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Plant Metabolomics - Maximizing Metabolome Coverage by Optimizing Mobile Phase Additives for Non-Targeted Mass Spectrometry in Positive and Negative Electrospray Ionization Mode

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ABSTRACT: Non-targeted screening methods with ultra-high-performance liquid chromatography-electrospray ionization/quadrupole-time-of-flight mass spectrometry have been extensively applied to plant metabolomics to very diverse scientific issues in plant metabolomics. In this study different mobile phase additives were tested in order to improve electrospray ionization process and to detect as many metabolites as possible with high peak intensities in positive and negative ionization mode. Influences of modifiers were examined for nonpolar and polar compounds, as optimal conditions are not always the same. By combining different additives, metabolite coverage could be significantly increased. Best results for polar metabolites in positive ionization mode were achieved by using 0.1% acetic acid and 0.1% formic acid in negative ionization mode. For measurements of nonpolar metabolites in positive ionization mode, the application of 10 mmol/L ammonium formate led to best findings, while the use of 0.02% acetic acid was more appropriate in negative ionization mode.

Abbreviations:

DG	Diglycerides
FA	Formic acid
FAA	Fatty acid amides
FFA	Free Fatty acids
HAc	Acetic acid
MG	Monoglycerides
LPA	Lysoglycerophosphate
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPI	Lysoglycerophophoinositol
NH_4Ac	Ammonium acetate
NH_4Cl	Ammonium chloride
NH_4F	Ammonium formate
NH_4Fl	Ammonium fluoride
PA	Glycerophosphate
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Glycerophosphoglycerols
PI	Glycerophophoinositol
TG	Triglycerides

INTRODUCTION

The number of metabolites in a single plant varies, depending on the amount of genes, ranging from 5.000 to 25.000 different compounds[1]. Moreover, the total number of metabolites in the plant kingdom is estimated at 200.000 including various other compounds[2]. From a chemical point of view, metabolites are small molecules (<1.500 Da[3]) of many various substance classes, which can be present in many different concentrations. Due to their high diversity, it is not possible to detect all metabolites by a single analytical method. The higher the resolution and performance of a technology, the more molecules can be registered. This is particularly important for non-targeted metabolomics studies free from hypothesis, as they offer a way to detect simultaneously many other possible metabolites and therefore increase the likelihood of identifying all relevant compounds. Non-targeted metabolomics applications are employed in various fields. In the food sector they enable the proof of authenticity parameters like geographical origin[4], organic cultivation, biological or chemical identity[5,6].

Currently, one of the most widespread analytical platforms for non-targeted metabolomics approaches is liquid chromatography connected with an electrospray ionization quadrupole-time-of-flight-mass spectrometer (LC-qToF-ESI-MS(/MS)). Because of the complexity of plant extracts it is recommended to separate analytes prior to detection to avoid matrix effects and ion suppression as well as splitting up isobars, otherwise some information might be lost. For separation of nonpolar substances, RP-phases are commonly applied, but for investigation of polar compounds, RP-phases are rather unsuitable, since they have poor retention- and separation-capabilities for highly polar analytes. Therefore, numerous alternatives have been developed[7]. Several research groups achieved good results, using aqueous normal phase chromatography (ANP) for the analysis of highly polar compounds[8-11]. In contrast to frequently applied hydrophilic interaction liquid chromatography columns (HILIC), ANP-columns do not require much equilibration time after gradient elution, to obtain reproducible findings, which is important for high-throughput analysis and large batches of measurements. The ANP-column that was applied in this study, is marketed by Microsolv as the Cogent

Diamond Hydride™ column. The stationary phase consists of a silica hydride surface (Type C silica) with less than 2% Si-OH and around 2% of bonded carbon to achieve better peak shapes and to enhance retention of less polar compounds, too[9,10,12-16]. Silica hydride-based columns can be operated in reversed-phase, normal phase and aqueous normal-phase retention mode depending on the amount of water and organic solvents that are added to eluents and properties of analytes.

Electrospray ionization is the preferred ionization source in metabolomics studies and enables the combination of liquid chromatography and mass spectrometry. This technology has been introduced 50 years ago[17,18] and despite frequent application, individual ionization and fragmentation processes are often unknown, therefore fundamental studies are needed. However, some important insights have been gained by several research groups[19-21]. To enhance ionization and MS-sensitivity as well as liquid chromatography separation, different organic solvents and mobile phase additives are applied. The selection of a suitable additive depends on the metabolites that should be detected, mobile and stationary phase as well as ionization mode. Such modifiers need to be soluble in solvents used, volatile to avoid contamination of the ion source and if possible less toxic. Most studies on this issue were carried out for targeted applications by using a limited set of standard compounds in order to better understand electrospray ionization process[21-27]. To the best of our knowledge there are only very few publications, in which the authors compared the influence of different additives for non-targeted metabolomics studies[25,28]. Most of them do not represent numbers of features, peak areas as well as signal intensities of the detected compounds as it is common practice in the evaluation of extraction procedures or column selection[29,30]. Furthermore, none of them refer to plant metabolomics analysis. In the selection of a suitable modifier, there are many different and contradictory data caused by the high number of metabolites, sample matrices, scientific problems and technologies. For this reason, the researchers of this publication wanted to check out, extensive series of tests with different additives which could help to significantly improve the information content of non-targeted plant metabolomics data.

The most widely employed modifier in non-targeted metabolomics is 0.1% formic acid[8,31] which is considered to be the gold standard[32]. In addition, other alternatives such as acetic acid[28], ammonium acetate[33], ammonium formate[34] in various concentrations and at different pH-values have been also applied[35]. In this study pH-values of 3.5 and 6.5 were applied, with some research groups achieving good results in alkaline pH range for some metabolites[21,26,36], but the columns utilized here are not stable under basic conditions. Moreover, with substances like ammonium fluoride[22,37] or ammonium chloride[24] could be achieved with positive deliverables especially in negative ionization mode as well.

EXPERIMENTAL SECTION

Plant Material

White asparagus, the most cultivated vegetable in Germany, was used as plant material, because this study was carried out in the scope of an IGF project, funded by the German Ministry of Economics and Energy for verifying the geographical origin (AiF 18349 N). For this analysis fresh asparagus of the Gijnlim variety from the north of Germany (Spargelhof Schäfer, Wiemersdorf, Schleswig-Holstein), harvested in April 2015, was applied.

Chemicals

Methanol, acetonitrile, isopropanol (LC-MS-grade), ammonium formate (≥95% puriss.) and ammonium acetate (≥97% p.a.) were obtained from Carl Roth GmbH (Karlsruhe, Germany). Formic acid (99% p.a.) and acetic acid (≥99% p.a.) were purchased from Acros Organics (Geel, Belgium). Ammonium fluoride (LC-MS-grade) was purchased from Sigma Aldrich and ammonium chloride (≥99% p.a.) was drawn from Merck (Darmstadt, Germany). Water was purified by using a Merck Millipore water purification system (Direct-Q 3 UV-R system) with a resistance of 18 MΩ (Darmstadt, Germany). All standard substances employed for identification were of analytical grade and purchased from different suppliers.

Sample Preparation

Fresh white asparagus stalks (at least 3 kg) were flash-frozen in liquid nitrogen and stored at -80°C until use. The individual asparagus serve whole were shorten to 15 cm long pieces and after pre-crushing with a ceramic knife, were cut into 5 cm pieces, finely ground together with dry ice at a ratio of 1:1 in a knife mill (GM 300, 140 s, 4000 U/min; Retsch, Haan, Germany). The homogenized pulp was freeze-dried (Christ Alpha 1-4 LD plus, Christ, Osterode, Germany) and applied for solvent extraction.

50 mg of the received powder were weighed into a 2.0 mL reaction tube (Eppendorf, Hamburg, Germany). According to the extraction method of Folch et al.[38], 1 mL of ice cold chloroform/methanol mixture (2:1, v/v) was added for the analysis of nonpolar compounds. Polar substances were separated by addition of 1 mL of ice cold methanol/water mixture (2:1, v/v). Plant cells were disrupted using a ball mill (Bead Ruptor 24, 3 min, 3 m/s, Omni International IM, GA, USA) with two steel beads (3 mm). Upon homogenization and protein precipitation, samples were centrifuged (Sigma 3-16PK, 16000g, 20 min, 4 °C, Sigma, Osterode, Germany), the supernatant liquids were transferred into 1,5 mL vials (Macherey-Nagel, Düren, Germany) and directly used for measurement.

Liquid Chromatography Conditions

Analyses were performed on a Dionex Ultimate 3000 UPLC-system (Dionex, Idstein, Germany), consisting of binary pump system (UltiMate3000RS Pump), degasser (UltiMate3000 degasser), autosampler (UltiMate3000 Autosampler WPS-3000) and temperature controlled column compartment (UltiMate3000 Colum Compartment TCC-3000). For separation of the nonpolar metabolites RP C-18 column (150 mm x 2.1mm, 1.7 µm particles, Phenomenex, Aschaffenburg, Germany) was applied. The column was maintained at 50 °C and the flow rate was 0.3 mL/min. The mobile phases consisted of (A) water and (B) isopropanol/acetonitrile (3:1, v/v) with different modifiers (Supporting Information 1, table S-1). Polar compounds were separated on the Cogent Diamond Hydride column (150 mm x 2.1 mm, 2.2 µm particles, MicroSolv Technology, Leland, NC, USA). The flow rate was 0.4 ml/min and the temperature was kept constant at 50 °C. The mobile phases were (A) water and (B) acetonitrile/methanol (9:1, v/v) with the same different modifiers as shown in Supporting Information 1, table S-1. The gradients are presented in Supporting Information 1, table S-2. Sample volume of 4 µL was injected successively in triplicate and the autosampler temperature was maintained at 5 °C. Instrumental control was operated with Chromeleon software (ver. 6.80, Dionex). For securing reliable and reproducible results, the system was purged extensive-

ly and one blank sample was injected after having switched eluents. Analytical investigations were made with regard to:

I.) non-polar compounds in positive ionization mode

II.) non-polar compounds in negative ionization mode

III.) polar compounds in positive ionization mode

IV.) polar compounds in negative ionization mode

All measurements were performed consecutively over a period of 3-4 days without interruption by other experiments or samples, to ensure the comparability. Buffer solutions (800 mM/L of ammonium formate and ammonium acetate, 80 mM/L of ammonium chloride and ammonium fluoride) were prepared in water and corresponding amounts of those solutions were added to eluents. According to Silvester[26], pH-values were adjusted with formic acid and respectively with acetic acid, prior mixing with mobile phase solvents as by adding organic solvents pH-shifts may occur. This approach is also more reproducible, which can be important for metabolomics experiments. To be able to compare retention times, lost volumes were substituted with water and mixtures of solvents were initially prepared without additives. For preparing eluents with 0.1% and 0.02% formic acid and acetic acid, the required amounts were added directly to solvents and filled up with water. Millipore water was freshly prepared, because sodium ions, dissolved out of glass bottles, can influence the retention process on the Cogent Diamond Hydride column[39] as well as the ionization process. pH-measurements were performed on a Sartorius PB-11 pH-meter, which was calibrated with pH 4 and pH 10 reference solutions directly from prior use.

Mass Spectrometry Conditions

Mass spectrometric detection was employed on a ultra-high resolution quadrupole-time-of-flight mass spectrometer equipped with an electrospray ionization source (maXis, Bruker Daltonics, Bremen, Germany). Data were acquired in a mass range from 80 to 1300 m/z with a spectra rate of 1 Hz. The mass spectrometer was operated in positive and negative ionization modes with following the parameters: end plate offset -500 V, capillary -4500 V (positive mode) / +4500 V (negative mode), nebulizer pressure 4.0 bar, dry gas 9.0 L/min at 200 °C dry temperature. Mass spectrometer calibration was performed externally, using sodium formate clusters with a mixture of formic acid/1 M NaOH in water/isopropanol (0.1:1:100, v/v/v). Additionally, sodium formate cluster solution was automatically injected in the end of each run via a 6-port divert valve to enable internal mass calibration and hexakis(1H,1H,2H-perfluoroethoxy)phosphazene (Santa Cruz Biotechnology Dallas, TX, USA) dissolved in isopropanol at a concentration of 1mg/mL, was used for lock-mass calibration. The system was controlled with Hystar software (ver. 3.2, Bruker) and micrOTOFcontrol software (ver. 3.4, Bruker). MS/MS spectra were performed in both ionization modes with collisions energies from 20 to 60 eV.

Raw data processing and metabolite identification

For data processing DataAnalysis software (ver. 2.1, Bruker) was applied. Raw data were calibrated internally, using sodium formate or sodium acetate clusters, depending on the mobile phase modifiers that were employed. Subsequently, peak finding was conducted by applying the "Find Molecular Features"-algorithm with the following parameters: S/N threshold 2.5, correlation coefficient 0.7, minimum compound length 8 spectra, smoothing width 2. LC-MS-Data from 80 to 1300 m/z were included in a time range from 2.0 min to 18.5 min for polar methods and from 2.0 min to 24.5 min for nonpolar methods. Besides $[M+H]^+$ suspected major adducts like $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$, $[M-H_2O+H]^+$, $[M-CO_2+H]^+$, $[M-NH_3+H]^+$ were additionally included for positive ionization measurements. $[M-H]^-$, $[M-H_2O-H]^-$, $[M+Cl]^-$, $[M+Na-H_2]^-$, $[M+K-H_2]^-$, $[M+HCOOH-H]^-$ and $[M+CH_3COOH-H]^-$ were used for negative ionization measurements. The choice of adducts was based on previous experiments with standard compounds.

Putative sum formulas, exact masses and isotope patterns for metabolite identification were calculated by applying the SmartFormula and the IsotopePattern tool of the Bruker Daltonics software package. Furthermore, structure information from some databases e. g. HMDB[40], METLIN[41], MassBank[42] and LIPID MAPS[43] were also considered as well as MS, MS/MS and retention data were compared with those of reference standards, when they were available (Supporting Information 2).

RESULTS AND DISCUSSION

The influences of 17 different additives compositions in positive and 23 in negative ionization mode were examined, which are commonly applied in LC-MS-based metabolomics studies. Some workgroups have preferred linear gradients or post column infusion approaches to evaluate ionization potential of additives, as solvents can influence ionization behavior, too[28,35,37,44,45]. But it should be noted, that linear gradients can be very time consuming to ensure, that all compounds are eluted completely from the column and therefore they are not suitable for high-throughput-screening experiments. Furthermore, the aim of this work consisted mainly in the development of methods, which are suitable for non-targeted metabolomics measurements and to detect as many metabolites as possible with high intensities to allow a clearly differentiation from signal to noise ratio in a limited time range. We also decided not to apply a post column approach, because the application of modifiers can influence retention behavior and peak shape of analytes, too. An example is shown in Supporting Information 1, Figure S-1. Furthermore, the original matrix and no standard compounds were used to take account of matrix effects, which can cause ion suppression or influence adduct formation.

Study of Polar Compounds in Positive Ionization Mode

An overview about the elution profile of different substance classes on the silica hydride phase is shown in Supporting Information 1, Figure S-2. Initially, effects of different additives based on total peak numbers, total peak intensities and total peak areas were evaluated, which were determined by DataAnalysis software. The results of the detection of polar compounds in positive ionization mode are summarized in (Figure 1).

To gain a deeper insight, different metabolites from various compound classes were examined in detail (Figure 2). For the sake of simplicity, only the peak intensities are shown and discussed here. In the past different working groups employed either peak intensities[23,27,44] or peak areas[26,28,35] in comparable studies. We used peak intensities, because if compounds are only present in low concentrations, a small but height peak allows a better detection (Supporting Information 1, Figure S-1).

The selected compounds were chosen due to their chemical and physical diversities and they should represent as many

analyte classes as possible[46,47]. When it was feasible, two chemically similar substances were analyzed for verifying results. For evaluation of data the most intensive adduct was always used.

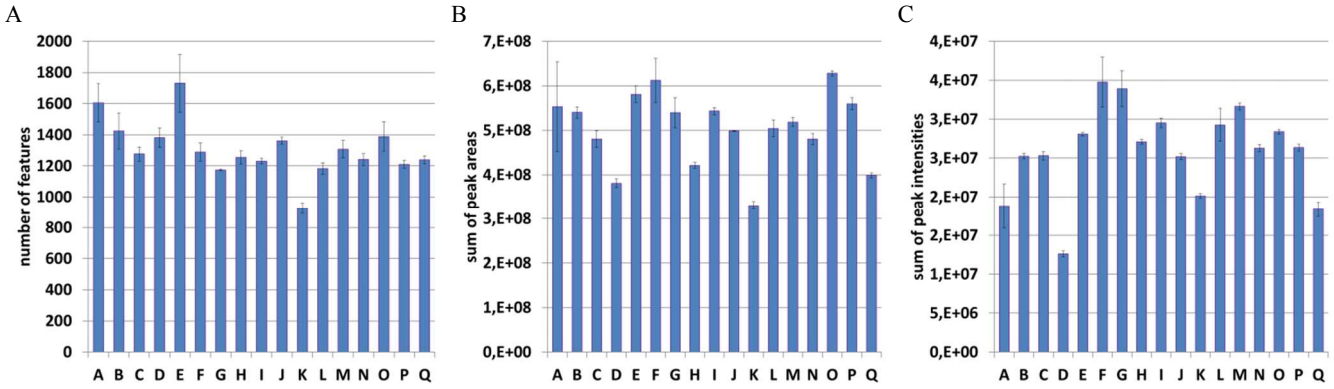

Figure 1. Effect of mobile phase additives on (A) number of detected features, (B) sum of peak areas and (C) sum of intensities for polar metabolites in positive ionization mode. **A,** without additives; **B,** 0.02% FA; **C,** 0.1% FA; **D,** 0.02% HAc; **E,** 0.1% HAc; **F,** 5 mmol/L NH_4F, pH 6.5; **G,** 10 mmol/L NH_4F, pH 6.5; **H,** 20 mmol/L NH_4F, pH 6.5; **I,** 5 mmol/L NH_4F, pH 3.5; **J,** 10 mmol/L NH_4F, pH 3.5; **K,** 20 mmol/L NH_4F, pH 3.5; **L,** 5 mmol/L NH_4Ac, pH 6.5; **M,** 10 mmol/L NH_4Ac, pH 6.5; **N,** 20 mmol/L NH_4Ac, pH 6.5; **O,** 5 mmol/L NH_4Ac, pH 3.5; **P,** 10 mmol/L NH_4Ac, pH 3.5; **Q,** 20 mmol/L NH_4Ac, pH 3.5.

Influence of the pH-value in Positive Ionization Mode on Polar Compounds

There are a lot of different mechanisms that are responsible for the ionization of analytes during the ESI process: electrolytic oxidation or reduction, acid or basic reactions within the electrospray droplets and gas-phase proton transfer as well as the formation of ions in solvents prior to the ESI-process. Generally, it is known, that highest peak intensities can be obtained, when the pH-value of solvents is in a slightly acidic range between 3.5-4.0, because the presence of acids facilitate protonation if analytes are more basic than solvents[28]. Therefore, it is recommended to adjust the pH-value of solvents two units below the pK_a of analytes[48]. However, some compounds do not show this conventional behavior and can be better detected under basic conditions[26], or are not influenced at all by different pH-values[49]. Various studies have shown, that protonated adducts occur in basic conditions as well as deprotonated molecules which can be produced in acidic composition. This effect is defined with the term "wrong-way-round ionization". In this study similar findings were noticed, while some analytes showed higher peak intensities by using acids as additives e.g. most of amino acids, the ionization efficiency of some compounds could not be improved e.g. sugars (Figure 2B).

Further, a relative equal ionization behavior was observed for most compounds that were analyzed in detail by using formic acid or acetic acid in a concentration of 0.1%, except for pantothenic acid, riboflavin and fatty acid amides. The pK_a-value of pantothenic acid is 4.4, whereas the pK_a-value of acetic acid is 4.8 and of formic acid 3.8. Obviously, the acid strength of acetic acid was not sufficient to protonate pantothenic acid. The same characteristics show fatty acid amides, which are weak bases and difficult to protonate, too. An influence of solvent composition could be excluded as the retention times were the same. In contrast, riboflavin should provide similar results by applying acetic or formic acid. However, riboflavin was only present in low concentrations and eluted very early, together with many other compounds, therefore we assumed that matrix effects influenced ionization capacity or, that other chemical or physical parameters were responsible for this behavior. We assumed that the occurrence of matrix effects was also responsible for the different behavior of LPA 16:0 and LPA 18:2, when 0.1% acetic acid was the additive in the solvents.

Influence of Adduct Formation in Positive Ionization Mode on Polar Compounds

Ammonium salts are often used for formation of adducts in ESI-process, because they can significantly enhance signals as they prevent the formation of sodium and potassium adducts, which can cause a reduction in signal intensities and are always present. Moreover, sodium adducts show poor reproducibility and stability, which complicate analyses. It is assumed, that modifiers like ammonium buffers reduce surface tension of the solvent droplets that are formed during the ESI process and thus improve the ionization process. However, in accordance with this hypothesis signals of all analytes should be improved. As shown in Figure 2 many but not all signal intensities increased by adding ammonium salts. This effect became particularly obvious for the sugars. In the case of sucrose, utilization of ammonium salts in concentrations of 5 mmol/L or 10 mmol/L led to significant signal enhancement, whereas the signals of glucose and fructose were only changed slightly (Figure 2 B). The ammonium adducts of sucrose delivered the most intensive signals. In contrast, ammonium adducts of glucose and fructose were also formed, but not as the most intense signals. Here the sodium adducts were much more intensive. Moreover, this effect should be also visible in negative ionization mode, but that was not the case. In compliance with Yang et al. it is more likely, that a gas-phase proton transfer is the cause for signal improvement, which strongly depends on the chemical and physical properties of the analytes as well as concentrations of modifiers[44,50].

Beside the influence of ammonium buffers to ionization process, a change in retention time could also be the relevant factor for improvement of signal intensities, as the use of high concentrations of ammonium salts were expressed in changes of retention times, too (Supporting Information 1, table S-4). The later analytes eluted, the higher the content of water, which is a poor solvent for ionization process, because of its high viscosity and low volatility[35]. However, the effect of

retention time shifts was not quite so pronounced, when ammonium formate at a pH-value of 3.5 was applied. Therefore, the influence of different concentrations of ammonium must predominate.

Ionization capacity of phospholipids (PA, PC, PE, PG, PI) by applying different modifiers were relatively similar, but larger deviations occurred at the ionization behavior of the lyso-forms, especially for the two lysophosphoethanolamines, as ionization efficiency is influenced by the nonpolar rest, too (Figure 2 G-I). There are different models that try to explain this behavior on the basis of pK_a-values, gas phase properties, solution energy, polarity (log P values, which are based on the octanol-water coefficient) or surface[26,45]. Based on these models and on our own findings, we expected that the both lyso-phosphoethanolamines would display the same behavior as well as the phospholipids and their lyso-forms, but this was not the case. As the ionization process is not fully understood yet and many different factors are involved, which are mutually dependent, it can be quite difficult to prognosticate, which modifier is the best one and which effect acts.

If modifier concentration was too high, ion suppression was observed, which could result of a reduced transfer of capillary voltage to solvent and compounds or chemical suppression. Simultaneously, high buffer concentration can also cause contaminations of ionization source and high background interferences.

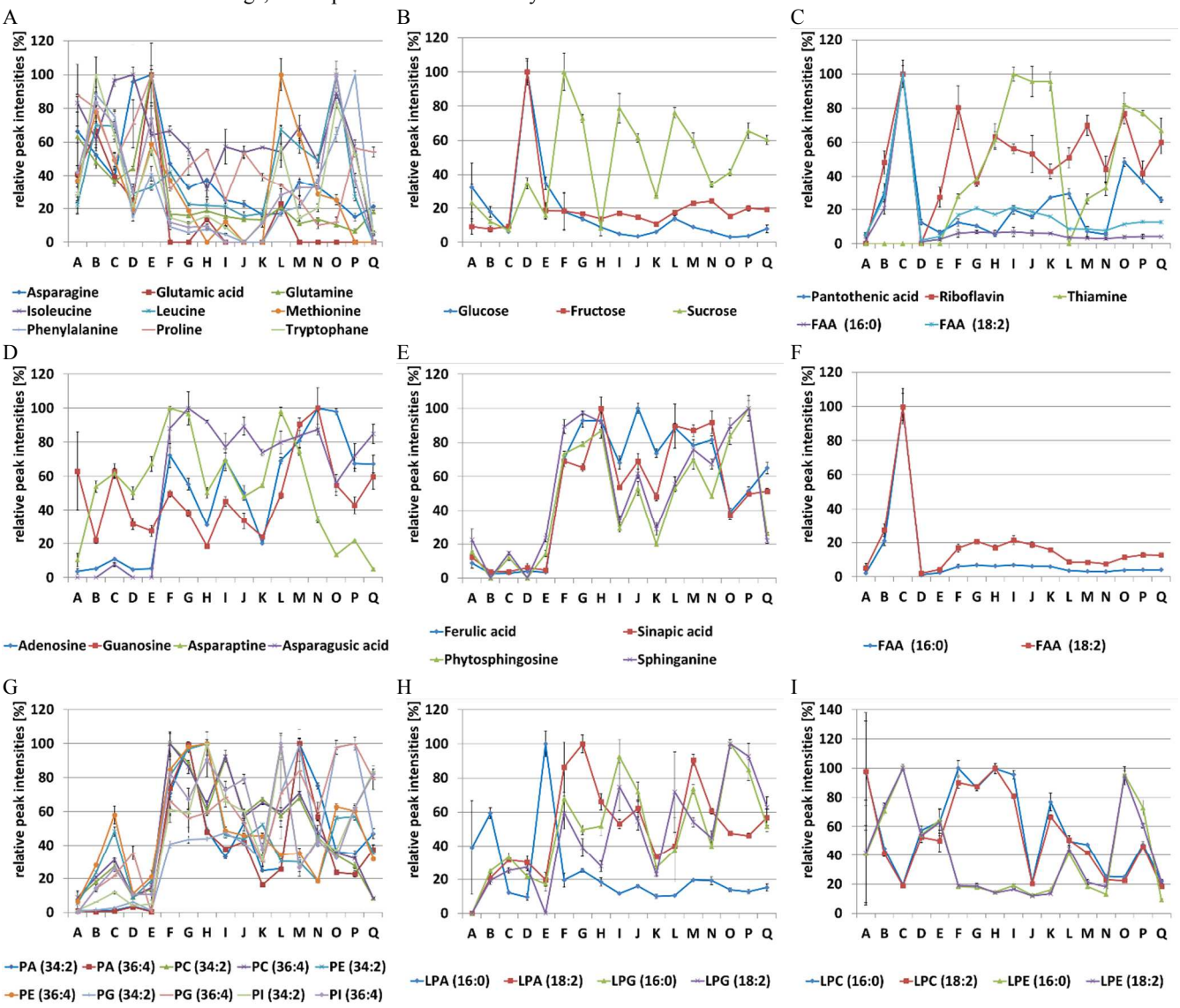

Figure 2. Relative intensities of selected polar compounds by using different solvent modifiers in positive ionization mode. The highest intensity of an analyte was set to 100%, all other intensities were subsequently normalized. **A,** without additives; **B,** 0.02% FA; **C,** 0.1% FA; **D,** 0.02% HAc; **E,** 0.1% HAc; **F,** 5 mmol/L NH_4F, pH 6.5; **G,** 10 mmol/L NH_4F, pH 6.5; **H,** 20 mmol/L NH_4F, pH 6.5; **I,** 5 mmol/L NH_4F, pH 3.5; **J,** 10 mmol/L NH_4F, pH 3.5; **K,** 20 mmol/L NH_4F, pH 3.5; **L,** 5 mmol/L NH_4Ac, pH 6.5; **M,** 10 mmol/L NH_4Ac, pH 6.5; **N,** 20 mmol/L NH_4Ac, pH 6.5; **O,** 5 mmol/L NH_4Ac, pH 3.5; **P,** 10 mmol/L NH_4Ac, pH 3.5; **Q,** 20 mmol/L NH_4Ac, pH 3.5.

Influence of Retention Time on Polar Compounds

As mentioned before and according to different research groups, retention mechanism of silica C phases is not fully understood, but there are various hypotheses[9,15,51-54]. Depending on proportion of organic or aqueous mobile-phase different hydrophilic or hydrophobic mechanisms predominate. In aqueous normal phase mode the order in decreasing solvent strength is water > methanol > isopropanol > acetonitrile[55] and therefore most working groups prefer acetonitrile as organic solvent while working with this column. In our own experiments, only poor results were achieved by using methanol, therefore acetonitrile was preferred. But later a mixture of 90% acetonitrile and 10% methanol as organic phase was used. First methanol should only ensure solubility of buffers, as ammonium acetate is not complete solvable in acetonitrile, but then it became obvious, that the application of small amounts of methanol had a significant influence on the retention mechanisms and much better deliverables could be achieved (Supporting Information 1, Figure S-3). Additional, it is well known that methanol strongly increases ionization compared to acetonitrile. Due to this observation, the solvent composition was maintained and further experiments under this aspect could be helpful to understand the retention mechanism better. The developers of this stationary phase initially assumed, that mainly adsorptive interactions are relevant for the separation mechanism. Probably, the protic effect of methanol has also an influence on the retention mechanisms. These results and further findings suggest, that contrary to expectations also putative additional ionic interactions exist[53,54].

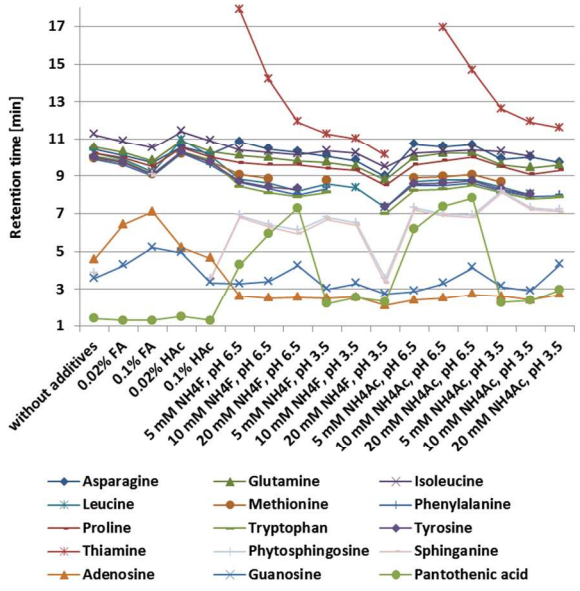

Figure 3. Shifts of retention times, using different additives of selected polar compounds.

By comparing acetic acid and formic acid, the working group of Pesek achieved better retention of amino acids using 0.1% acetic acid in contrast to 0.1% formic acid, too. We obtained the same result (Supporting Information 1, Figure S-4), but vice versa some analytes e.g. the nucleic bases adenosine and guanosine or nonpolar compounds like sphinganine and phytosphingosine were hold back less (Figure 3). Presumably, due to the different pK_a-values of formic acid and acetic acid as well as the chemical properties of compounds, the protonation occur differently and was responsible for this behavior. As described above, addition of ammonium buffer with different concentrations can also influence the retention mechanism on this column. Various effects depending on chemical properties of analytes were achieved. The use of ammonium buffers led to a better retention for some compounds, mainly with many OH-groups e.g. sugars, glycerophosphates, glycerophosphoinositols, glycerophosphoglycerols as well as phytosphingosine and sphinganine or pantothenic acid, but most analytes showed similar or sometimes worse results. Huge differences were observed for thiamine and pantothenic acid, especially at a pH-value of 6.5 (Figure 2). While an increase of the ammonium concentration caused a gain of the retention time of pantothenic acid, the exact opposite was the case for thiamine.

Until now, no studies have been done to evaluate the best concentration of ammonium salts by using this column. Furthermore, the employment of acetate and formate salts, which have different ionic strengths, has not been examined to our knowledge before, but also led to different findings.

Recommendation for the detection of polar compounds in positive ionization mode

Dependent on food materials, metabolite patterns and composition of metaboloms of different species can be quite diverse. Therefore, we recommend a large scale trying different modifiers together with the indicated solvent composition and gradient to ensure the best achievements, because the resulting effects are difficult to prognosticate. However, in many cases it should be sufficient to apply solvent composition with 0.1% acetic acid, as we detected the most substances with this modifier, because the chromatographic retention was more suitable. Most peak intensities were not so intensive but detectable, therefore ion suppression was probably reduced and the competition with analytes, which were present in lower concentration, was less pronounced.

Furthermore, 5 mmol/L ammonium formate at a pH-value of 6.5 should be tried too, as most signal intensities were significantly higher. The acidification of the buffers gave no better findings and is very cumbersome just as reproducible. Moreover, the pH-value is not constant, if solvents are stored for longer periods. Therefore, this practice is not appropriate. An increase of buffer salts is also not necessary as no significant improvements could be detected. Additionally, we noticed salt contaminations of the ESI source, when higher buffer concentrations than 5 mmol/L where used.

Study of polar compounds in negative ionization mode

Generally positive ionization mode is often preferred for non-targeted analysis, because in contrast to negative ionization mode more compounds are ionized. Contrariwise many of small compounds, in particular with anionic groups, are better ionized in negative mode, therefore it can be important, to optimize the negative ionization mode, too.

The evaluation of the data sets in negative ionization mode were carried out analogous to measurements of positive ionization mode by comparing number of features, sum of peak areas and sum of peak intensities (Figure 4). It is obvious, that the use of 0.1% formic acid was superior to all other compositions. This result was clearly confirmed, when the selected compounds were evaluated in detail (Figure 5).

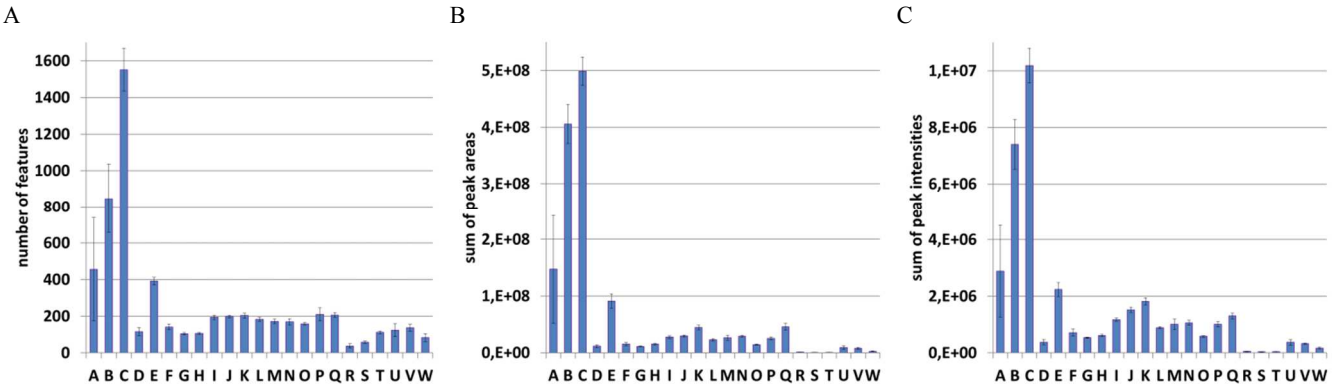

Figure 4. Effect of mobile phase additives on (A) number of detected features, (B) sum of peak areas and (C) sum of intensities for polar metabolites in negative ionization mode. **A,** without additives; **B,** 0.02% FA; **C,** 0.1% FA; **D,** 0.02% HAc; **E,** 0.1% HAc; **F,** 5 mmol/L NH_4F, pH 6.5; **G,** 10 mmol/L NH_4F, pH 6.5; **H,** 20 mmol/L NH_4F, pH 6.5; **I,** 5 mmol/L NH_4F, pH 3.5; **J,** 10 mmol/L NH_4F, pH 3.5; **K,** 20 mmol/L NH_4F, pH 3.5; **L,** 5 mmol/L NH_4Ac, pH 6.5; **M,** 10 mmol/L NH_4Ac, pH 6.5; **N,** 20 mmol/L NH_4Ac, pH 6.5; **O,** 5 mmol/L NH_4Ac, pH 3.5; **P,** 10 mmol/L NH_4Ac, pH 3.5; **Q,** 20 mmol/L NH_4Ac, pH 3.5; **R,** 0.02 mmol/L NH_4Cl; **S,** 1 mmol/L NH_4Cl; **T,** 2 mmol/L NH_4Cl; **U,** 0.02 mmol/L NH_4Fl; **V,** 1 mmol/L NH_4Fl; **W,** 2 mmol/L NH_4Fl.

Influence of the pH-value in negative ionization mode on polar compounds

The "wrong-way-round ionization" effect can also occur in negative ionization mode, as common $[M-H]^-$-ions are also present in acidic solutions, and in the past different results were achieved depending on acid concentrations by analyzing different analyte classes in negative ionization mode[24,56]. It is postulated, that the addition of acids improve the reduction of analytes during the ionization process, as the spray droplets are better able to carry negative charges, and therefore an environment is obtained, that is more appropriate for the reduction process, because during the evaporation the pH-value of electrospray droplets decreases[57-59]. Furthermore the conjugate anion of the acid is also involved within the ionization process, as it carries the negative charge and takes part during gas-phase transfer as well as the molecular volume of the modifier[56]. Our results support this theory as the application of 0.1% formic acid improved signal intensities of almost all analytes. However, different working groups achieved the best deliverables by using lower concentrations of formic acid or acetic acid[28,56,60]. We obtained similar findings but mainly by analyzing nonpolar compounds in negative ionization mode as discussed below. The findings, when 0.02% and 0.1% acetic acid were applied also suggest, that a higher acid concentration should be employed, when mainly polar compounds are to be analyzed.

Some analytes, especially compounds with an acid group, showed better intensities, when no modifiers were used, but then standard deviations were comparable high, which could cause problems in routine analysis. Due to the addition of the acids to the solvent these acid analytes were protonated and fewer molecules were present in their ionic structures. This effect is contrary to the above explained impacts and depends on the properties of analytes[61].

Influence of adduct formation in negative ionization mode on polar compounds

Analog to the positive ionization mode in negative ionization mode, adduct formation can support signal responses, too. Besides formate or acetate adducts, chloride adducts are commonly observed. Some research groups prefer to employ ammonium fluoride as modifier, then analog to chloride, fluoride ions have a higher electronegativity and therefore the formation of $[M-H]^-$ is supported. But chloride and fluoride salts are less volatile compared to ammonium acetate or ammonium formate and can cause contamination of the ion source. Furthermore, exposure to ammonium fluoride must be done carefully due to the potential of the formation of hydrofluoric acid.

In accordance with some other groups relative good signal intensities were achieved regarding the amino acids by using ammonium fluoride[22,37]. For most other compounds only poor signal intensities could be observed (Figure 5) and many compounds hardly could be detected. These findings were different to the results of other working groups, which was probably because the matrices are not directly comparable. The same and even worse results were achieved, when ammonium chloride was employed. Although ammonium chloride and ammonium fluoride were of highest purity, strong noise ratio was observed. That reason could be responsible for these bad performances, too.

The application of ammonium formate and ammonium acetate at a pH-value of 3.5 led for the most compounds to better intensities compared to neutral solvent composition. This effect was not surprising as explained above the acidification caused significant signal improvement. Conversely, this means that the use of ammonium formate or ammonium acetate, which could potentially contaminate the ionization source, is not necessarily needed.

Recommendation for the detection of polar compounds in negative ionization mode

For the detection of polar compounds in negative ionization mode the influence of trying different additives was most clearly and the best results were received unambiguously with 0.1% formic acid. As other groups made different conclusions in the past, we suppose that the solvent composition had a significant influence, which additive should be applied.

Most groups in the metabolomics field prefer the application of the same additives for measurements in positive and negative ionization mode. As shown above and as published just recently[27], it is reasonable to try different additives, because the data quality can be improved in many cases.

Almost all selected analytes that represent the most important polar compound classes, could be detected in negative ionization mode, when using the proposed method together with 0.1% formic acid. Therefore, it should be sufficient to detect the polar metabolites only in negative ionization mode, when measuring time is limited. But for a non-targeted approach it could also be helpful to detect other than the selected compound classes and therefore we recommend using 0.1% acetic acid in positive ionization mode, even if not all the analytes, that were observed in detail, could be detected, too. By the combination of these two different methods the maximum of information should be observable.

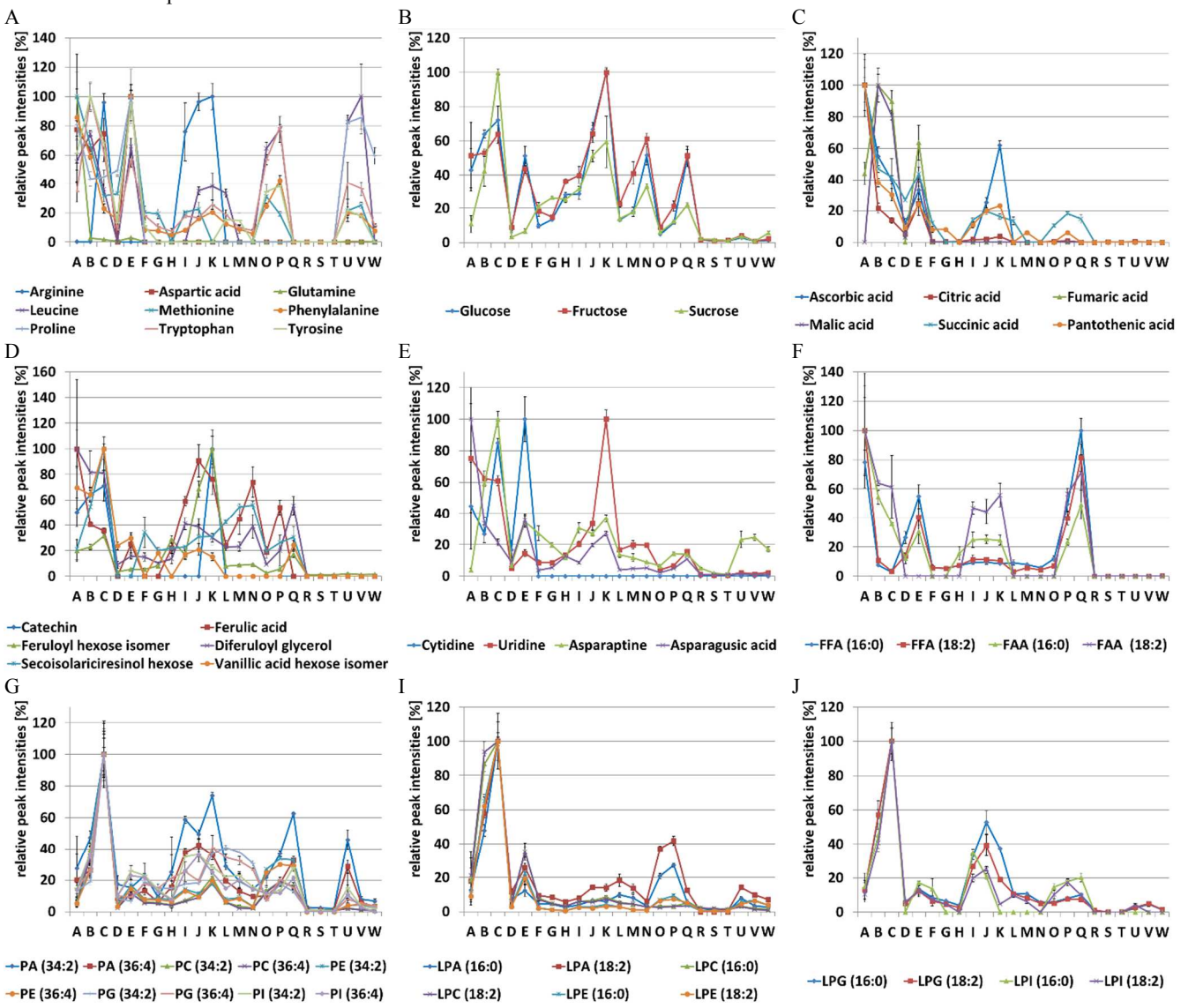

Figure 5. Relative intensities of selected polar compounds by using different solvent modifiers in negative ionization mode. The highest intensity of an analyte was set to 100%, all other intensities were subsequently normalized. **A,** without additives; **B,** 0.02% FA; **C,** 0.1% FA; **D,** 0.02% HAc; **E,** 0.1% HAc; **F,** 5 mmol/L NH_4F, pH 6.5; **G,** 10 mmol/L NH_4F, pH 6.5; **H,** 20 mmol/L NH_4F, pH 6.5; **I,** 5 mmol/L NH_4F, pH 3.5; **J,** 10 mmol/L NH_4F, pH 3.5; **K,** 20 mmol/L NH_4F, pH 3.5; **L,** 5 mmol/L NH_4Ac, pH 6.5; **M,** 10 mmol/L NH_4Ac, pH 6.5; **N,** 20 mmol/L NH_4Ac, pH 6.5; **O,** 5 mmol/L NH_4Ac, pH 3.5; **P,** 10 mmol/L NH_4Ac, pH 3.5; **Q,** 20 mmol/L NH_4Ac, pH 3.5; **R,** 0.02 mmol/L NH_4Cl; **S,** 1 mmol/L NH_4Cl; **T,** 2 mmol/L NH_4Cl; **U,** 0.02 mmol/L NH_4Fl; **V,** 1 mmol/L NH_4Fl; **W,** 2 mmol/L NH_4Fl.

Study of nonpolar compounds in positive ionization mode

Within the scope of analyzing non-polar compounds with a C-18 column and organic solvent compositions, consisting of acetonitrile and propanol, diverse nonpolar analyte classes could be detected (Supporting Information 1, Figure S-5). The chosen classification of the different lipid categories (Supporting Information 1, table S-5) bases on the definition of the International Lipid Classification and Nomenclature Committee as well as in accordance to the LIPID MAPS Structure database[62,63].

Compared to the polar methods significant more nonpolar compounds could be detected, although the fat content of asparagus is only 0.16%[64]. Nevertheless, lipids are highly diverse molecules and many isomers and structures are known. The achieved results of compounds, sum of peak areas and sum of peak intensities are shown in Figure 6. The most

compounds were detected with 0.1% formic acid, but significant higher peak areas and peak intensities were achieved with ammonium salts and especially ammonium formate. Comparable findings of structural similar compounds by a targeted analyses were recently shown by the research group of Fiehn[27].

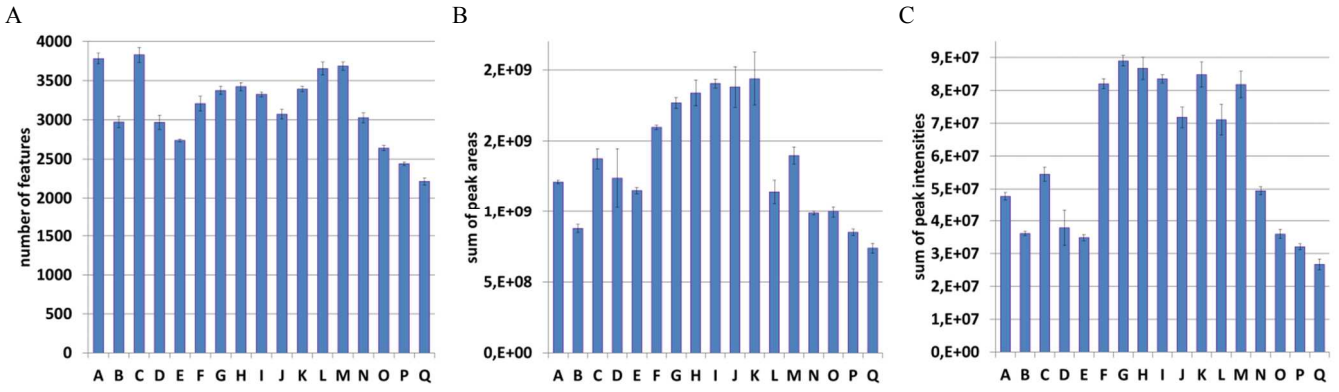

Figure 6. Effect of mobile phase additives on (A) number of detected features, (B) sum of peak areas and (C) sum of intensities for nonpolar metabolites in positive ionization mode. **A,** without additives; **B,** 0.02% FA; **C,** 0.1% FA; **D,** 0.02% HAc; **E,** 0.1% HAc; **F,** 5 mmol/L NH_4F, pH 6.5; **G,** 10 mmol/L NH_4F, pH 6.5; **H,** 20 mmol/L NH_4F, pH 6.5; **I,** 5 mmol/L NH_4F, pH 3.5; **J,** 10 mmol/L NH_4F, pH 3.5; **K,** 20 mmol/L NH_4F, pH 3.5; **L,** 5 mmol/L NH_4Ac, pH 6.5; **M,** 10 mmol/L NH_4Ac, pH 6.5; **N,** 20 mmol/L NH_4Ac, pH 6.5; **O,** 5 mmol/L NH_4Ac, pH 3.5; **P,** 10 mmol/L NH_4Ac, pH 3.5; **Q,** 20 mmol/L NH_4Ac, pH 3.5.

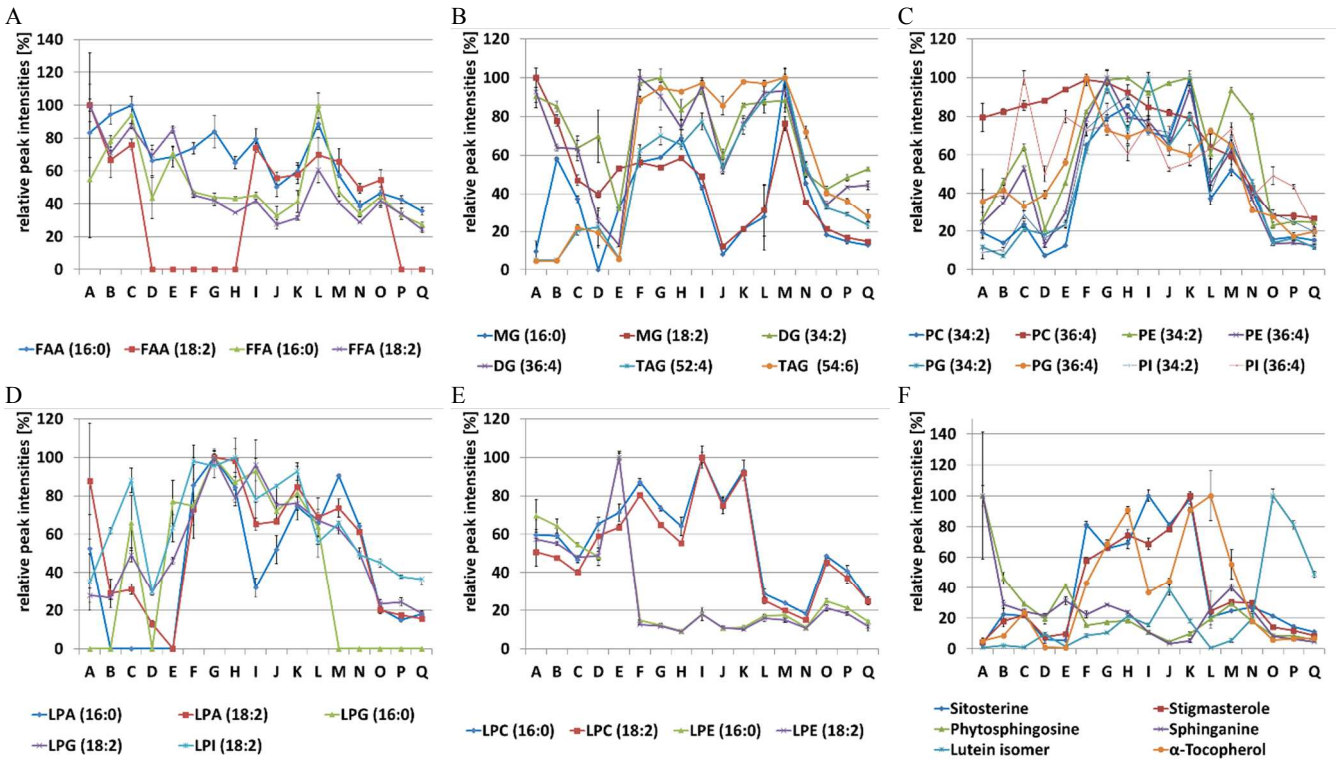

Figure 7. Relative intensities of selected nonpolar compounds by using different solvent modifiers in positive ionization mode. The highest intensity of an analyte was set to 100%, all other intensities were subsequently normalized. **A,** without additives; **B,** 0.02% FA; **C,** 0.1% FA; **D,** 0.02% HAc; **E,** 0.1% HAc; **F,** 5 mmol/L NH_4F, pH 6.5; **G,** 10 mmol/L NH_4F, pH 6.5; **H,** 20 mmol/L NH_4F, pH 6.5; **I,** 5 mmol/L NH_4F, pH 3.5; **J,** 10 mmol/L NH_4F, pH 3.5; **K,** 20 mmol/L NH_4F, pH 3.5; **L,** 5 mmol/L NH_4Ac, pH 6.5; **M,** 10 mmol/L NH_4Ac, pH 6.5; **N,** 20 mmol/L NH_4Ac, pH 6.5; **O,** 5 mmol/L NH_4Ac, pH 3.5; **P,** 10 mmol/L NH_4Ac, pH 3.5; **Q,** 20 mmol/L NH_4Ac, pH 3.5.

Influence of the pH-value in positive ionization mode on nonpolar compounds

Generally, compounds that could be detected with the polar and with the nonpolar method in positive ionization modes, like some phospholipids and lysophospholipids showed a relative similar ionization behavior (Figure 2 and Figure 7). In contrast to the experiments for the detection of polar compounds, the most of nonpolar metabolites showed a lower or relatively weak ionization efficiency, when acids were used as modifier compared to the complete absence of additives. This effect became especially evident by the evaluation of glycerides and is due to

the fact, that all glycerides formed sodium or ammonium adducts. Whereas, for example the most intensive signals of the phospholipids and lysophospholipids were dependent on the formation of $[M+H]^+$-adducts.

Influence of adduct formation in positive ionization mode on nonpolar compounds

As shown in Figure 7, peak intensities of the selected compounds were better for almost all compounds, when ammonium salts were chosen.

For the most nonpolar metabolites, differences by using ammonium salts with various pH-values or different concentrations were less pronounced in contrast to the polar substances, because the chemical structures of nonpolar metabolites are more similar and the most of them do not change their charge state due to the absence of polar groups. Obviously, it is better to take a moderate buffer concentration, as signal intensities become lower, which was also recognized by analyzing polar metabolites.

Influence of retention time on nonpolar compounds

For the separation of nonpolar compounds, a C-18 column with 1.7 µm particles was used. Columns packed with sub-2 µm particles produce high backpressures, a factor that requires an UHPLC system, which can overcome backpressures up to 1.000 bar. But at the same time these columns enable a higher number of theoretical plates and therefore a better separation, shorter analysis time and higher peak capacities as well as superior peak sensitivities[36]. Furthermore, high backpressures can be reduced via using core-shell-particles[65]. Compounds eluted, as expected, according to their chain length and lipophilicity. Unsaturated substances left the column before the saturated agents. Compared with the polar method retentions time shifts were less pronounced, except for phytosphingosine and sphinganine, too (Supporting Information 1, Figure S-6).

Recommendation for the detection of nonpolar compounds in positive ionization mode

Summarized, all relevant lipid classes could be detected with relatively good peak intensities in positive ionization mode, when ammonium salts were used. For the most analytes observed in detail, ammonium formate delivered better results than ammonium acetate. Furthermore, it is not necessary to adjust an acidic pH-value, although the use of ammonium formate at a pH-value of 3.5 led to a slightly decrease in relation to peak intensities. These findings are in accordance to previous publications[23,27]. Even though the most compounds were detected by using 0.1% formic acid we therefore recommend employing 10 mmol/L ammonium formate as the signal intensities and peak areas were considerable to be better.

Study of nonpolar compounds in negative ionization mode

In contrary to the findings of the measurements of polar substances in negative ionization mode, most compounds with relative good peak intensities and areas were detected, when acids in lower concentrations were employed (Figure 8). That peak intensities and areas by using ammonium acetate and ammonium fluoride were sometimes comparatively higher, was owed to the fact that the use of these additives led to a higher signal to noise ratio, which was not observed in positive ionization mode and the polar methods (Supporting Information 1, Figure S-7). Nevertheless, by using the negative mode some saponins could be detected additionally, but compared to the other methods, in total far fewer compounds could be recognized. **Influence of the pH value in negative ionization mode on nonpolar compounds**

Following the explanations in the previous sections many compounds could be better detected in an acidic environment at negative ionization mode (Figure 9). However, some compounds, particularly with regard to the anionic lysophospholipids like LPA, LPG and LPI, could not be detected at all, respectively these signals were too small. Usually, these compounds should be easily detectable in negative ionization mode by using small amounts of acetic acid or ammonium salt buffers[23]. Probably, the concentrations of these analytes in the sample matrix were just too low and compared to the results of the polar measurements in negative ionization mode, there is also an effect of organic solvent composition and the water ratio, which also could be responsible for these different findings. For estimating those influences, we also recommend confirming such experiments with the final LC-MS-method and the original matrix.

Influence of adduct formation in negative ionization mode on nonpolar compounds

The use of ammonium salts led to various indications. While some compounds showed an increase of ionization efficiency e.g. fatty acids, PCs and saponins, other analytes like the anionic phospholipids PA, PG and PI were rather worse ionized. In comparison with the findings of the polar method comparable results were realized for compounds that could be detected with both methods. It seems that the application of ammonium acetate is slightly better than ammonium formate for the most compounds. However, a lot of these substance e.g. fatty acids or saponins did not form intensive acetate adducts and rather the $[M-H]^-$-adducts were the most intense products.

Recommendation for the detection of nonpolar compounds in negative ionization mode

Based on our findings that the major nonpolar analyte classes could better detected in positive ionization mode, we recommend selecting the negative method according to other criteria, because it should not be necessary to recognize the same metabolite twice. Therefore, the use of 0.02% acetic acid should be the best alternative as the most compounds could be detected, when this composition was chosen.

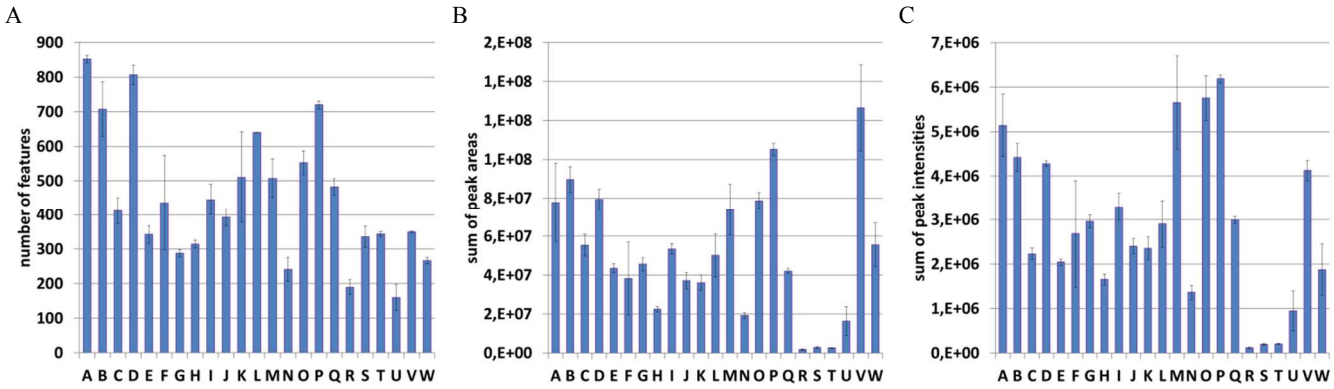

Figure 8. Effect of mobile phase additives on (A) number of detected features, (B) sum of peak areas and (C) sum of intensities for nonpolar metabolites in negative ionization mode. **A,** without additives; **B,** 0.02% FA; **C,** 0.1% FA; **D,** 0.02% HAc; **E,** 0.1% HAc; **F,** 5 mmol/L NH_4F, pH 6.5; **G,** 10 mmol/L NH_4F, pH 6.5; **H,** 20 mmol/L NH_4F, pH 6.5; **I,** 5 mmol/L NH_4F, pH 3.5; **J,** 10 mmol/L NH_4F, pH 3.5; **K,** 20 mmol/L NH_4F, pH 3.5; **L,** 5 mmol/L NH_4Ac, pH 6.5; **M,** 10 mmol/L NH_4Ac, pH 6.5; **N,** 20 mmol/L NH_4Ac, pH 6.5; **O,** 5 mmol/L NH_4Ac, pH 3.5; **P,** 10 mmol/L NH_4Ac, pH 3.5; **Q,** 20 mmol/L NH_4Ac, pH 3.5; **R,** 0.02 mmol/L NH_4Cl; **S,** 1 mmol/L NH_4Cl; **T,** 2 mmol/L NH_4Cl; **U,** 0.02 mmol/L NH_4Fl; **V,** 1 mmol/L NH_4Fl; **W,** 2 mmol/L NH_4Fl.

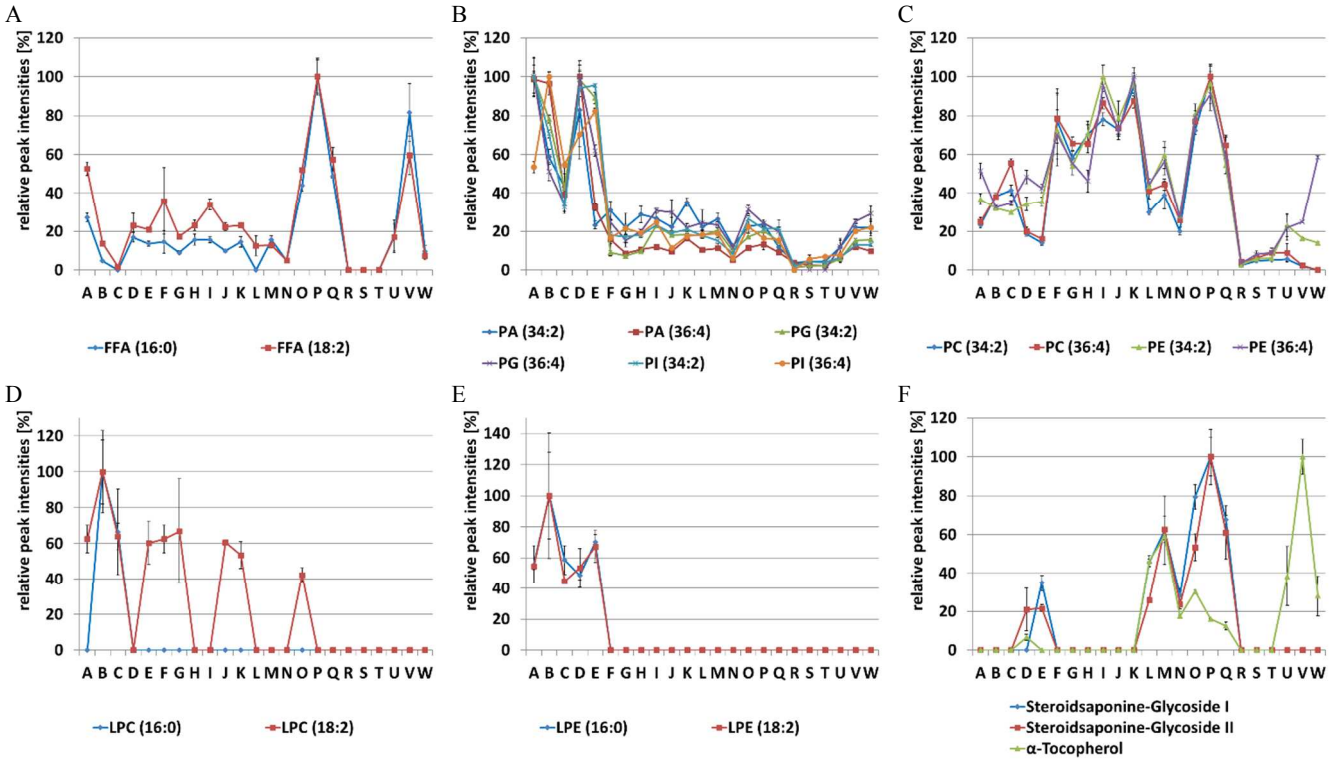

Figure 9. Relative intensities of selected nonpolar compounds by using different solvent modifiers in negative ionization mode. The highest intensity of an analyte was set to 100%, all other intensities were subsequently normalized. **A,** without additives; **B,** 0.02% FA; **C,** 0.1% FA; **D,** 0.02% HAc; **E,** 0.1% HAc; **F,** 5 mmol/L NH_4F, pH 6.5; **G,** 10 mmol/L NH_4F, pH 6.5; **H,** 20 mmol/L NH_4F, pH 6.5; **I,** 5 mmol/L NH_4F, pH 3.5; **J,** 10 mmol/L NH_4F, pH 3.5; **K,** 20 mmol/L NH_4F, pH 3.5; **L,** 5 mmol/L NH_4Ac, pH 6.5; **M,** 10 mmol/L NH_4Ac, pH 6.5; **N,** 20 mmol/L NH_4Ac, pH 6.5; **O,** 5 mmol/L NH_4Ac, pH 3.5; **P,** 10 mmol/L NH_4Ac, pH 3.5; **Q,** 20 mmol/L NH_4Ac, pH 3.5; **R,** 0.02 mmol/L NH_4Cl; **S,** 1 mmol/L NH_4Cl; **T,** 2 mmol/L NH_4Cl; **U,** 0.02 mmol/L NH_4Fl; **V,** 1 mmol/L NH_4Fl; **W,** 2 mmol/L NH_4Fl.

CONCLUSIONS

An extensive evaluation of commonly different used mobile phase additives was performed to maximize data quality of non-targeted LC-MS metabolomics approaches. The received results in table 1 demonstrate, that no single solvent modifier was appropriate to give the best performances, but an extensive validation can improve significantly data quality and hence also information content. Furthermore, a skillful combination of different modifiers for the various ionization modes is able to extend the analytic spectrum that can be detected as it should not be necessary to measure the same analyte twice. Electrospray ionization process is not fully understood at the moment; therefore, it is especially difficult for non-targeted approaches to prognosticate, which modifier is the best one, and therefore we recommend checking the best results, which were achieved in this study.

It has advantages to use the same modifiers in positive and negative ionization mode, as compound identification is facilitated as well as manufacturing of mobile phase. But due to the achieved results we recommend using different modifiers within different ionization modes and to measure some quality control samples consisting of aliquots of all samples in the other ionization mode (MS and MS/MS) for saving time and efforts, but without having a loss of information as the complementary ionization mode can deliver additional information, which can be also important for compound identification.

Table 1. Summary of the best results that could be achieved in this study.

Method	Best modifier in this study
I.) nonpolar metabolites in positive ionization mode	10 mmol/L ammonium formate, pH-value of 6.5
II.) nonpolar metabolites in negative ionization mode	0.02% acetic acid
III.) polar metabolites in positive ionization mode	0.1% acetic acid respectively 5 mmol/L ammonium formate at a pH-value of 6.5
IV.) polar metabolites in negative ionization mode	0.1% formic acid

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supporting Information 1: PDF-file with further tables and figures for a more detailed description.

Supporting Information 2: XSLX-file with details of exact masses and fragments of compounds, which were analyzed in this study.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript

Notes

The authors declare no competing financial interest.

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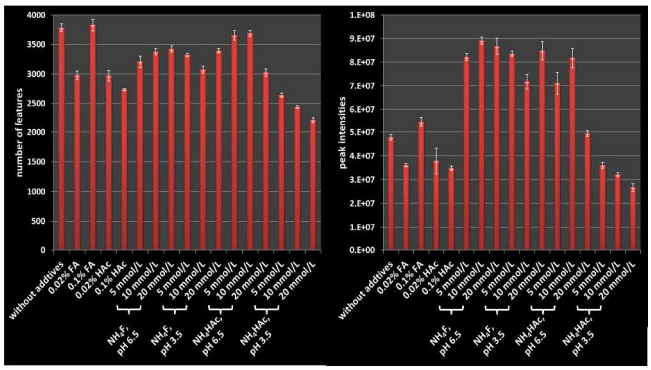
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without additives
0.02% FA
0.1% FA
0.02% HAC
0.1% HAC
5 mmol/L
10 mmol/L
20 mmol/L
NH4F, pH 6.5
NH4F, pH 3.5
NH4HAC, pH 6.5
NH4HAC, pH 3.5