Standard-free Quantification of Dicarboxylic Acids: Case Studies with Salt-rich Effluents and Serum

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Abstract

The present study evaluates the ionization efficiency (IE) of linear and branched C_2 - C_{14} dicarboxylic acids (DCAs) by electrospray ionization under different conditions. The influence of the concentration of organic modifier (MeOH), mobile phase additive, and its concentration, pH, and DCAs structure on IE values is studied using the flow injection analysis. The IE values of DCAs increase with the increase of MeOH concentration but also decrease with an increase of pH. The former is due to the increase in solvent evaporation rates; the latter is caused by an ion-pairing between the diacid and the cation (ammonium), confirmed by the study with different amines. The investigation of DCAs ionization in the presence of different acidic mobile phase additives showed that a significant improvement in the (-)ESI responses of analytes was achieved in the presence of weak hydrophobic carboxylic acids, such as butyric or

propanoic acid. Conversely, the use of strong carboxylic acids, such as trichloroacetic acid, was found to cause signal suppression. The results of the IE studies were used to develop the liquid chromatography – high resolution mass spectrometry method that provided instrumental limits of detection in the range from 6 to 180 pg. Furthermore, by applying the non-parametric Gaussian process, a model for the prediction of IE values was developed, which contains the number of carbons in the molecule and MeOH concentration as model parameters. As a case study, dicarboxylic acids are quantified in the salt-rich effluent and blood serum samples using the developed LC-HRMS method.

Introduction

The application of the liquid chromatography - high resolution mass spectrometry analysis with electrospray ionization (LC-ESI-HRMS) is becoming more and more common in various fields of science due to the improvement in the capabilities of modern mass-spectrometers as well as simplification of gaining results with the use of modern computational tools such as machine learning methods.[1–6] The comprehensive qualitative evaluation of the sample can be easily performed. However, the quantitative data, concentrations of the compounds, are typically missing due to the lack of authentic standards.[2,3,7] This data is necessary to draw a meaningful conclusion regarding the significance of the observed compounds and compare the results obtained in different studies or different laboratories.[2,3] Another possible approach to overcome the limitation mentioned above is to develop the so-called "authentic standard-substance-free" method, which is based on the prediction of the ionization efficiency (IE) as a function of the physicochemical parameters of the compound of interest and the mobile phase composition.[1,2,8] The concentration can be calculated by the following formula, where the **Area** represents the mass spectrometry response:

$$\text{Concentration} = \frac{\textbf{Area}}{\text{IE}}$$

According to IUPAC, the term "ionization efficiency" is defined as the ratio of the number of generated ions to the total number of molecules injected into the ion source of a mass spectrometer.[9] However, in practice, the direct calculation of ionization efficiency values is challenging; therefore, the slopes of the calibration curves are used instead.[1–3,8,10–12]

Several studies were performed to find the relationship between ionization efficiency and molecular properties. The positive correlations between the ionization efficiency and octanol/water partition coefficient (LogP) values for sartans (angiotensin-II-receptor antagonists) and phenolic compounds, the length of the non-polar side chains for lignin model compounds, retention times, and non-polar surface areas for small peptides were observed.[13–17]

Further development of the IE prediction models showed that the implementation of the factors calculated by quantum chemistry provide a good correlation between the predicted and measured ionization efficiency values. Five independent variables representing hydrogen bond acidity, highest occupied molecular orbital energy, the number of hydrogen bond donating groups, the concentration of organics in the mobile phase, and the polar solvent accessible surface area were the major contributors to the ionization efficiency of aromatic organic acids.[18] In the work of Hermans et al.[19], the combination of the atomic composition, structural groups, and molecular size descriptors, generated by Dragon 5.5 software, provided a good estimation of ionization efficiency of the acylated amino acids, with $R^2 > 0.9$. Kruve and Kaupmees[8] suggested another combination of the molecular descriptors for the prediction of the ionization efficiency. They demonstrated that the best fit of the data was achieved with a model containing parameters for the degree of charge delocalization, the ionization degree, the hydrogen bond acceptor ability, and the concentration of the organics in the mobile phase. The first three parameters were calculated by COSMO-RS[20] and Turbomole program packages.[21]

The qualitative and quantitative analysis of the specific compound classes, such as dicarboxylic acids (DCAs), is of particular interest as these compounds are the products of the corn stover and rice straw valorization[22] and lignin conversion.[23] They are also considered

as markers for monitoring the progress of the Kraft pulping process[24] and are known as intermediate products during the decomposition of toxic aromatic hydrocarbons.[25] DCAs can be found as degradation markers of modern painting oils,[26,27] biodegradable plastics,[28] and biomass burning.[29] Also, dicarboxylic acids were known to be important components in the process of secondary organic aerosols formation[4,30–34] and pristine atmosphere.[35]

The desirable "authentic standard-substance-free" method for profiling the dicarboxylic acids should be twofold. Firstly, it provides high sensitivity and selectivity for wide range of different diacids. Secondly, it predicts the concentration of the compound without the knowledge of the structural formula, which is now the main drawback of the existing "authentic standard-substance-free" methods.[2,3,6]

Our study aims to investigate the ionization efficiency of the dicarboxylic acids and understand the factors responsible for the anion formation during the electrospray ionization process. The study includes the evaluation of the influence of the mobile phase composition and instrument parameters on IE values. Particular attention is paid to the role of the mobile phase components in the ionization process of the dicarboxylic acids, which allows us to form the basis for a rational design of the analytical procedure. By the evaluation of the data with the use of Gaussian regression process, the "authentic standard-substance-free" quantification method, which is based on the prediction of the IE as a function of the molecular descriptors (number of carbon atoms in the molecule) and mobile phase composition (MeOH concentration), has been developed. In addition, the transferability of the "authentic standard-substance-free" method between different instruments, as well as the applicability of the method for the quantification of the DCAs in the salt-rich effluent from the biomass treatment plant and blood serum samples, is examined.

Materials and Methods

Chemicals

All chemicals had purity grade >99%. phthalic, malonic, glutaric, adipic, pimelic, sebacic, dodecanedioic, tetradecanedioic, concentrated aqueous ammonium (28-30% solution), formic acid, glacial acetic acid, monochloroacetic, dichloroacetic, trichloroacetic, propanoic acid, butyric acid were purchased from Merck (Switzerland). Succinic, methylsuccinic, 3-methylglutaric, 3-methyladipic, suberic and azelaic acids were purchased from TCI Europe N.V. (Belgium), Oxalic acid was purchased from Fluka. 2,2-Dimethylsuccinic acid was purchased from Apollo Scientific (Switzerland), 3,3-dimethylglutaric acid was purchased from Alfa Aesar (Switzerland). Methanol (MeOH) (HPLC grade) was purchased from Avantor VWR International (Switzerland). Ultra-pure water (18.2 MΩ.cm, Milli-Q) was used throughout the experiments.

Human serum from platelet poor human plasma, sterile-filtered was purchased from Merck (Switzerland). The brine sample was obtained by supercritical water desalination process from process water of hydrothermal liquefaction of pine wood.[36]

The aqueous phase pH was measured with a pH meter (Handylab pH/LF 12, Schott instruments) using a glass electrode (BlueLine 24 pH).

Sample preparation

All the sample preparation procedures, including dilutions, were performed using an analytical balance. Three independent sets of individual diacids stock solutions were prepared in MeOH-Water (v/v, 50:50) mixture and kept at -20℃. For dissolving suberic, dodecanedioic and tetradecanedioic acids pure MeOH was used.

The work solutions for the flow injection analysis were prepared by dilution of the stock solutions of individual acids with MeOH-Water (v/v, 50:50) mixture to the concentration of 25, 50 and 100 μM. For the LC-HRMS analysis, individual acids stock solutions were

mixed and then diluted with (v/v, 50:50) MeOH-Water mixture to the same concentrations as for the FIA experiments. All working solutions were kept at -20℃ and equilibrated in the autosampler of the LC system prior to the experiments.

Flow injection analysis (FIA)

The flow injection analysis was performed with the use of Agilent 1290 Infinity 2 LC system connected to the Thermo Scientific Q-Extractive hybrid quadrupole-Orbitrap mass spectrometer controlled by Xcalibur^{TM} 3.1 software (Thermo Fisher Scientific, Switzerland) equipped with heated electrospray ionization source. The following ESI-MS and LC parameters were set: sheath gas flow rate 55 a.u., aux gas flow rate 20 a.u., sweep gas flow rate, 0 a.u., the capillary voltage was -3 kV, capillary temperature 281℃, aux gas heater temperature 450℃. Mass spectra were acquired in full scan mode with an isolation window of 1 m/z from 50-750 m/z. The resolution was set to 70000. The injection volume was 1 µL, and the eluent flow was 0.7 mL/min. For the estimation of instrument stability during measurement, the reference compound 10 µM phthalic acid, was analyzed in the beginning and end of each experimental run. The influence of MeOH concentration on the ionization efficiency was evaluated using mixtures of MeOH and water (1:99, 20:80, 40:60, 60:40, 80:20, and 99:1 v/v) with the addition of 0.2 vol. % formic acid. The influence of pH on IE was studied using mobile phases containing mixtures of MeOH. The buffer solution was obtained by adjusting the pH of 10 mM formic or acetic acid solutions by ammonium hydroxide solution and then mixed with MeOH in a ratio of 1 to 1 by volume. If only formic acid, acetic acid, or ammonium was used as pH modifiers, they have directly added to the (v/v, 50:50) MeOH-Water mixture The transferability of the IE values between different instruments was tested with the use of ACQUITY QDa single quadrupole mass spectrometer equipped with ACQUITY UPLC system (both are from Waters AG, Switzerland) in FIA mode. As the Single Quadrupole MS has a lower sensitivity than Orbitrap MS, the concentration range of the diacids was from 100 µM to 1000 µM, concentration of phthalic acid was 1 mM. The injection volume

was 10 µL and flow was 0.7 mL/min. The FIA experiments were conducted using 5 mM formic acid in (v/v, 50:50) MeOH/Water and 5 mM ammonium hydroxide in (v/v, 50:50) MeOH/Water. For the inter-instrument comparison of the IE values, they were anchored by the IE value of phthalic acid.

LC-HRMS analysis

The separation of diacids was carried out on a Thermo Scientific UltiMate 3000 UHPLC system connected to the same MS device as in FIA experiments with the same parameter settings. Analysis was performed with an ACCUCORE RP-MS LC column (150 mm x 2.1 mm, particle size 2.6 µm) from Thermo Fisher Scientific (Switzerland) with an Uniguard precolumn (Accucore RP-MS Defender Guards included; 2.1 x 10 mm, 2.6 µm). The mobile phase consisted of (A) 1mM formic acid in water and (B) 1 mM formic acid in MeOH. The following gradient program was applied: 1 % B (0-1 min) 1 to 99 % B (1-6 min), 99 % B (6-8 min), followed by equilibration step and 99 to 1 % B (8-8.2 min), 1 % B (8.2-10 min).The injection volume was 1 µL, the flow rate was 0.7 mL/min.

The instrumental limits of detection were calculated by the following formula: LOD = $\frac{3S_y}{b}$, where b is a slope of the calibration curve and S_y is the standard error of the predicted y-value for each x in the regression.[37] For the determination of LOD values and estimation of a linear dynamic range of the mass spectrometer response, a wider concentration range, from 1 nM, 0.1 pg, to 1 mM, 100 000 pg, was studied.

Data processing and ionization efficiency calculations

After the acquisition, data were imported into Xcalibur^{TM} 3.1 software (Thermo Fisher Scientific, Switzerland)and processed with the mass tolerance of 10 ppm for the extraction of peaks areas. Typical extracted ion chromatogram (XIC) from the FIA data and used m/z ranges can be found in the ~~ESI~~ **Figure S1**. The areas of the single and double (if detected) charged ions were summarized. Peak detection and integration were done using the following

parameters: baseline window 40, area noise factor 5, peak noise factor 10. Smoothing of the detected peaks was performed using the Gaussian algorithm with 11 points. The ionization efficiency values were calculated from the slope of the calibration curve obtained at three concentrations: 25, 50, 100 μM by the following formula:

$$\mathrm{IE} = \mathrm{Log10}(\frac{\mathrm{Slope}}{\mathrm{V_{inj}}})$$

where Slope and V_{inj} represent the slope of the analyte signal versus concentration estimated via linear regression in the linear range of the signal-concentration plot and injection volume, respectively. The intra-day instrument stability was checked by measuring the response of 10 μM solution of phthalic acid in water-MeOH-formic acid (v/v, 80:20:0.2) mobile system. The obtained values were used for the normalization of the signal for comparison of the results from different experimental days.

Peak detection in the LC-HRMS data from the LOD study was performed using XcaliburTM 3.1 software (Thermo Fisher Scientific, Switzerland) with the mass tolerance of 10 ppm and retention times of DCAs obtained from the analysis of standard substances. Prior to the ion chromatograms extraction, the blank background was subtracted from the samples' data using "Subtract Background" in XcaliburTM 3.1 software with the scaling factor of 1. For the peak detection, S/N was set to 10, and integration was performed with the following parameters: baseline window 100, area noise factor - 50, and peak noise factor - 1. The number of smoothing points was set to 11. An example of an XIC from the LC-HRMS data can be found in ESI.

The LC-HRMS data evaluation from the analysis of brine and serum samples was done using the Compound Discoverer^{TM} 3.2 software (Thermo Scientific^{TM}, Switzerland). The data were processed with standard settings except for mass tolerance (set to 2.5 ppm). The composition (of a general formula $C_cH_hO_o$ was predicted based on exact mass and isotopic patterns. The data analysis workflow of Compound Discoverer can be found in ESI

Investigation of the influence of different acidic mobile phase additives on the dicarboxylic acids response

The influence of different acidic mobile phase modifiers on dicarboxylic acids response with the use of 10 μM solutions of malonic, adipic, and sebacic acids in equimolar solutions of formic, acetic, propionic, butyric, chloroacetic, dichloroacetic and trichloroacetic in MeOH-Water (v/v, 50:50) mixtures by flow injection analysis.

Model development for the IE prediction

The model of relative ionization efficiency is based only on the experimentally observed values: number of carbon atoms and concentration of organic modifier (MeOH). The model was developed using a non-parametric Gaussian process (GP) as implemented into GPflow 2.0 package[38,39] using TensorFlow.[40] GP regression was optimized using Scipy minimize method.[41] The following strategy was used: 1) as the number of carbon atoms is a discrete variable - one model per carbon number is generated. 2) The linear kernel was implemented using a polynomial kernel of degree one. 3) A squared exponential kernel was used to fit nonlinear functions. Given the limited number of data per model, the goodness of the model was validated using the leave-one-out cross-validation strategy. The error is reported as the mean relative error in percent. The test of the model was performed using independently collected IE of the branched and non-branched diacids after chromatographical separation and non-branched diacids from FIA experiments.

Brine and Serum case study

As a case study, dicarboxylic acids were quantified by the standard edition method in serum and salt-rich effluent from the biomass treatment plant (brine)[36,42] samples and the results were compared with the quantification by the "authentic standard-substance-free" method. For the preparation of the serum sample, a standard protein precipitation method was used:

100 μL of the serum was mixed with 800 μL of MeOH and spiked with 100 μL solution of dicarboxylic acids with the concentrations of 100 μM, 50 μM, and 25 μM in (v/v, 50:50) MeOH-Water. Then it was centrifuged for 10 min at 13 000 g and then the supernatant was analyzed. For the quantification of dicarboxylic acids in the brine, the sample was firstly diluted 100 and 1000 times with water and then spiked (1:1, v/v) with 100 μM, 50 μM, and 25 μM solutions of dicarboxylic acids. The acid-free brine matrix was obtained by removal of anionic species using the Chromabond HR-XA polypropylene column (Macherey-Nagel AG, Switzerland). The column was prepared according to the procedure recommended by the manufacturer: flush with 10 mL of methanol and then equilibration with 10 mL of water. The load of the brine sample was 150 μL of sample per 500 mg of the adsorbent. Then the acid-free brine sample were spiked with 100 μM, 50 μM, and 25 μM solutions of dicarboxylic acids in the ratio 1:1 (v/v). As a reference matrix for the estimation of matrix effect (v/v, 50:50) MeOH-Water solution was used. The mean absolute percentage error (MAPE) between the calculated and measured concentrations was utilized to assess the quality of "authentic standard-substance-free" method: MAPE $= \frac{100\%}{n} \sum_{t=1}^{n} |\frac{A_t - F_t}{A_t}|$, where A_t and F_t stand for the measured and predicted value, respectively.

Results and Discussions

A comprehensive evaluation of the IE of dicarboxylic acids under different conditions is performed. Understanding the influence of organics concentration (MeOH), type of mobile phase additive and its concentration, pH, and DCAs structure on the efficiency of anion formation by electrospray ionization is done. The results are used to develop a simple, sensitive, and robust "authentic standard-substance-free" quantification method. MeOH was selected as an organic mobile phase modifier because it provides the highest (-)ESI response of acidic compounds compared to acetonitrile or acetone.[43]

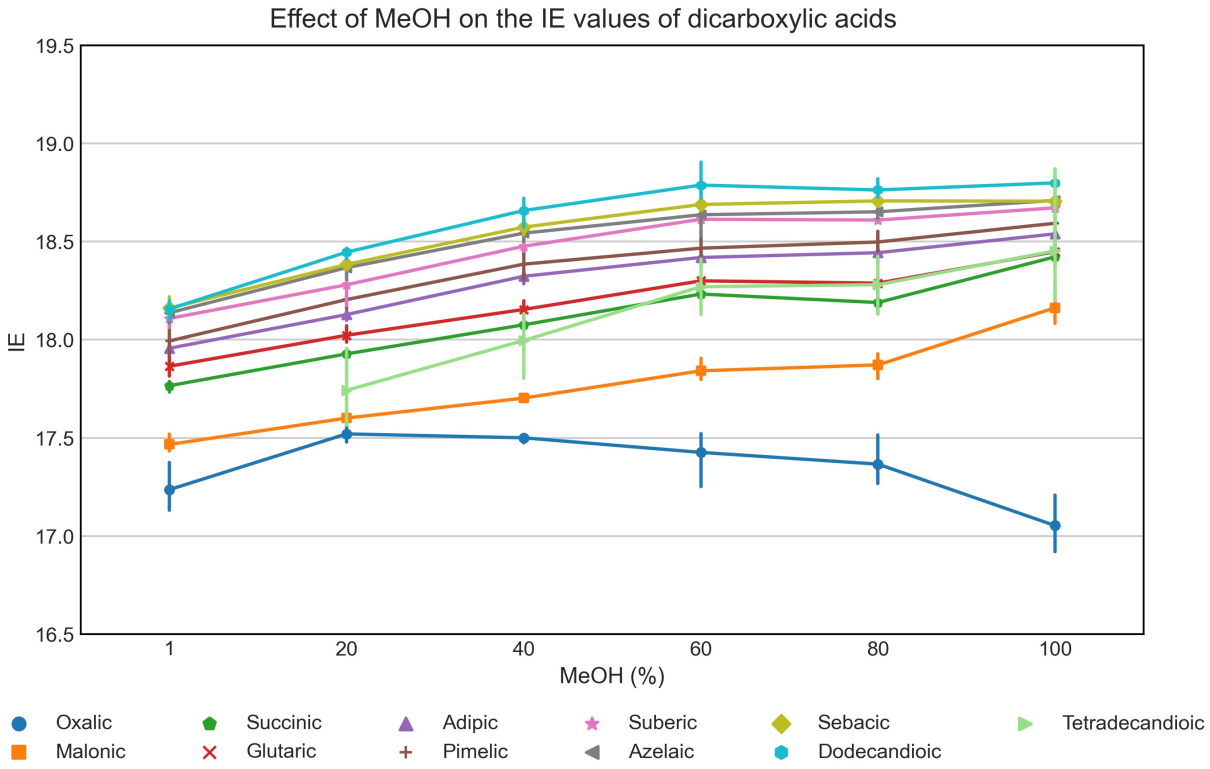

Figure 1: IE values of dicarboxylic acids measured at different MeOH concentrations. The values, slopes of the calibration curves and R^2 can be found in Table S1.

Figure 1 shows the influence of MeOH concentration on IE values of DCAs. A positive correlation between the MeOH content and ionization efficiency is observed. The linear increase in the IE values from 1% to 100% of MeOH is found for the short-chain dicarboxylic acids, such as malonic and succinic diacids. The long-chain diacids, from glutaric to dodecanedioic acid, show a linear increase in the IE values from 1% to 60% of MeOH. The further increase in the concentration of organics did not cause any statistically significant changes in their IE values. However, the IE values for the oxalic acid appear to be significantly different from the other diacids. The IE values increased from 1 to 20% of MeOH and then decreased from 20 to 100%.

The increase of the MeOH concentration increases the speed of the ESI droplets drying, leading to the faster formation of smaller droplets facilitating the ion evaporation and thus increasing the IE values.[10,44] With the increasing size of the molecule by one $-CH_2-$ group,

the IE values increase by 0.07±0.01 IE unit on average, Figure S17. This phenomenon is related to the increase of the non-polar character and volume of the molecule, which leads to the increased concentration of the analytes on the surface of the droplets and thus facilitating the gas phase ion formation.[8,10,43,45] This trend is observed for the dicarboxylic acids with a chain length from 2 to 12 carbon atoms, from oxalic to dodecanedioic diacid. The longer homologous tetradecanedioic acid with 14 carbon atoms shows lower IE values than the dodecandioic acid with 12 carbon atoms. This fact is in contrast to the findings reported for the n-monocarboxylic acids where the linear increase of response with an increase of the chain-length was observed for the molecules with up to 30 carbon atoms.[32,43,46] One of the possible explanations is the thermodynamically favored formation of the intramolecular hydrogen bonds between –COOH groups due to the flexible carbon backbone of the long-chain (more than 8 carbon atoms in the molecule) dicarboxylic acids, thus stabilizing the cyclic structure of the molecule and decreasing the ionization efficiency.[47]

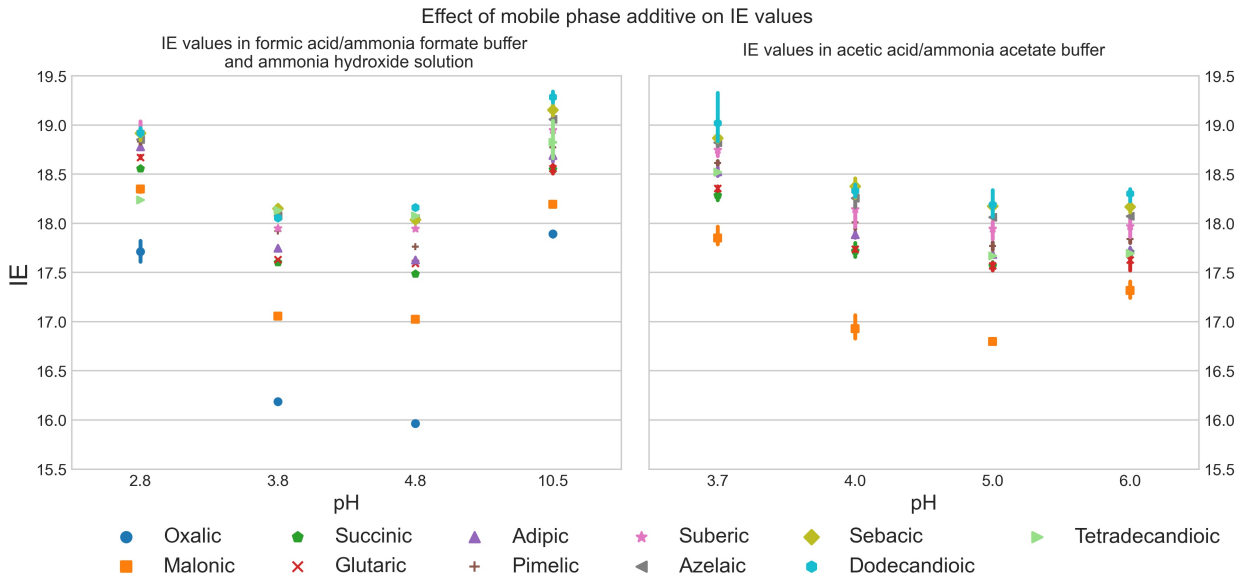

Figure 2: IE values of dicarboxylic acids at different pH in 5 mM formic acid (pH 2.8), 5mM ammonium formate (pH 3.83 and 4.81), 5 mM ammonium hydroxide (pH 10.55), 5 mM acetic acid (pH 3.7) and 5 mM ammonium acetate solutions (pH 4.01, 5.0, 6.0). Some errors bars are smaller than the size of the point.

Figure 2 shows the influence of the mobile phase additive and pH on IE values. The

highest IE values are obtained using the solution of formic acid, pH 2.8. The IE values decreased with an increase in pH, from 2.8 to 4.81 in the formic acid/ammonium formate solutions, and from 3.7 to 5 in the acetic acid/ammonium acetate solutions. Surprisingly, the signal of the oxalic acid is only detectable in the formic acid/formate solutions. Also, the IE values observed at low pH (2.8) in the formic acid solution are found to be even higher for the mid and long-chain dicarboxylic acids than the ones measured in the ammonium hydroxide solution, pH 10.55, where those compounds are expected to show the highest response due to the complete deprotonation, see figure S18.

Elucidation of the ionization mechanism of dicarboxylic acids

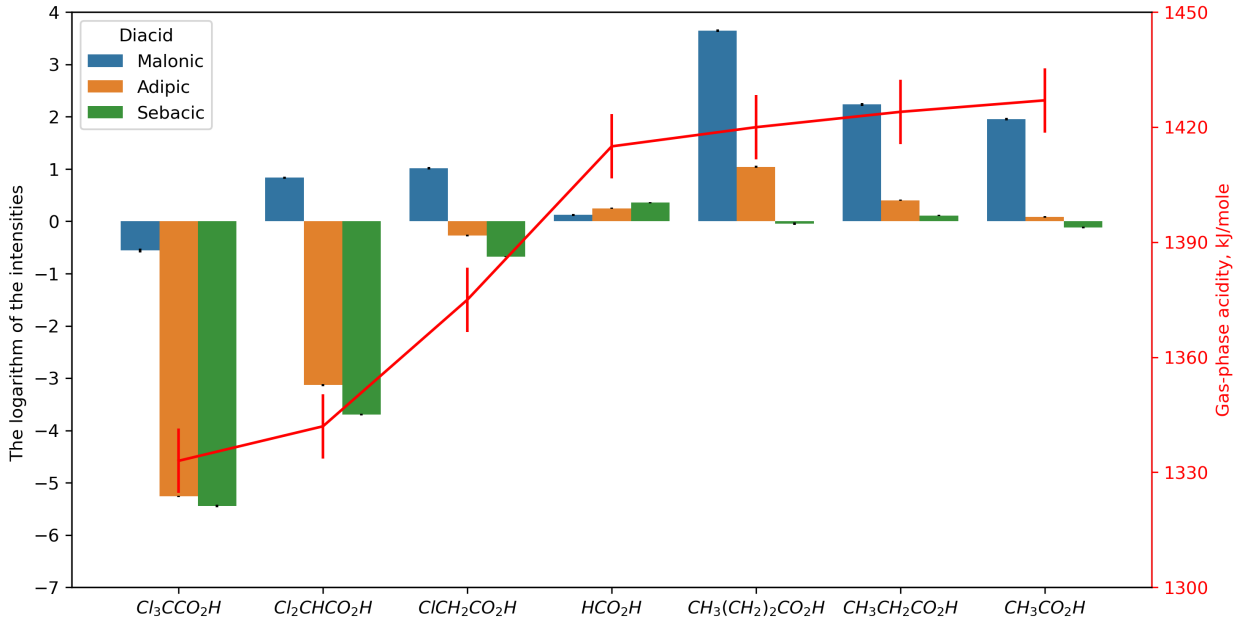

Figure 3: The logarithm of the relative intensities of 10 μM solutions of malonic, adipic and sebacic diacids in 10 μM solutions of formic (HCO_2H), acetic (CH_3CO_2H), propionic ($CH_3CH_2CO_2H$), butyric ($CH_3(CH_2)_2CO_2H$), mono- ($ClCH_2CO_2H$), di- (Cl_2CHCCO_2H) and trichloroacetic (Cl_3CCO_2H) acids obtained by flow injection analysis. The intensity values were normalized by the intensity of diacids in the MeOH/Water (50/50 v/v) solution.

Table 1: Gas-phase acidity values, LogP values, pK_a, and Van der Waals volumes of the dicarboxylic acids and acidic mobile phase additives.

Compound	Gas-phase acidity constant, $\Delta_r G°$ **(kJ/mole)** [48–50]	Octanol-water partion coefficient, (**LogP**) [51,52]	pK_a [52]	Van der Waals volume ($1\,Å^3$) [52]
Formic	1415±8.4	-0.272	3.75	36.81
Acetic	1427±8.4	-0.223	4.75	53.5
Propanoic	1424±8.4	0.477	4.88	70.56
Butyric	1420±8.4	0.922	4.82	89.91
Monochloroacetic	1377±8.4	0.314	3.06	67.55
Dichloroacetic	1342±8.4	1.058	2.3	84.09
Trichloroacetic	1322±8.4	1.529	1.72	95.77
Malonic	1318±8.4	-0.331	2.43	78.82
Adipic	1343±8.4	0.491	4.43	129.84
Sebacic	1345±8.4	2.27	4.72	202.5

The interpretation of the results shown in figure 2 requires a detailed study of the ionization process if the DCAs. Investigation of the effect of the acidic mobile phase modifiers on the ionization of dicarboxylic acids was carried out by direct injection analysis of the malonic, adipic, and sebacic diacids in the equimolar solutions of weak carboxylic acids, namely formic, acetic, propionic, butyric, and strong carboxylic acids, namely mono, di- and trichloroacetic. The concentration of 10μM is utilized to minimize the effect of charge competition on the droplet surface.[53,54] Those acidic mobile phase modifiers were chosen as they cover a wide range of gas-phase acidity values, octanol-water partition coefficients, and Van der Waals volumes, table 1. Figure 3 shows that the weak carboxylic acids, namely acetic, propanoic, and butyric, enhanced the response of the malonic and adipic diacid. However, they do not affect the response of sebacic diacid, see table S3 for the results of the t-test analysis and figure S16 for the absolute values. The use of formic acid as an acidic modifier caused a slight increase in the intensities of all diacids. The stronger mono- and dichloroacetic acids enhance the signal of malonic acid while suppressing the signals of the adipic and sebacic diacids. Significant suppression of all diacids signals was observed in the solution containing

trichloroacetic acid. The ion evaporation model describes the electrospray ionization of the low molecular weight compounds (IEM).[55] Under this model, the ionization occurs via the ejection of small solvated ions from the droplet surface. This gas-phase cluster, while traveling through the sampling interface of the mass spectrometer, undergoes collisions with background gas and loses its solvation shell.[56] Alternatively, the ionization of compounds is also possible via the gas-phase reactions.[57–59] The ionization of analytes in the negative-ion ESI is affected by the gas-phase acidity and acid dissociation constants, pK_a and $\Delta_r G$ respectively, and hydrophobicity, logP of the acidic mobile phase additive.[51,60,61] The results of the current study, in terms of the influence of the weak carboxylic acids on the negative-ion ESI, were not in line with the findings of Wu et al.[51]. Wu et al. noted that the high gas-phase acidity and small molecular volumes of the acidic mobile phase additive facilitated the ionization of the analytes. In contrast, we observe that the highest response of the malonic and adipic diacids was using butyric acid with a low gas-phase acidity value and the highest molecular volume among tested weak carboxylic acids. It seems that the ionization process of dicarboxylic acids in the presence of the acidic mobile phase additive included the formation of the diacid-additive adduct by the hydrogen bonding. Subsequent ejection of this adduct from the droplet surface and its dissociation in the gas phase forms the deprotonated diacid. The efficient ionization of the diacids via the proposed pathway depends both on the gas-phase and solution acidity, as well as the hydrophobicity of the mobile phase additive. It is known that the hydrophobic molecules with high LogP values prefer positions close to the droplet surface, which simplify the subsequent ejection to the gas phase, while more hydrophilic compounds prefer the interior part of the droplet.[45,62] Thus, when the butyric acid (LogP = 0.922) was used for the ionization of the malonic (LogP = -0.331) or adipic (LogP = 0.491) diacid, it can efficiently produce an adduct that can easily be ejected into the gas phase according to the IEM. The proposed mechanism is in line with the results for the sebacic acid, where the addition of weak carboxylic acids (acetic, propionic, or butyric) had no impact on the IE of this compound, as it possesses the highest LogP value than any

weak carboxylic acid used. The increase in the IE of sebacic acid using formic acid can be associated with the low molecular volume of the formate ion and thus a higher probability to be present on the droplet surface, simplifying the formation of the diacid-additive adduct. The competition for the interior/exterior parts of the droplet between the analyte and mobile phase additive can explain the positive effect of formic acid (LogP = -0.272) on the ionization of oxalic acid (LogP = -0.264). The higher concentration of the acidic mobile phase additive promotes the competition for the droplet surface, explaining the highest response of the DCAs in the formic than in the acetic acid solution, Figure 2.

When the strong acidic mobile phase modifier is used in negative-ion ESI, the signal suppression of the analytes can be observed, and it associates with the protonation of the analyte in the solution meaning that the negative excess charge on the droplet surface is carried mainly by the additive.[60,61] The pK_a values of the mono, di- and trichloroacetic acids are significantly lower than the pK_{a1} values of the adipic and sebacic diacid, and, as expected, the signal suppression of these diacids was observed. The pK_{a1} value of the malonic acid is lower than the pK_a value of the monochloroacetic acid and comparable with the pK_a of the dichloroacetic acid. The increase in the intensity of malonic acid is likely associated with the proposed ionization mechanism described above as the gas-phase acidity, and LogP values of the monochloroacetic and dichloroacetic are higher than the gas-phase acidity and LogP values of the malonic acid. When trichloroacetic acid was used, a signal suppression of all three dicarboxylic acids is observed as the pK_a and gas-phase acidity values of trichloroacetic are much lower than any of the DCAs.

The pH dependencies of the IE values shows that the diacids' response decreased with pH increase. This observation can be associated with the formation of an ion pair between the $–COO^-$ groups of diacids and ammonium (NH_4^+).[63] To investigate this phenomenon, the determination of the IE values in the mobile phases containing amines with different proton affinity values ranging from 891 kJ/mole for the ammonium to 951 kJ/mole for the triethylammonium as pH modifiers are performed. It was expected that the increase

of the proton affinity of the amine would decrease the IE values as the stability of the $COO^- \cdots NR_3H^+$ ion-pair increases due to the higher energy of the proton binding to the nitrogen. This trend is confirmed with experiments shown in Figure 4a for the adipic to sebacic diacids. The result for the long-chain diacids is found to be scattered, and a huge deviation in the signal for the tetradecanedioic acid is observed. Figure 4b shows that depending on the molecular size of the diacid and amine used, the analytes were detected both as $[M-H]^-$ and $[M-2H]^{2-}$ ions. The share of $[M-H]^-$ ion decreased both with the increase of the chain length and proton affinity values of the amine likely by the increase of the $COO^- \cdots NR_3H^+$ ion pair stability and increase of the molecular volume of the diacid thus limiting the space for the amine in the droplet. The increase of the $[M-H]^-$ share in the triethylamine solution can be associated with structural difficulties in the formation of $COO^- \cdots NEt_3H^+$ ion pair. Continuing the investigation of the ion-pairing effect of the base on the diacids ionization process, the experiments with the use of tetramethylammonium hydroxide as a basic mobile phase additive are performed. It was found that this additive led to the strong suppression of the signal, and no diacids were detected in the mass spectra, neither as $[M-H]^-$ nor $[M-2H]^{2-}$ ions. Moreover, the precipitation of the salts on the surface of the MS inlet was observed, figure S20, supporting our hypothesis.

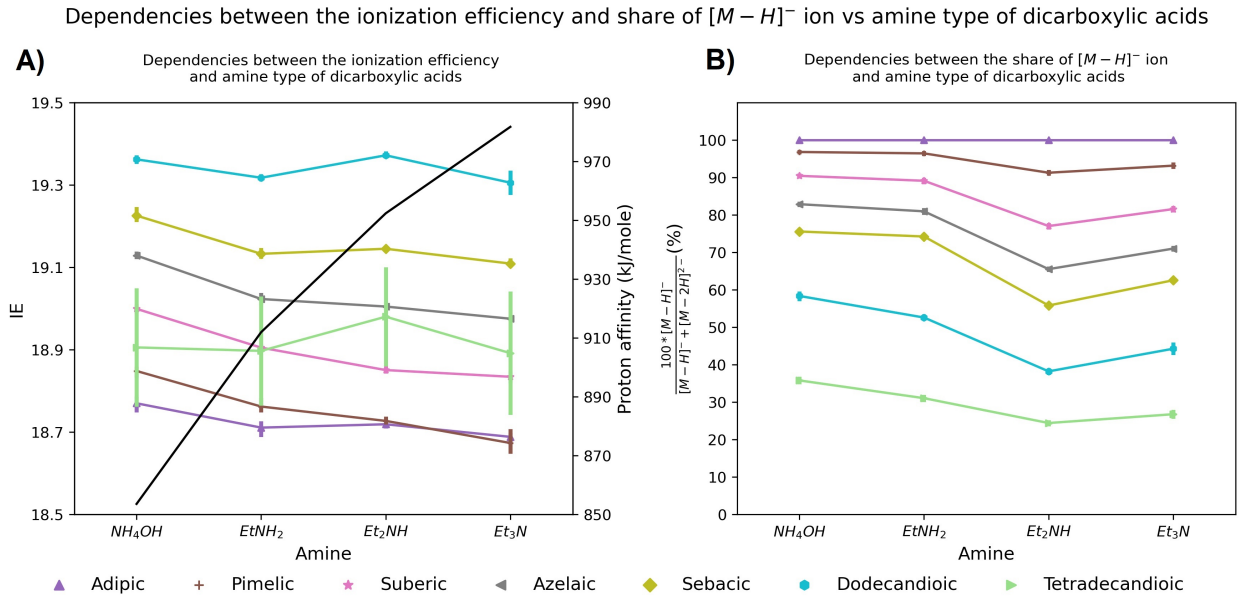

Figure 4: The IE values and share of $[M-H]^-$ ion of dicarboxylic acid as a functions of amine type. The black curve shows the proton affinity of the amine.

From the results of the analysis of performed IE studies, formic acid was chosen as the most suitable mobile phase additive. The optimum concentration of the additive that, on the one hand, provides efficient separation of the compounds on a chromatographic column, and on the other hand, gains the highest ionization efficiency values was found to be 1 mM, table S4 and figures S21, S24. The developed liquid chromatography – high resolution mass spectrometry method (LC-HRMS) was compared in terms of the instrumental quantification limits (LODs) with those, reported in the works of Mirivel et al.[30] and Štávová et al.[64], Table 2, and showed better performance for the C_2 - C_7 dicarboxylic acids. The results for the C_8 - C_{14} were found to be comparable to the previous studies, see figures S24 and S25 for the obtained calibration curves and QQ-plot analysis of the residues from the regression analysis.

Table 2: Comparison of the limits of detection (LODs) for dicarboxylic acids obtained in the current work with previously reported data.

Diacid	**Tested range, pg**	**Instrumental LODs, pg Orbitrap, m/z range of 50–750**	**LODs, pg Štávová et al.[64], TOF-MS, m/z range of 30–1000**	**LODs, pg Mirivel et al.[30], TOF-MS, m/z range of 50–1000**
Malonic	0.1-100 000	30	2926	
Succinic	0.1-100 000	180	6735	273
Glutaric	0.1-100 000	8.5	3070	86
Adipic	0.1-100 000	7.4	322	59
Pimelic	0.1-100 000	7.1		32
Suberic	0.1-100 000	2.7	47	18
Azelaic	0.1-100 000	13.7	65	11
Sebacic	0.1-100 000	9.6		8
Dodecanedioic	0.1-100 000	15.1		16
Tetradecanedioic	0.1-100 000	8.8		

The Gaussian process (GP) model for the predicting of dicarboxylic acids ionization efficiency as MeOH dependency per discrete carbon number was adequately accurate using the squared exponential covariance (SEC) function (Figure 5). The baseline model, first-order polynomial, for the same data is depicted in SI (Figure S26). The mean squared error (MSE) for the polynomial and squared exponential covariance function was determined as 0.23 and 0.11 IE units, respectively. The overall matrix of MSE for SEC is depicted in Figure 6, and the first-order polynomial in SI Figure S27, respectively. It can be observed that the error increases at the "borders," as expected from GP, as data at the border is only defined from the inner site.

Transferability test of the DCAs IE scale between different devices: Brine and Serum case studies

To evaluate the influence of the mass-spectrometer configuration on the DCAs IE values, the IE measurements were repeated using the single quadrupole mass spectrometer, ACQUITY QDa, equipped with the ESI Z-spray source. Figure 7 shows that the data was found to be comparable between both devices, which is in line with the previous results obtained by Liigand et al.[65].

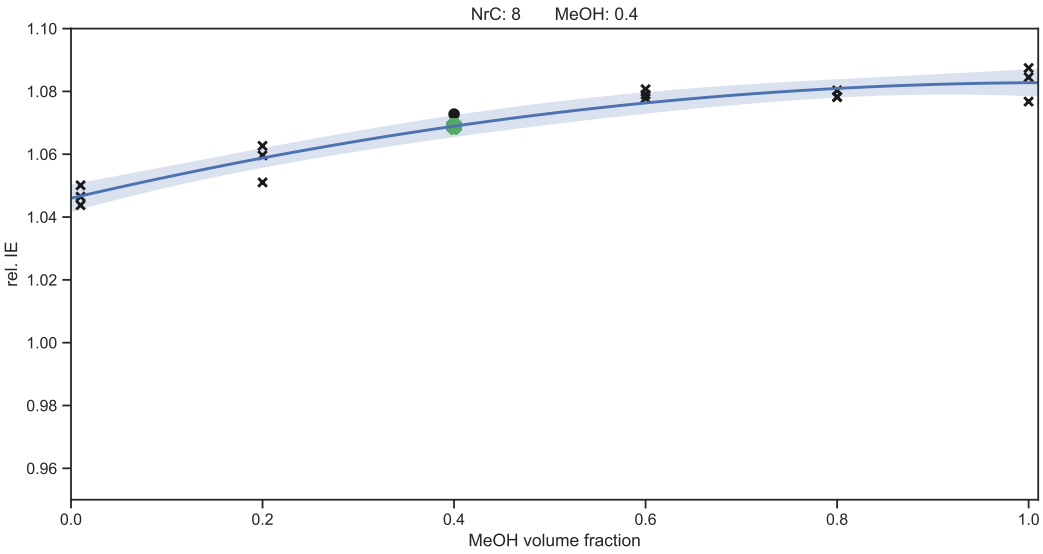

Figure 5: Gaussian Process model fit and error estimation (grayed area) of the IE for the adipic acid using squared exponential covariance function. The x's represent the measured values and a green dot - predicted value.

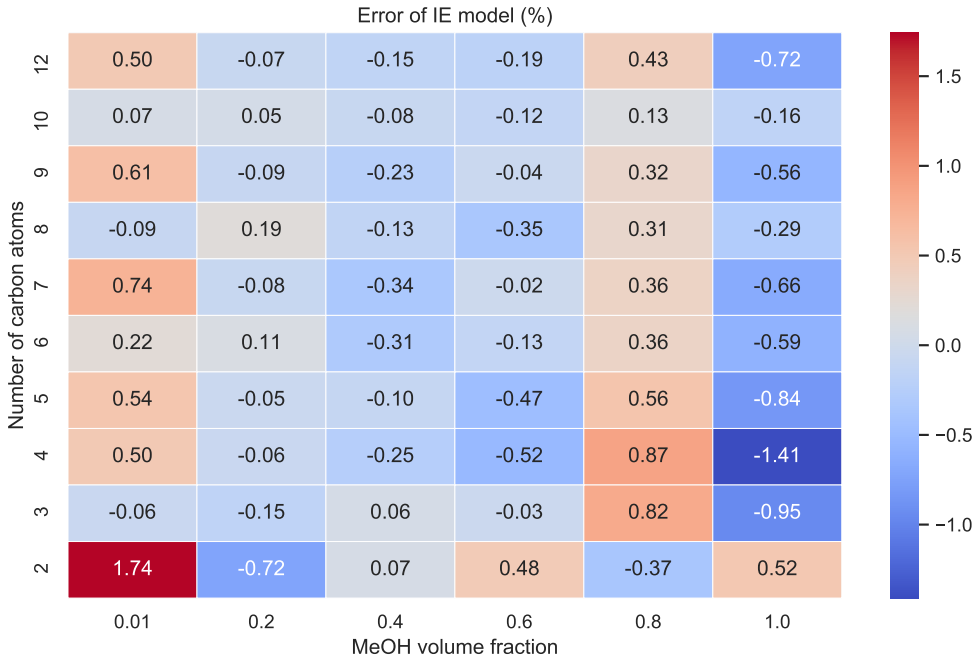

Figure 6: The Heat map of the mean squared error(MSE) for the data fited using the squared exponential covariance (SEC) function.

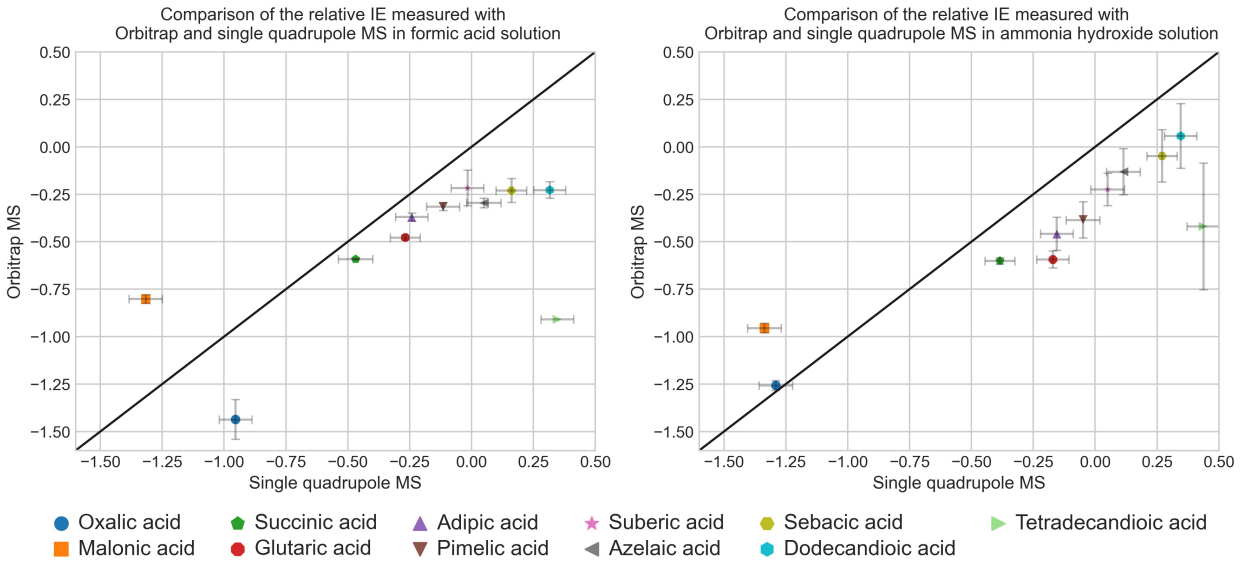

Figure 7: Comparison of the relative ionization efficiency values obtained with Orbitrap and Single quadrupole MS in formic acid (left) and ammonium hydroxide (right) solutions. The DCAs IE values are anchored to the IE value of phthalic acid.

The developed LC-HRMS "authentic standard-substance-free" method was used for the quantification of the dicarboxylic acids in serum and brine samples and the results were compared with those obtained by the standard addition method, Figure 8 A. In the brine sample, 16 dicaboxylic acids, with the carbon number in the range from 4 to 10, were detected, see figure S28 for the chromatogram. The comparison of the quantification results showed a good correlation between the measured and calculated values. The mean absolute percentage error (MAPE) between the measured and calculated concentrations was found to be 30%. The analysis of the serum sample revealed the presence of succinic, glutaric and adipic diacids at μg/mL levels. The comparison between the measured and calculated values showed a good agreement between the measured and calculated values for the succinic and adipic diacid with the MAPE equal to 40% and 26%, respectively. The concentration of the glutaric acid was found to be significantly overestimated by calculation with the MAPE equal to 202%, which can be associated with the matrix effect.

To evaluate the influence of the matrix component on ionization of DCAs in brine and serum samples, the acid-free brine and serum spiked with the standard solution of DCAs were

analysed, Figure 8 B. The obtained intensities were compared with those obtained in the reference matrix, Water/MeOH (50/50, v/v). Values around 0% correspond to the absence of the matrix effect, while values which are lower than 0% corresponds to the suppression and higher than 0% to enhance the signal, respectively. For the serum sample, the estimation of the matrix effect was performed only for the non-detected diacids, e.g. ranging from the pimelic to tetradecanedioic acids. The results showed that for succinic acid the matrix component in the acid-free brine sample caused a significant signal suppression of about 35%, while for the rest of diacids the matrix effect was $\leq 10\%$. The suppression of succinic acid can be associated with the presence of inorganic salts in the acid-free brine sample. To minimize the impact of such matrices, a proper dilution of the sample is required[66,67]

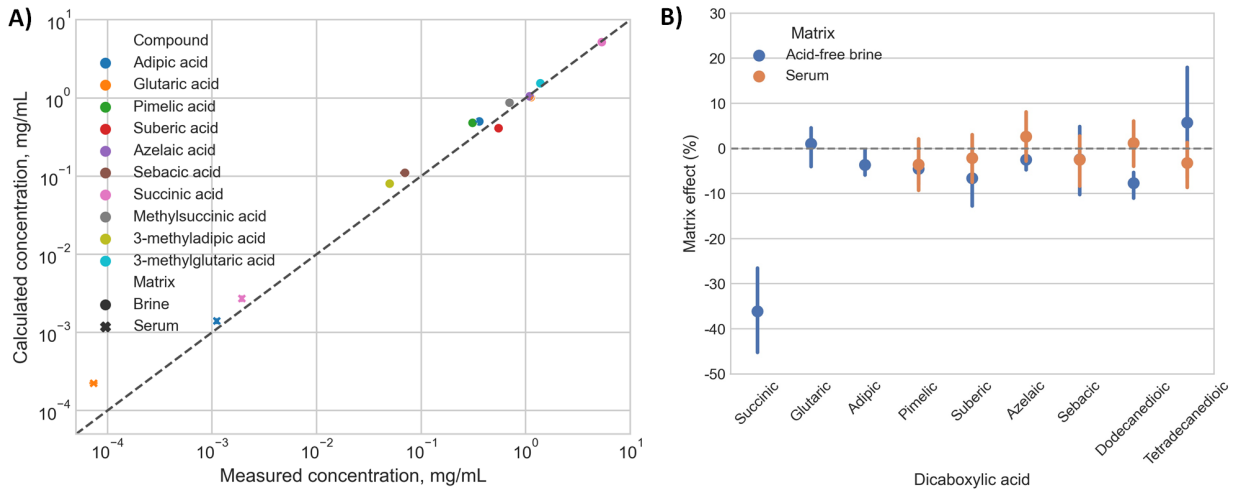

Figure 8: Calculated versus measured concentrations of dicarboxylic acids in brine sample (A) and the scatter plot illustrating the the matrix effect observed in acid-free brine and serum samples (B). The errors bars are smaller than the size of the points. The values can be found in table S5

Conclusions

In this work, the evaluation of the ionization efficiency of DCAs is performed at different concentrations of organics (MeOH), type of mobile phase additive, and pH by means of flow injection analysis and chromatography experiments. It is found that the increase of MeOH

concentration increased the IE values of DCAs. On the opposite, the increase of pH caused the decrease of the IE values. The decrease is due to formation of the ion-pairs between the diacid and cation (ammonium), which was confirmed by the studies using different amines. The investigation of the influence of acidic mobile phase additive on ionization of the DCAs showed that the optimum mobile phase additive that provides an enhance of diacid response should possess both high gas-phase acidity and pK_a value as well being surface active. This phenomenon is explained by the proposed ionization mechanism of dicarboxylic acids, which includes the formation of the diacid-additive adduct during the ionization process. The results of IE studies were used to design liquid chromatography - high-resolution mass spectrometry method, which provided instrumental detection limits of dicarboxylic acids in the range from 6 to 30 pg. The chromatographic resolution between the diacids, α , was ≥ 1.1. An accurate prediction of the IE values for linear and branched dicarboxylic acids is achieved using a model containing the number of carbons in the molecule and MeOH concentration. The developed LC-HRMS "authentic standard-substance-free" method is suitable for the quantification of dicarboxylic acids in complex samples, such as brine and serum, as the influence of the matrix components on diacid response was found to be negligible. This study provides a simple, sensitive, and robust method for screening and quantifying dicarboxylic acids in real-world samples.

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Supporting Information Available

Associated content

- Summary tables containing slopes of calibration curves, R^2 of regression analysis and IE values of the DCAs measured at different concentrations of MeOH, pH, and formic acid
- Additional plots representing the recorded mass spectra, chromatograms, calibration curves of the DCAs obtained by FIA and chromatography experiments and Compound Discover workflow
- IE prediction model errors

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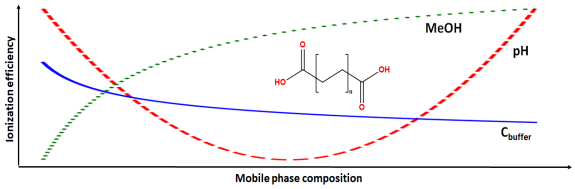

~~Figure 9:~~ The picture shows how different parameters of mobile phase, e.g. MeOH, pH, concentration of the additive affect the ionization efficiency of dicarboxylic acids.