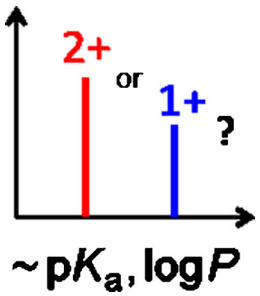
J. Am. Soc. Mass Spectrom. (2016) 27:1211–1218
DOI: 10.1007/s13361-016-1384-2

RESEARCH ARTICLE

Ionization Efficiency of Doubly Charged Ions Formed from Polyprotic Acids in Electrospray Negative Mode

Piia Liigand, Karl Kaupmees, Anneli Kruve

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



Abstract. The ability of polyprotic acids to give doubly charged ions in negative mode electrospray was studied and related to physicochemical properties of the acids via linear discriminant analysis (LDA). It was discovered that the compound has to be strongly acidic (low pK_{a1} and pK_{a2}) and to have high hydrophobicity ($logP_{ow}$) to become multiply charged. Ability to give multiply charged ions in ESI/MS cannot be directly predicted from the solution phase acidities. Therefore, for the first time, a quantitative model to predict the charge state of the analyte in ESI/MS is proposed and validated for small anions. Also, a model to predict ionization efficiencies of these analytes was developed. Results indicate that acidity of the analyte, its octanol-water partition coefficient, and charge delocalization are important factors that influence ionization efficiencies as well as charge states of the analytes. The pH of the solvent was also found to be an important factor influencing the ionization efficiency of doubly charged ions.

Keywords: Electrospray, Multiple charging, Ionization efficiency

Received: 14 December 2015/Revised: 3 March 2016/Accepted: 14 March 2016/Published Online: 4 April 2016

Introduction

Mass spectrometry with electrospray ionization source (ESI/MS) is increasingly the method of choice for determination of various biological substances [1]. Many analytes (e.g., peptides) form multiply charged ions in ESI source. The multiply charged ions are very beneficial for compounds which form singly charged ions that have too high *m/z* values for most mass analyzers. Multiple charging and its reasons have been studied; however, much is still unclear in this field. No studies have been done on ionization efficiency (IE) of multiply charged analytes to our knowledge, although its relevance has been pointed out [2].

It has been shown that ionization efficiency (ESI response) of large multiply charged molecules depends on the distance between chargeable sites [3] and on the structure of the molecule (how well the ionized form is stabilized) [4]. Nevertheless, it is still not fully clear how to predict the charged state of the molecule in ESI/MS analyses. Wong et al. [5] developed a model for predicting the maximum possible number of charges of polyethylene glycol depending on the number of monomers and their affinity towards the ion that is merging with it. Later, Schnier et al. [6] and Smith et al. [7] studied the number of protons that bind to the peptide in the gas phase and found that the number of protons is very similar to the number of basic amino acid residues in the peptide. They also noticed that close proximity of basic residues decreases the basicity of the observed residue. The same tendency was also observed by Felitsyn et al. [8] who studied native proteins. Furthermore, the availability of basic sites has been shown to be important [6, 7, 9, 10] for determining the charged state of the analyte.

It has been observed that the charge state of an analyte in solution does not always correlate with the charge state observed in MS analysis [11]. Iavarone et al. was the pioneer in studies of supercharging [12]. They observed an increase in the charge state for peptides if additives such as diethylamine, 2-methoxyethanol, ethylene glycol, glycerol, or m-nitrobenzyl alcohol were added to the solution [12–14]. Although the mechanism of supercharging is yet to be fully explained, the authors related this effect with the additives being less volatile than water and thus increasing surface tension. Supercharging has also been observed in negative ion mode [15, 16] where organic bases were used as supercharging reagents and a positive correlation was found between gas-phase basicities (GB) and charge state distributions. It was observed that in negative ionization mode the correlation was linear, and in positive

Electronic supplementary material The online version of this article (doi:10.1007/s13361-016-1384-2) contains supplementary material, which is available to authorized users.

Correspondence to: Piia Liigand; *e-mail:* piia.liigand@ut.ee

mode the correlation had a maximum value at GB of approximately 800 kJ/mol [16].

To study ionization efficiency of different compounds, an ionization efficiency scale approach has been introduced for singly charged compounds. As the evaluation of absolute IEs is difficult if not impossible, the term relative ionization efficiency (RIE) has been introduced [17] (see Formula 1 in Experimental).

In contrast to absolute IEs, RIEs can be measured reliably. This approach has been previously used to develop a model for predicting IE of singly charged analytes both in positive [18] and negative mode [19]. In both sources, the RIEs were transformed to log*IE* values by anchoring the obtained values to one specific compound. In positive mode this compound was methyl benzoate, and in negative mode benzoic acid. Anchoring was used for the compounds to give comparable values for all compounds.

For singly charged compounds, IEs of various analytes in different solvents with varying equipment have been studied. Most studies have been made in positive mode. Tang and Kebarle [20] found that the signal of analyte is affected by surface tension of the solvent and ion evaporation rate constant of the analyte. Cech and Enke [2] found that in the case of selected tripeptides, the nonpolar area affects their signal in mass-spectrometer. They concluded that analytes need to have a large enough nonpolar area to move to the surface of the droplet and also a structural element that allows charging (protonation, deprotonation, adduct formation, etc.). This tendency was also confirmed by Leito et al. [17] based on a study of esters and aromatic amines. Ehrmann et al. [21] tried to predict analyte signal in ESI/MS and observed the best predicting power while using the solution phase basicity of analyte (pK_b). The effect of gas-phase proton affinities was smaller than previously stated for the studied compounds [22]. Oss et al. [18] calculated six physicochemical properties and found that ionization efficiency correlates best with pK_a value and molecular volume of the analyte. Chalcraft et al. [23] modeled response factors for polar metabolites and found that important descriptors are molecular volume, octanol-water distribution coefficient ($logD_{ow}$), and absolute mobility of the ion. Nguyen et al. [24] found a positive correlation between ESI signal and adjusted mass. In negative ionization mode, Henriksen et al. [25] found that for phenols and phenoxy alkanoic acids ionization was more efficient in methanol than in acetonitrile and that analytes $logP_{ow}$ (octanol-water partition coefficient) was in better correlation with ESI/MS signal than acidity of the analyte (pK_a). Huffman et al. [4] found that in negative mode, compounds that are more acidic and hydrophobic ionize better. Kruve et al. [19] discovered in the negative ionization mode that ionization efficiency can be best predicted by using degree of dissociation for the analyte and charge delocalization (*WAPS* parameter) that describes the delocalization of the charge in the anion. However, high ionization efficiency of the anions in media where acidic compounds are expected to be present as neutrals has been observed. This phenomena, called wrong-way-round ionization, has been described by Zhou et al. [26].

As the ionization efficiency of multiply charged ions has not been studied, the aim of this paper is to study multiple charging in negative ionization mode in acidic and basic solvent. In the literature, there is much ambiguity of the charge states of the compounds and therefore we aim to reveal the factors that affect the ability to give multiple charging in ESI/MS based on analysis of small molecules in negative ESI ionization mode. We aim to propose a quantitative model that can be used to predict the charge state. The compounds included in this study cover a wide acidity range (pK_a values from –3 to 11 in water) and wide hydrophobicity range ($logP_{ow}$ from –0.5 to 7.5). Small molecules allow determining conditions and parameters that affect multiple charging based on a relatively simple system. This is a good starting point to move on to bigger systems such as proteins.

Experimental

Instrumental

The measurements were carried out using Agilent XCT ion trap mass spectrometer. Default settings for ESI source were used: nebulizer gas pressure 15 psi, drying gas flow rate 7 L/min, drying gas temperature 300 °C. The capillary voltage between the MS and nebulizer was –3500 V. All remaining ion transport parameters were determined by the Target Mass (TM) parameter, set by the operator. The TM was optimized for each component and the optimal value used for during measurements of ionization efficiency [27].

The ion trap parameters were: Smart Target (parameter characterizing the number of ions accumulated in the trap) 100,000, maximum accumulation time 300 ms. Each spectrum was scanned from *m/z* 30 to 1000. Depending on the compound one ion $[M - H]^-$ or two ions $[M - H]^-$ and $[M - 2H]^{2-}$ were observed in mass spectra.

For every RIE measurement of two compounds, two syringe pumps connected with a "T-piece" were used. The concentration ratio was varied by changing the infusion rates of two pumps from 1.7 µL/min to 6.7 µL/min so that the sum of the infusion rates of two pumps would always be 8.4 µL/min. Concentrations of compounds in the sprayed solutions were between 0.2 and 60 µmol/L depending on the compounds. During every measurement, the linearity of signal to concentration graph was checked and thereby it was verified that the signal was not saturated in any of the measurements [see example Supplementary Figure S1 in Supplementary Information (SI)].

Two solvents were used: (1) acidic solvent with the addition of formic acid (80/20 MeCN/0.1% formic acid solution), and (2) basic with the addition of ammonia (80/20 MeCN/0.1% ammonium hydroxide solution). The solvent was prepared from acetonitrile (HPLC grade, J. T. Baker, Deventer,

The Netherlands), ultrapure water (18.2 MΩ·cm, TOC 1–2 ppb, prepared by a Millipore Milli-Q Advantage A10 water purification system), concentrated aqueous ammonia (25% solution; Lach:Ner, Czech Republic), and formic acid (≥98%, Sigma, Steinheim, Germany).

As it is complicated to measure log*IE* values, we measured the *RIE* values between compounds 1 and 2:

$$RIE\frac{A_1}{A_2} = \frac{IE(A_1)}{IE(A_2)} = \frac{K_1^{'}}{K_2^{'}} = \frac{R_1 C_2}{R_2 C_1} \quad (1)$$

where R_1 and R_2 are the responses of the compounds and C_1 and C_2 the respective concentrations of the compounds in the spray. For each compound pair, the *RIE* was measured on five concentration ratios and the obtained *RIE* values were averaged. First, in both solvents the log*IE* values were temporarily assigned to compounds by minimizing the sum of squares (*SS*) of differences between measured log*RIE* values and the assigned log*IE* values [18]:

$$SS = \sum_{k=1}^{n_m} \left\{ \log RIE_k\left(A_i, A_j\right) - \left[\log IE(A_i) - \log IE\left(A_j\right)\right] \right\}^2 \rightarrow \min \quad (2)$$

where n_m is the number of measurements and $\log RIE_k(A_i, A_j)$ is the result of *k*-th measurement that has been conducted between compounds A_i and A_j. For this step, the log*IE* value of benzoic acid was temporarily taken as zero in both solutions.

Second, to obtain the log*IE* values that could be compared between two solvents the log*IE* values from Formula 2 were anchored to benzoic acid in solution (2), for which the log*IE* value of zero has been previously assigned [19]. To anchor the scale obtained in solvent (1) to the solvent (2), the MS signal intensities for benzoic acid solution (concentration in both solutions approximately 60 μmol/L) were measured on the same day and the log*IE* for benzoic acid in solvent (1) was calculated as:

$$\log IE_{\text{benzoic acid,solvent (1)}} = \log \frac{R_{\text{solvent (1)}} \cdot C_{\text{solvent (2)}}}{R_{\text{solvent (2)}} \cdot C_{\text{solvent (1)}}} \quad (3)$$

The $\log IE_{\text{benzoic acid,solvent(1)}} = 0.84$ was observed by averaging over 3 day measurements (one measurement per day, mean standard deviation of measurements was 0.085 logarithmic units). Thereafter, the $\log IE_{\text{benzoic acid,solvent(1)}}$ was used to obtain log*IE* values for other compounds in solvent (1).

In the following discussion and results, the log*IE* values are used, as these provide the possibility to compare ionization efficiencies of all the compounds without having to measure each relative ionization efficiency value for all of the compounds. Also, the anchoring procedure between two solvents [Formula 3 followed by the re-anchoring for solvent (1)] allows comparing the log*IE* values for different solvents.

Consistency of the scale is expressed as consistency standard deviation:

$$s = \left(\frac{SS}{n_m - n_c}\right)^{1/2} \quad (4)$$

where n_c is the number of assigned log*IE* values [i.e., number of analyzed substances – number of anchors (here: 1)].

Compounds Studied compounds were (structures can be found in SI) bought from Reakhim, Russia: 3-nitrophthalic acid, adipic acid, eosin B, bromophenol blue, bromothymol blue, phenol-2,4-disulfonic acid, phenolphthalein, cresol red, *m*-cresol purple, tiron, thymolphthalein, benzoic acid; from Chemapol, Czech Republic: bathocuproinedisulfonic acid, SPADNS; from Schering AG, Berlin, Germany: bromocresol purple; from Sigma-Aldrich, USA: bromocresol green, eosin Y; from E. Merck, Darmstadt, Germany: maleic acid, thymol blue; from Lach-ner, Czech Republic: 5-sulfosalicylic acid; and from Aldrich, USA: glutaric acid, pimelic acid, suberic acid. Obtained as a kind gift from the Institute of Pharmacology, Estonia were phthalic acid, fumaric acid, isophthalic acid, itaconic acid, succinic acid, mesaconic acid, and terephthalic acid.

Calculations COSMO-RS method [28] was used for calculating various parameters: aqueous pK_a, $\log P_{ow}$ (octanol-water), charge delocalization parameters (*WAPS*) and Klamt parameters. Degree of dissociation α of the compounds was calculated from the computed pK_a values and the water phase pH.

First, full geometry optimization and energy calculation were carried out at the DFT BP TZVP level with the RI approximation and applying the COSMO continuum solvation model for all compounds using Turbomole, ver. 6.4 [29]. For most compounds, several conformers corresponding to different local energy minima were found. All of these were taken into account by statistical weighing inherent in the COSMO-RS procedure. The default convergence criteria of Turbomole were used: wave function convergence max difference 10^{-6} Hartree, geometry convergence max gradient $|dE/dxyz|$ 10^{-3} Hartree/Bohr. This first computation step yields for every conformer the following data: the geometry of the conformer, detailed data on the shape of molecular cavity, the polarization charge densities mapped onto the cavity surface, the total electronic energy of the species submerged into a virtual conductor ($\varepsilon = \infty$), and molecular surface area and volume. Molecular cavity refers to the cavity constructed for the particular conformer within the COSMO solvation theory—constructed utilizing smoothed spheres using atomic radii ~20% larger than van der Waals radii. This cavity was later used as the molecular volume. The cavity surface refers to the so-called sigma-surface–polarization charge density on the molecular surface. For

further information about the COSMO-RS theory, see reference [28].

Second, COSMO-RS calculation was carried out on all compounds using the above-listed data as input data with the COSMO*therm*, ver. C3.0, release 14.01 [30]. COSMO-RS calculations take into account the interactions between species and the solvent/medium molecules, as well as between the solvent molecules themselves (implicit solvation model). The solvent composition is a required input parameter for COSMO-RS calculations, and in these calculations water was used as a solvent. Zero concentrations were used for the studied molecules. This way the interaction between the studied compounds and the solvent is taken into account but not the interaction between the molecules of studied compounds themselves. This situation corresponds well to the reality of very low concentrations used in the experiments and is common practice for carrying out such calculations. Both van der Waals interactions (electrostatic interactions: dipole–dipole, ion–dipole, etc. forces as well as dispersion forces) and hydrogen bonds (implicitly) are taken into account. These interactions are quantified via statistical counting and averaging of energies of pairwise interactions of molecular surface segments using polarization charge density maps of compounds created in the first step, taking into account the concentrations of the respective species in the solution. Terms accounting for vibrational contributions to the G_{tot} are also added in this step. This is done implicitly, as these are represented through the experimental data used for parameterization of the method. The energetics of these interactions are calculated at the 298 K, using statistical thermodynamics procedure whereby also the conformers of all the interacting molecules are taken into account and statistically weighted based on their relative stabilities. This way, entropy effect of the same species present in multiple conformers is also accounted for. As a result, G_{tot} value is found for every compound.

Model Development To find out why certain substances give multiply charged species in mass spectrum and why others do not, it is necessary to know the physicochemical properties of all of the substances. In addition, the properties of the solvent need to be taken into account. It is known that some processes that lead to ionization of the analyte occur in the solvent phase and some in the gas phase [1]. Since it is difficult to account for processes in the spray that lead to the change in solvent properties, a simplification is usually done and the properties of analyte in the water phase are used [18]. In the ESI spray during droplet evaporation water content increases as the more volatile organic component vaporizes. Also, it is significantly less complicated to measure water phase pH-s. The same assumption is used in the current work. For model development, physicochemical properties obtained by COSMO-RS calculation were used. All statistical tests were carried out at 95% confidence level. Linear discriminant analysis (LDA) was carried out with statistical program R using the package Mass.

Results

The ionization efficiency scale was compiled for both basic and acidic solvents, and for both doubly and singly charged ions. Altogether 29 compounds were studied, nine of them gave multiply charged ions in addition to singly charge ions in mass spectrometer in both solvents (given in Table 1 with the corresponding log*IE* values). In addition to the compounds brought in Table 1, also phenol-2,4-disulfonic acid gave doubly charged ions, but due to spectral interferences it was not possible to measure the corresponding log*IE* value. Benzoic acid was a reference substance, its log*IE* value taken to be zero in basic solvent as defined earlier in literature [19]. Altogether, 23 measurements in basic and 17 measurements in acidic solvent were made. In basic solvent, the ionization efficiency scale range was about 3 logarithmic units for doubly charged ions and 4 logarithmic units for singly charged ions. In acidic solvent the corresponding values were 4 and 3 logarithmic units. For singly charged ions the ionization efficiency values did not statistically differ in two solvents according to the *t*-test (95% confidence level was used). For doubly charged ions the log*IE* difference in two solvents was statistically significant for all compounds, except for tiron and SPADNS.

Parameters calculated with COSMO-RS method were correlated with measured log*IE* values in basic solvent. Correlation was poor ($R^2 < 0.55$) with Klamt parameters (polarity, polarizability, hydrogen bond accepting capability, hydrogen bond donating capability; see SI for details). It was not possible to correlate α_2 to ionization efficiency values obtained in basic solvent because all the analytes were fully deprotonated and degree of dissociation therefore equal to one in the case of all analytes. With the rest of the parameters, the correlation was good. Best correlation was observed between pK_{a1} and ionization efficiencies corresponding to singly charged analytes ($R^2 = 0.79$, correlation was positive). Good correlation was also obtained with *WAPS* and hydrogen bond donating capability for neutral (R^2 is 0.68 and 0.72 accordingly). For doubly charged ions, good correlation with *WAPS* parameter, molecular volume, and hydrogen bond donating capability was observed. In acidic solvent the correlations were similar; however, there was no correlation between doubly charged analytes ionization efficiency and *WAPS* parameter.

It was also of interest whether a predictive model for log*IE* values can be obtained based on physicochemical properties of analyzed substances. Best quantitative model (both for basic and acidic solvent) for predicting singly charged analytes

Table 1. Log*IE* Values in Negative Ionization Mode for Molecules that Gave Doubly Charged Species

				Doubly charged					Singly charged		
	pK_{a1}[a]	pK_{a2}[a]	$logP_{ow}$[a]	α_2[a] pH=10.74	α_2[a] pH=2.68	$logIE_2$[b] pH=10.74	$logIE_2$[b] pH=2.68	*WAPS* $\cdot 10^{5}$[a]	$logIE_1$[b] pH=10.74	$logIE_1$[b] pH=2.68	*WAPS* $\cdot 10^{5}$[a]
Bromophenol blue	0.99	2.96	6.86	1.00	0.34	2.63	0.28	1.86	2.34	1.93	1.54
Bathocuproinedisulfonic acid	–1.70	5.05	–0.06	1.00	0.00	1.95	1.06	1.92	0.45	0.42	2.02
Bromocresol green	0.55	3.49	6.63	1.00	0.13	1.71	–0.15	1.83	1.45	2.01	1.62
Bromothymol blue	1.15	5.76	7.47	1.00	0.00	1.23	–0.25	1.70	2.95	2.16	1.44
Eosin Y	3.30	4.54	5.03	1.00	0.00	1.15	0.27	1.90	1.50	1.47	1.60
SPADNS	–2.32	–2.19	1.41	1.00	1.00	0.66	0.16	2.57	–0.20	–0.58	2.34
Sulphosalicylic acid	–1.53	2.31	2.68	1.00	0.70	–0.38	–2.54	2.60	0.22	0.22	2.38
Tiron	–2.66	–2.15	3.07	1.00	1.00	–0.62	–0.29	5.01	–0.68	–0.34	3.98
s[c]						0.16	0.15		0.40	0.08	

[a]Calculated pK_a values, degrees of dissociation (α_2), $logP_{ow}$ values for neutrals and *WAPS* parameters for singly and doubly charged species can be found.
[b]Two log ionization efficiency values can be found in the table: $logIE_1$ is the log*IE* of the molecule via formation of singly charged ion; $logIE_2$ of the molecule via formation of doubly charged ion. Reference substance is taken to be benzoic acid.
[c]*s* – consistency standard deviation, see Formula 4.

ionization efficiency was obtained by using *WAPS* values for singly charged ions:

$$\log IE_1 = (-1.14 \pm 0.23) \cdot WAPS + (3.36 \pm 0.51) \quad (5)$$

Prediction precision of this model can be estimated by root mean error of 0.71 log*IE* units and square of correlation coefficient (R^2) of 0.64. The only substance to deviate significantly from the model was tiron. However, for doubly charged ions the obtained models for predicting log*IE* values quantitatively gave inacceptable results.

Linear discriminant analysis (LDA) was carried out to find properties to predict whether an analyte gives or does not give doubly charged ions in negative ionization mode. LDA model was found using training set composed of 18 randomly chosen compounds. Among training set there were five substances that gave doubly charged ions and 13 that did not give doubly charged ions (see SI, Supplementary Table S3). Different combinations of molecular parameters calculated with COSMO*therm* were used ($logP_{ow}$, *WAPS* parameters for anions and dianions, molecular area, pK_a values). A model was chosen so that it would give the highest prediction precision for training set. This model was based on pK_{a2} and $logP_{ow}$ values:

$$F = -0.48 \cdot pK_{a2}(A) + 0.60 \cdot \log P_{ow}(A) \quad (6)$$

If $F > 0$, then analyte A gives doubly charged ions,
If $F < 0$, then analyte A does not give doubly charged ions.

This model has a prediction precision of 94%. An only slightly worse model (89%) was obtained with a model that contained molecular volume or *WAPS* instead of $logP_{ow}$ value. This model applies in solvent (1) as well as in solvent (2) since the same compounds gave doubly charged ions in both solvents, although with different ionization efficiency. The prediction model gave false positive result in the case of eosin B. This model was tested on a validation set containing 11 substances of which four gave doubly charged ions. The prediction precision was 82% for validation set. The model was incorrect for bathocuproinedisulfonic acid (false negative) and bromocresol purple (false positive). Possible reasons for incorrectness of the model for these substances are explored in the Discussion section.

Discussion

It was observed that analytes with certain functional groups tended to give doubly charged ions. All substances that gave doubly charged ions had at least one sulfo group; also, in most cases (except bathocuproinedisulfonic acid) one or several electronegative groups (hydroxyl or bromo) and were aromatic. Dicarboxylic acids did not give doubly charged species in the MS independent of the pK_a values and distance between carboxyl groups. Maximum distance between carboxylic groups in the case of dicarboxylic acids was about 10 Å in the case of suberic acid (octane diacid). In the case of tiron or sulfosalicylic acid, for example, which formed doubly charged ions, the distance between charged groups was about 5 Å.

From the LDA model it can be seen that the main properties that determine multiple charging are acidity and hydrophobicity. One important parameter is pK_{a2} that describes dissociation of the second acidic group. However, several substances that according to pK_a value of the substance and pH of the solvent should be charged, did not give doubly charged ions (for example eosin B, phthalic acid, pimelic acid). Only analytes that had substantially lower pK_a than solvent pH gave doubly charged ions. A similar result was also obtained by Felitsyn et al. [8], who studied multiple charging of peptides. They showed that obtaining a second charge in ESI process is hindered by electrostatic repulsion of charges and influenced by the hydrophobicity of the compound. Dissociation makes acids more hydrophilic, whicht in turn makes them stay inside the droplet. This means that only substances with substantial

hydrophobic character can move to the surface of the droplet in ESI process. As a result, only substances with reasonable acidity (in this study pKa_2 value below 5.8) combined with remarkable hydrophobic character can give doubly charged ions in ESI process.

LDA gave incorrect results for three substances (eosin B, bathocuproinedisulfonic acid, and bromocresol purple). The false positive result for eosin B and bromocresol purple may be caused by additives in the substance that could suppress signal and make mass spectrum noisy. Therefore, it is possible that eosin B could give doubly charged ions but its intensity remains below the limit of detection. On the other hand, it is possible that some other parameter is affecting the formation of doubly charged species that has not been considered in this study. The logP_{ow} value of bathocuproinedisulfonic acid is very similar to substances that did not give doubly charged species and probably this is the main reason that the prediction is false negative. In calculation of physicochemical properties of bathocuproinedisulfonic acid, zwitterionic conformers were included and observed to be the most favored conformers in the liquid phase. It has been shown by Teesch et al. that the structure of gas-phase ions and solvent-phase ions are; however, different [31]. Consequently, it can be presumed that logP_{ow} is not the best parameter to describe distribution of zwitterionic compounds such as bathocuproinedisulfonic acid on the surface of the droplet.

Span of the log*IE* scale and order of the substances in the scale was found to depend on solvent pH. In acidic solvent, the range of the log*IE* scale for doubly charged species is one logarithmic unit wider than in basic solvent. Therefore, the pH is a suitable tool to increase or decrease MS sensitivity for doubly charged species. The order of the substances is also dependent on solvent pH. Generally the ionization efficiencies for doubly charged species are about one logarithmic unit lower in basic solvent than for acidic solvent. The ionization efficiency values for singly charged species are less influenced by solvent pH (see Figure 1).

In basic solvent, more doubly charged species are formed in the solution phase (pK_{a2} is substantially lower than solvent pH allowing the second protonation step) and, therefore, the signal corresponding to doubly charged species and corresponding log*IE* values are significantly higher. In acidic solvent, the formation of doubly charged ions is not favored and, therefore, more singly charged ions are formed. As a result, the IEs of doubly charged ions are lower than in basic solvent. Only for SPADNS and tiron the log*IE* values in basic and acidic solvent do not change, which can be explained by their very low pK_{a2} values (negative) and degrees of dissociation (α_2 value) of 1 in both solvents.

In basic solvent only bromothymol blue and eosin Y give higher IE for singly charged species than for doubly charges species. As mentioned before, the changes between acidic and basic solvent for singly charged species are, according to *t*-test, statistically insignificant.

In both solvents, bromothymol blue has the highest log*IE* value for singly charged species. This is probably because it has the highest hydrophobic character and, therefore, the singly charged species will move to the surface of the droplet easily, even if not a lot of them are formed in solution. The lowest log*IE* value belongs to tiron, which is a relatively small molecule with high hydrophilic character compared with other studied compounds. Also, another similar small and hydrophilic molecule, sulfosalicylic acid, has a low log*IE* value.

In acidic solvent, higher logIE_1 for singly charged species were observed for analytes that (1) have higher molecular volume, and (2) give doubly charged species with lower logIE_2 (bromothymol blue, bromocresol green, and bromophenol blue). Among the previously mentioned compounds, the highest logIE_1 value belongs to bromothymol blue, which also has the highest hydrophobicity. Substances with lower log*IE* values either give doubly charged ions with high logIE_2 value or are relatively hydrophilic substances, such as sulfosalicylic acid and tiron. This phenomenon can be explained that if the compound forms extensively doubly charged species, there is

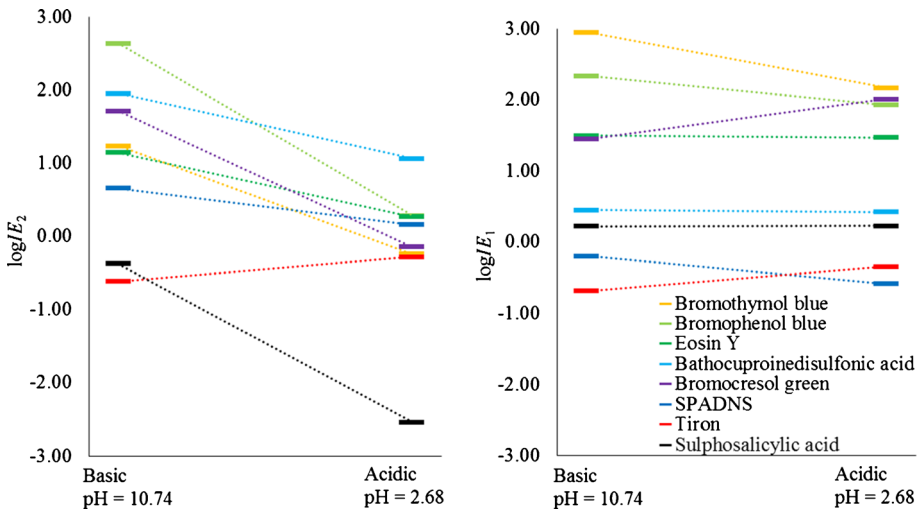


Figure 1. Ionization efficiencies of analytes in acidic and basic solvents, where logIE_1 is the log*IE* of the molecule via formation of singly charged ion; logIE_2 of the molecule via formation of doubly charged ion

only a limited amount of compound left for forming singly charged species. Relatively large eosin Y has medium ionization efficiency and is different from others in hat it does not contain sulfo groups.

To formulate a model for predicting logIE_1 via formation of singly charged ions, different parameters were tested that had the best correlation with logIE_1 (pK_{a1}, *WAPS*, and hydrogen bonding capacity for neutral substance) and also their combinations. The best parameter to describe ionization efficiencies (logIE_1) was charge delocalization parameter *WAPS*. It seems that charge delocalization is most important.

All in all, it can be seen that logIE_2 values in basic and acidic solvents differ for doubly charged species, which shows that log*IE* values depend on pH values. Solvent pH is therefore an important parameter to take into account to form a universal log*IE* prediction model and also to reach higher selectivity in analysis. Also, it can be seen from previous the discussion that formation of singly and doubly charged species are strongly related processes.

Conclusions

A model for predicting whether a compound gives or does not give doubly charged ions in negative ionization mode was developed based on pK_{a2} and logP_{ow} values with the prediction precision of 82%. Also, a model was developed for predicting ionization efficiencies (logIE_1) corresponding to singly charged species of multiply chargeable substances based on charge delocalization (*WAPS*) parameter. It was also shown that double charging is most influenced by acidity of a substance, its hydrophobicity (octanol-water partition coefficient), and charge delocalization. It was observed that solvent pH affects the ionization efficiencies of analytes and its optimal choice is a good tool to enhance selectivity and sensitivity. Therefore, the factors influencing the ionization efficiency of doubly charged ions are similar to the ones that have been reported to influence ionization efficiencies of singly charged analytes. However, it is shown that the solution phase degree of dissociation alone does not relate to the appearance of doubly charged ions in ESI/MS spectra. This demonstrates the complexity of ESI processes and the need to further investigate ESI mechanism.

Acknowledgments

The authors acknowledge support for this work by Personal Research Funding Project 34 from the Estonian Research Council.

References

1. Cech, N.B., Enke, C.G.: Practical implications of some recent studies in electrospray ionization fundamentals. Mass Spectrom. Rev. **20**, 362–387 (2001)
2. Cech, N.B., Enke, C.G.: Relating electrospray ionization response to nonpolar character of small peptides. Anal. Chem. **72**, 2717–2723 (2000)
3. Douglass, K.A., Venter, A.R.: Investigating the role of adducts in protein supercharging with sulfolane. J. Am. Soc. Mass Spectrom. **23**, 489–497 (2012)
4. 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)
5. Wong, S.F., Meng, C.K., Fenn, J.B.: Multiple charging in electrospray ionization of poly (ethylene glycols). J. Phys. Chem. **92**, 546–550 (1988)
6. Schnier, P.D., Gross, D.S., Williams, E.R.: On the maximum charge state and proton transfer reactivity of peptide and protein ions formed by electrospray ionization. J. Am. Soc. Mass Spectrom. **6**, 1086–1097 (1995)
7. Smith, R.D., Loo, J.A., Loo, R.R.O., Busman, M., Udseth, H.R.: Principles and practice of electrospray ionization-mass spectrometry for large polypeptides and proteins. Mass Spectrom. Rev. **10**, 359–451 (1991)
8. Felitsyn, N., Peschke, M., Kebarle, P.: Origin and number of charges observed on multiply-protonated native proteins produced by ESI. Int. J. Mass Spectrom. **219**, 39–62 (2002)
9. Loo, J.A., Edmonds, C.G., Udseth, H.R., Smith, R.D.: Effect of reducing disulfide-containing proteins on electrospray ionization mass spectra. Anal. Chem. **62**, 693–698 (1990)
10. Loo, J.A., Udseth, H.R., Smith, R.D.: Solvent effects on the charge distribution observed with electrospray ionization-mass spectrometry of large molecules. Biol. Mass Spectrom. **17**, 411–414 (1988)
11. Cole, R.B.: Electrospray and MALDI mass spectrometry: fundamentals, instrumentation, practicalities, and biological applications. Wiley, Hoboken (2010)
12. Iavarone, A.T., Jurchen, J.C., Williams, E.R.: Effects of solvent on the maximum charge state and charge state distribution of protein ions produced by electrospray ionization. J. Am. Soc. Mass Spectrom. **11**, 976–985 (2000)
13. Iavarone, A.T., Jurchen, J.C., Williams, E.R.: Supercharged protein and peptide ions formed by electrospray ionization. Anal. Chem. **73**, 1455–1460 (2001)
14. Iavarone, A.T., Williams, E.R.: Mechanism of charging and supercharging molecules in electrospray ionization. J. Am. Chem. Soc. **125**, 2319–2327 (2003)
15. Ganisl, B., Taucher, M., Riml, C., Breuker, K.: Charge as you like! Efficient manipulation of negative ion net charge in electrospray ionization of proteins and nucleic acids. Eur. J. Mass Spectrom. **17** (2011)
16. Douglass, K., Venter, A.: On the role of a direct interaction between protein ions and solvent additives during protein supercharging by electrospray ionization mass spectrometry. Eur. J. Mass Spectrom. **21**, 641 (2015)
17. Leito, I., Herodes, K., Huopolainen, 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)
18. Oss, M., Kruve, A., Herodes, K., Leito, I.: Electrospray ionization efficiency scale of organic compounds. Anal. Chem. **82**, 2865–2872 (2010)
19. Kruve, A., Kaupmees, K., Liigand, J., Leito, I.: Negative electrospray ionization via deprotonation: predicting the ionization efficiency. Anal. Chem. **86**, 4822–4830 (2014)
20. Tang, L., Kebarle, P.: Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution. Anal. Chem. **65**, 3654–3668 (1993)
21. Ehrmann, B.M., Henriksen, T., Cech, N.B.: Relative importance of basicity in the gas phase and in solution for determining selectivity in electrospray ionization mass spectrometry. J. Am. Soc. Mass Spectrom. **19**, 719–728 (2008)
22. Amad, M.H., Cech, N.B., Jackson, G.S., Enke, C.G.: Importance of gas-phase proton affinities in determining the electrospray ionization response for analytes and solvents. J. Mass Spectrom. **35**, 784–789 (2000)
23. 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)
24. Nguyen, T.B., Nizkorodov, S.A., Laskin, A., Laskin, J.: An approach toward quantification of organic compounds in complex environmental samples using high-resolution electrospray ionization mass spectrometry. Anal. Methods **5**, 72–80 (2013)
25. 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)

26. 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)
27. Kruve, A., Kaupmees, K., Liigand, J., Oss, M., Leito, I.: Sodium adduct formation efficiency in ESI source: sodium adduct formation efficiency in ESI source. J. Mass Spectrom. **48**, 695–702 (2013)
28. Klamt, A.: COSMO-RS from quantum chemistry to fluid phase thermodynamics and drug design. Elsevier Science Ltd., Amsterdam (2005)
29. TURBOMOLE V6.4, 2012, a development of University of Karlsruhe and Forschungszentrum Karlsruhe GmbH, 1989–2007, TURBOMOLE GmbH, since 2007; available at http://www.turbomole.com. Accessed 2013
30. Eckert, F., Klamt, A.: COSMOtherm, Version C3.0, Release 13.01; COSMOlogic GmbH&CoKG, Leverkusen, Germany, 2013; available from http://www.cosmologic.de/. Accessed: 2013
31. Teesch, L.M., Orlando, R.C., Adams, J.: Location of the alkali metal ion in gas-phase peptide complexes. J. Am. Chem. Soc. **113**, 3668–3675 (1991)