH. For other compounds, the ionization efficiencies were independent of the mobile phase acetonitrile composition.

Table 2. The Distribution of Analytes According to the Behavior of Ionization Efficiency Values in ESI and Degrees of Ionization in Solution for 80:20 Acetonitrile:Buffer

$\log IE$	Dependent	Independent
α		
Dependent	Pyridine 2-Aminophenol 3-Aminobenzoic acid Aniline 2,6-Dimethylpyridine Quinoline 3-Methoxy- <i>N,N</i> -dimethylaniline <i>N,N</i> -Dimethylaniline 1-Naphthylamine 4-Aminobenzoic acid	Acridine <i>N,N</i> -Diphenylbispidine 3-Aminophenol 4-Amino- <i>N,N</i> -dimethylaniline 8-Aminoquinoline 2,6-Diaminopyridine 2-Aminopyridine 2-Aminobenzimidazole 3-Dimethylaminobenzoic acid 3-Hydroxypyridine 2,6-(NO ₂) ₂ -C ₆ H ₃ -P1 (pyrr) phosphazene Tris 4-Dimethylamino- <i>N,N</i> -dimethylaniline
Independent	3-Nitroaniline 2-Nitroaniline 2,4-Dinitroaniline	4-Nitroaniline 2,4,6-Trinitroaniline

**Figure 1.** The $\log IE$ values and degree of ionization (α) values of *N,N*-dimethylaniline in 80:20 acetonitrile:buffer and pH 2.1–7. The blue and red lines are fitted curves

To explain the pH-dependence in ESI source, a linear discriminant analysis was conducted based on physicochemical parameters calculated via the COSMO-RS method and on measured solution phase degrees of ionization. For 80:20 acetonitrile:buffer mobile phase, the best accuracy was achieved with Equation 5:

$$F = -1.501 \cdot N_{\text{charge centers}} - 0.210 \cdot \text{HBacc}3_n + 2.211 \quad (5)$$

where $N_{\text{charge centers}}$ is the number of potential charge centers, $\text{HBacc}3_n$ is the hydrogen bonding acceptor capacity of neutral form of the analyte. If $F > 0$, the analyte was considered to be in the pH-dependent group; if $F < 0$, the analyte was considered to be in the pH-independent group. The prediction precision of this model was 84.2% in the training set and 66.67% in the validation set. This means that 16 compounds of 19 were grouped correctly into corresponding groups in the training set and 6 of 9 correspondingly in the validation set. If three parameters were used, the prediction precision increased in the validation set (see Supplementary Figure S2) but the functions had the same precision using any of the parameters as the third parameter. It shows, additionally, that two parameter function describes the behavior well enough.

The best discrimination in the case of 20:80 acetonitrile:buffer was achieved using Equation 6:

$$F = -0.186 \cdot \text{p}K_a - 0.030 \cdot \text{sig}2_n + 3.500 \quad (6)$$

where $\text{sig}2_n$ is polarity of neutral form of analyte. The prediction precision was 78.9% for the training set and 77.8% for the validation set. If $F > 0$, the analyte was considered to be in the pH-dependent group; if $F < 0$, the analyte was considered to be in the pH-independent group.

Discussion

It has been previously shown in several studies (e.g., [1, 15, 20]) that ionization efficiency depends on the mobile phase composition. In a previous study [25], we observed that ionization efficiencies of analytes with $\text{p}K_a$ values in the range of studied aqueous phase pH were pH-dependent. In this study, we focus on analytes with different $\log P$ (−1.5 to 5.1 calculated with COSMO-RS method) and $\text{p}K_a(\text{aq})$ values, mostly in the range of studied aqueous phase pH. The analytes with these particular $\text{p}K_a$ values divide into two groups based on ESI response behavior (Table 2). However, the reasoning for such grouping is not self-explanatory: it cannot be determined solely based on $\text{p}K_a$ values of compounds or α determined in a particular mobile phase. Linear discriminant analysis (Equations 5 and 6) was used to explain the grouping of analytes based on their pH-(in)dependence in ESI source, and the best fit was achieved by using two parameters: number of potential charge centers and hydrogen bonding acceptor capacity (in case of 80% acetonitrile) or polarity of neutral form of analyte and $\text{p}K_a$ (in case of 20% acetonitrile). These parameters can be related to three stages occurring during electrospray ionization: (1) protonation of

analyte in the droplet interior, K_{a_i} , and on the droplet surface, K_{a_s} , (see Figure 2), (2) ejection of charged analyte from the droplet, and (3) protonation of analyte in the gas phase, K_{a_g} . The possible processes occurring in the droplet are shown in Figure 2.

The number of potential charge centers and $\text{p}K_a$ can be related to the process of ionization in solution. Analyte polarity and possibility to accept hydrogen bonds influence the ability to move to the droplet surface and eject from droplet. The number of potential charge centers describes the analyte probability of becoming charged in the solution phase. Analytes with more than one potential charge center tend to be in the pH-independent group; however, this group also contains compounds that have only one charge center. The analytes with more than one potential charge center are more likely to be ionized, as these compounds have more possibilities to become protonated. Similar observations have been made by Wang et al. [36], who observed that the fragmentation spectra of drugs with two basic functional groups may be significantly different at various pH. They explained this phenomenon by the change in the location of the charge on the molecule. This effect suggests that attaching the proton may be the limiting stage in ESI ionization for pH-independent compounds.

This phenomenon can be followed through the example of pyridine (one charge center), 2-aminopyridine (two charge

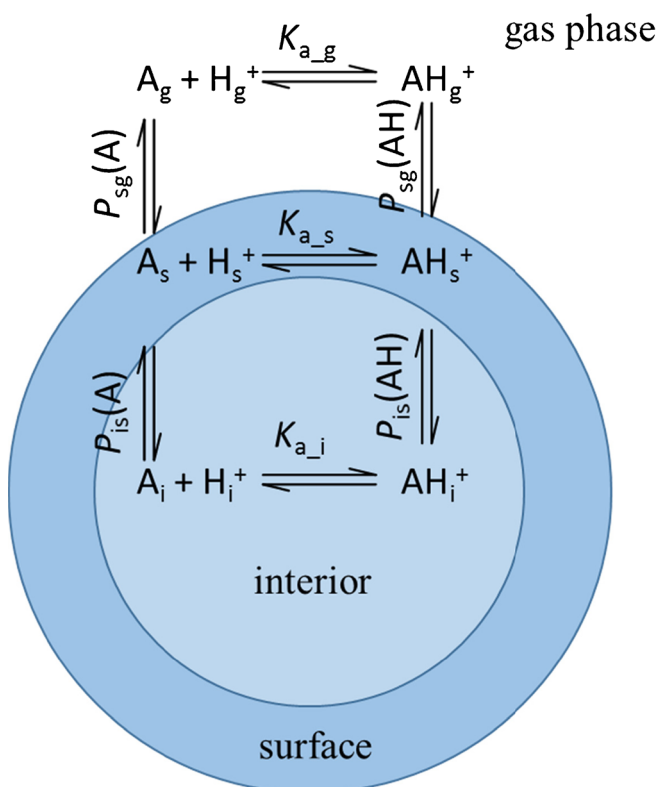


Figure 2. The possible processes affecting formation of a charged analyte. K_a denotes equilibrium constant of protonation, P denotes the partition between two phases, and subscripts i, s, and g stand, correspondingly, for interior of droplet, surface of droplet, and gas phase

centers), and 2,6-diaminopyridine (three charge centers); of these, only pyridine is in the pH-dependent group. This trend is also observed for two analyte pairs: aniline (1 charge center) and 4-amino-*N,N*-dimethylaniline (2 charge centers); and *N,N*-dimethylaniline (1 charge center) and 4-*N,N*-dimethylamino-*N,N*-dimethylaniline (2 charge centers). The aniline and *N,N*-dimethylaniline are in the pH-dependent group but 4-amino-*N,N*-dimethylaniline and 4-*N,N*-dimethylamino-*N,N*-dimethylaniline are in the pH-independent group.

However, other physicochemical parameters also change for these compounds with increasing number of potential charge centers. For example, the $\log P$ value changes from 0.61 (pyridine) to -0.69 (2,6-diaminopyridine). The effect of $\log P$ can also be followed through the example of related compounds like pyridine, quinoline, and acridine. The $\log P$ value increases from pyridine (0.61) to quinoline (1.74) and acridine (2.76). However, only acridine (the most hydrophobic of these compounds) is in the pH-independent group in case of 80:20 acetonitrile:buffer. For these two series of compounds, the $\log P$ effect on ionization efficiency is controversial. Therefore, it could be hypothesized that the analytes in the pH-independent group are either highly hydrophobic (tend to prefer droplet surface) or highly hydrophilic (tend to prefer droplets interior).

This hypothesis was tested by using quadratic discriminant analysis with the octanol-water partition coefficients of the neutral forms of analytes ($\log P_n$) calculated with COSMO-RS method as an input parameter to explain the pH-dependent behavior of compounds in 80:20 acetonitrile:buffer, Equation 7:

$$F = \log^2 P - 2.4 \cdot \log P + 0.4 \quad (7)$$

if $F > 0$, the analyte was considered to be in pH-dependent group; if $F < 0$, the analyte was considered to be in the pH-independent group.

The quadratic discriminant function is able to predict the classification into pH-dependent and pH-independent compounds with the precision of 82.1%. This phenomenon is visualized in Figure 3 and could be related to the two possible mechanisms for the analyte to form gas-phase ions (Figure 2). First, analyte can partition to the droplet surface in a protonated

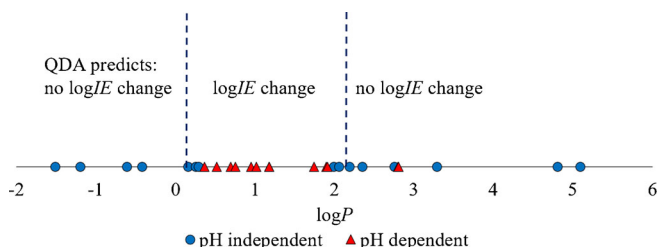


Figure 3. The relationship between pH-dependency and calculated octanol-water partition coefficients of the neutral form of an analyte ($\log P_n$) in the case of 80:20 acetonitrile:buffer. The dashed lines indicate the discriminating levels predicted with quadratic discriminant analysis

form [$P_{is}(AH^+)$]; this process is driven both by the charge-charge repulsion in the droplet but also by the hydrophobicity of the protonated analyte. Secondly, the analyte may partition to the droplet surface as a neutral [$P_{is}(A)$] and become protonated on the acidic surface of the droplet [37]. This process is driven solely by the hydrophobicity of the analyte (neutral form). However, the proportion of these effects is very complicated to estimate as the hydrophobicities of the protonated forms are not available. A similar effect has also been observed by Golubović et al. [8], who noted a Gaussian-shaped relation between the ESI/MS response and $\log P$ of the analytes. In the case of 20:80 acetonitrile:buffer, this phenomenon was not as evident.

Although the best discriminant function was obtained with two parameters, the size of analyte plays a role in ESI source. The LDA functions of three parameters with highest precision contained parameter that describes size (area or molecular volume). Larger (by volume) analytes tend to be in the pH-independent group; 2,6-(NO_2)₂- C_6H_3 -P1(pyrr) phosphazene, *N,N*-diphenylbispidine, 4-dimethylamino-*N,N*-dimethylaniline, acridine, 2,4,6-trinitroaniline, and 3-dimethylaminobenzoic acid are the six largest analytes among the studied compounds and all are in the pH-independent group. An explanation could be that, as a general trend, larger molecules tend to be more stable in the gas phase when protonated as they screen and stabilize the charge through the polar effect more efficiently if the nature of the protonation center does not change dramatically (e.g., for alkylamine versus pyridine the change in the nature of the protonation center is significant) [38]. However, these compounds are among the most hydrophobic of those studied and, therefore, the molecular volume effect cannot be fully separated from $\log P$ effect.

In addition to structurally similar compounds, the behavior of isomers can also be compared. For 3-nitroaniline, 2-nitroaniline, and 4-nitroaniline, some interesting phenomena arise. In the solution phase (UV-vis measurements), none of these compounds show any change in degree of ionization with changing pH. In terms of ionization efficiency, 3-nitroaniline and 2-nitroaniline are in the pH-dependent group whereas 4-nitroaniline belongs to pH-independent group. It is very interesting that 2-nitroaniline and 4-nitroaniline have similar resonance behavior but they behave differently in ESI ionization. Though 4-nitroaniline and 2-nitroaniline have the same resonance effect, the inductive and steric effects are different and that results in lower basicity of 2-nitroaniline. The 4-nitroaniline was continuously mispredicted, adding more evidence that stereochemistry is unpredictable with these molecular parameters. Interestingly, a similar mismatch between 2- and 4-isomers behavior has been observed in negative ESI mode for the 2-nitrophenol and 4-nitrophenol, where significantly lower ionization efficiency for 2-nitrophenol was observed [6]. These observations lead to the hypothesis that different steric effects can influence processes that kinetically limit the ionization. However, the kinetics of the processes occurring in ESI droplets are known to be significantly different from the ones observed in bulk solution [39]. Studying

kinetic effects on the ESI mechanism is challenging and out of the scope of this paper.

Comparing the results of measurements of degree of ionization with results of ionization efficiency measurements (an example is given in Figure 1, similar graphs were observed for other analytes) for 80:20 acetonitrile:buffer, we see that the ionization efficiency change occurs at 0.5 units higher pH (aqueous phase) than the ionization degree changes in the solution. This offset can be explained by the change of pH that occurs during the electrospray process. It has been shown in positive mode ESI that due to electrochemical reaction occurring on the ESI needle tip, the pH of the mobile phase is lower in the plume compared with the original solution [22]. During electrochemical reactions, additional hydrogen ions are generated in ESI positive mode [22]. Additionally, the evaporation of solvent from the ESI droplets increases the concentration of protons; therefore, pH decreases along the plume [24].

One of the solvents used (pH 2.1) contained trifluoroacetic acid (TFA) as a pH modifier. Previously, it has been observed in the literature [12, 15] that TFA may cause ionization suppression. In our case, several compounds showed lower $\log IE$ values in the mobile phase containing 0.1% TFA than in the mobile phase containing 0.1% formic acid, though the mobile phase with TFA had lower pH. The highest suppression was observed for 3-aminobenzoic acid (0.8 $\log IE$ units). However, the suppressive effect of TFA was rarely statistically significant. Interestingly, in both solvents, $\log IE$ values have been anchored to tetraethylammonium, for which the $\log IE$ did not change remarkably with pH. This means that suppressive effect of TFA is compound-dependent.

Conclusion

pH is a powerful way to change the sensitivity of ESI/MS method but only for some analytes. Some analyte's ionization efficiencies are completely unaffected by the mobile phase pH. However, the extent of ionization efficiency change with pH cannot be predicted based on the degree of ionization or pK_a . More important tend to be the number of potential charge centers revealing the fundamental difference between processes in bulk solution and in ESI droplets.

Acknowledgements

This work was supported by Personal Research Funding Project 34 from the Estonian Research Council. The authors thank Karl Kaupmees and Piia Liigand for fruitful discussions.

References

- Kostiainen, R., Kauppila, T.J.: Effect of eluent on the ionization process in liquid chromatography-mass spectrometry. *J. Chromatogr. A* **1216**, 685–699 (2009)
- Cech, N.B., Enke, C.G.: Practical implications of some recent studies in electrospray ionization fundamentals. *Mass Spectrom. Rev.* **20**, 362–387 (2001)
- Gao, S., Zhang, Z., Karnes, H.: Sensitivity enhancement in liquid chromatography/atmospheric pressure ionization mass spectrometry using derivatization and mobile phase additives. *J. Chromatogr. B* **825**, 98–110 (2005)
- Raji, M.A., Schug, K.A.: Chemometric study of the influence of instrumental parameters on ESI-MS analyte response using full factorial design. *Int. J. Mass Spectrom.* **279**, 100–106 (2009)
- Oss, M., Krueve, A., Herodes, K., Leito, I.: Electrospray ionization efficiency scale of organic compounds. *Anal. Chem.* **82**, 2865–2872 (2010)
- Krueve, A., Kaupmees, K., Liigand, J., Leito, I.: Negative electrospray ionization via deprotonation: predicting the ionization efficiency. *Anal. Chem.* **86**, 4822–4830 (2014)
- Wu, L., Wu, Y., Shen, H., Gong, P., Cao, L., Wang, G., Hao, H.: Quantitative structure–ion intensity relationship strategy to the prediction of absolute levels without authentic standards. *Anal. Chim. Acta* **794**, 67–75 (2013)
- Golubović, J., Birkemeyer, C., Protić, A., Otašević, B., Zečević, M.: Structure–response relationship in electrospray ionization-mass spectrometry of sartans by artificial neural networks. *J. Chromatogr. A* **1438**, 123–132 (2016)
- Cole, R.B., Harrata, A.K.: Solvent effect on analyte charge state, signal intensity, and stability in negative ion electrospray mass spectrometry; implications for the mechanism of negative ion formation. *J. Am. Soc. Mass Spectrom.* **4**, 546–556 (1993)
- Huffman, B.A., Poltash, M.L., Hughey, C.A.: Effect of polar protic and polar aprotic solvents on negative-ion electrospray ionization and chromatographic separation of small acidic molecules. *Anal. Chem.* **84**, 9942–9950 (2012)
- Kostiainen, R., Bruins, A.P.: Effect of solvent on dynamic range and sensitivity in pneumatically-assisted electrospray (ion spray) mass spectrometry. *Rapid Commun. Mass Spectrom.* **10**, 1393–1399 (1996)
- Mallet, C.R., Lu, Z., Mazzeo, J.R.: A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. *Rapid Commun. Mass Spectrom.* **18**, 49–58 (2004)
- Marwah, A., Marwah, P., Lardy, H.: Analysis of ergosteroids: VIII: enhancement of signal response of neutral steroidal compounds in liquid chromatographic–electrospray ionization mass spectrometric analysis by mobile phase additives. *J. Chromatogr. A* **964**, 137–151 (2002)
- Choi, B.K., Hercules, D.M., Gusev, A.I.: LC-MS/MS signal suppression effects in the analysis of pesticides in complex environmental matrices. *Fresenius J. Anal. Chem.* **369**, 370–377 (2001)
- Kamel, A.M., Brown, P.R., Munson, B.: Effects of mobile-phase additives, solution pH, ionization constant, and analyte concentration on the sensitivities and electrospray ionization mass spectra of nucleoside antiviral agents. *Anal. Chem.* **71**, 5481–5492 (1999)
- Dams, R., Benijts, T., Günther, W., Lambert, W., Leenheer, A.D.: Influence of the eluent composition on the ionization efficiency for morphine of pneumatically assisted electrospray, atmospheric-pressure chemical ionization and sonic spray. *Rapid Commun. Mass Spectrom.* **16**, 1072–1077 (2002)
- Silvester, S.: Mobile phase pH and organic modifier in reversed-phase LC-ESI-MS bioanalytical methods: assessment of sensitivity, chromatography and correlation of retention time with in silico $\log D$ predictions. *Bioanalysis* **5**, 2753–2770 (2013)
- Mansoori, B.A., Volmer, D.A., Boyd, R.K.: “Wrong-way-round” electrospray ionization of amino acids. *Rapid Commun. Mass Spectrom.* **11**, 1120–1130 (1997)
- Zhou, S., Cook, K.D.: Protonation in electrospray mass spectrometry: wrong-way-round or right-way-round? *J. Am. Soc. Mass Spectrom.* **11**, 961–966 (2000)
- Hua, Y., Jenke, D.: Increasing the sensitivity of an LC-MS Method for screening material extracts for organic extractables via mobile phase optimization. *J. Chromatogr. Sci.* **50**, 213–227 (2012)
- Peng, L., Farkas, T.: Analysis of basic compounds by reversed-phase liquid chromatography–electrospray mass spectrometry in high-pH mobile phases. *J. Chromatogr. A* **1179**, 131–144 (2008)
- van Berkel, G.J., Zhou, F., Aronson, J.T.: Changes in bulk solution pH caused by the inherent controlled-current electrolytic process of an electrospray ion source. *Int. J. Mass Spectrom. Ion Process.* **162**, 55–67 (1997)
- Girod, M., Dagany, X., Antoine, R., Dugourd, P.: Relation between charge state distributions of peptide anions and pH changes in the electrospray plume. A mass spectrometry and optical spectroscopy investigation. *Int. J. Mass Spectrom.* **308**, 41–48 (2011)

24. Zhou, S., Prebyl, B.S., Cook, K.D.: Profiling pH changes in the electrospray plume. *Anal. Chem.* **74**, 4885–4888 (2002)
25. Liigand, J., Kruve, A., Leito, I., Girod, M., Antoine, R.: Effect of mobile phase on electrospray ionization efficiency. *J. Am. Soc. Mass Spectrom.* **25**, 1853–1861 (2014)
26. Henriksen, T., Juhler, R.K., Svensmark, B., Cech, N.B.: The relative influences of acidity and polarity on responsiveness of small organic molecules to analysis with negative ion electrospray ionization mass spectrometry (ESI-MS). *J. Am. Soc. Mass Spectrom.* **16**, 446–455 (2005)
27. Toom, L., Kütt, A., Kaljurand, I., Leito, I., Ottosson, H., Grennberg, H., Gogoll, A.: Substituent effects on the basicity of 3,7-diazabicyclo[3.3.1]nonanes. *J. Org. Chem.* **71**, 7155–7164 (2006)
28. Rodima, T., Kaljurand, I., Pihl, A., Mäemets, V., Leito, I., Koppel, I.A.: Acid-base equilibria in nonpolar media. 2. ¹ Self-consistent basicity scale in THF solution ranging from 2-methoxypyridine to EtP₁ (pyrr) phosphazene. *J. Org. Chem.* **67**, 1873–1881 (2002)
29. Klamt, A., Eckert, F.: COSMO-RS: a novel and efficient method for the a priori prediction of thermophysical data of liquids. *Fluid Phase Equilib.* **172**, 43–72 (2000)
30. Eckert, F., Klamt, A.: Fast solvent screening via quantum chemistry: COSMO-RS approach. *AIChE J.* **48**, 369–385 (2002)
31. Kruve, A., Kaupmees, K., Liigand, J., Oss, M., Leito, I.: Sodium adduct formation efficiency in ESI source. *J. Mass Spectrom.* **48**, 695–702 (2013)
32. Leito, I., Herodes, K., Huopolahti, M., Virro, K., Künnapas, A., Kruve, A., Tanner, R.: Towards the electrospray ionization mass spectrometry ionization efficiency scale of organic compounds. *Rapid Commun. Mass Spectrom.* **22**, 379–384 (2008)
33. Gagliardi, L.G., Tascon, M., Castells, C.B.: Effect of temperature on acid-base equilibria in separation techniques. *Anal. Chim. Acta* **889**, 35–57 (2015)
34. Ahadi, E., Konermann, L.: Ejection of solvated ions from electrosprayed methanol/water nanodroplets studied by molecular dynamics simulations. *J. Am. Chem. Soc.* **133**, 9354–9363 (2011)
35. Chalcraft, K.R., Lee, R., Mills, C., Britz-McKibbin, P.: Virtual quantification of metabolites by capillary electrophoresis-electrospray ionization-mass spectrometry: predicting ionization efficiency without chemical standards. *Anal. Chem.* **81**, 2506–2515 (2009)
36. Wang, J., Aubry, A., Bolgar, M.S., Gu, H., Olah, T.V., Arnold, M., Jemal, M.: Effect of mobile phase pH, aqueous-organic ratio, and buffer concentration on electrospray ionization tandem mass spectrometric fragmentation patterns: implications in liquid chromatography/tandem mass spectrometric bioanalysis. *Rapid Commun. Mass Spectrom.* **24**, 3221–3229 (2010)
37. Enami, S., Stewart, L.A., Hoffmann, M.R., Colussi, A.J.: Superacid chemistry on mildly acidic water. *J. Phys. Chem. Lett.* **1**, 3488–3493 (2010)
38. Santos, L.S.: *Reactive Intermediates. MS investigations in solution.* Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim (2010)
39. Banerjee, S., Zare, R.N.: Syntheses of isoquinoline and substituted quinolines in charged microdroplets. *Angew. Chem. Int. Ed.* **54**, 14795–14799 (2015)