Version of accepted author manuscript, published in final form in
Organic Geochemistry, 59, 10-21 doi: 10.1016/j.orggeochem.2013.03.004

Application of two new LC-ESI-MS methods for improved detection of intact polar lipids (IPLs) in environmental samples

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ABSTRACT

Intact polar lipid distributions have become a valuable tool for the study of microbial ecosystems. In order to expand the detection and interpretation of the presence of these lipids, improved analytical methods are needed. Therefore, two high pressure liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS^2) methods, based on hydrophilic interaction chromatography (HILIC) and reversed phase (RP) chromatography were developed, taking advantage of new chromatographic possibilities such as smaller particle size and recently developed column fillings. Both were optimized to cover the broad range of compounds found in environmental samples and to cope with the associated complex sample matrices. The capabilities of the resulting methods were tested on pure standards and an environmental sample. Both methods offer improved peak resolution and detection limit, and reduced chromatographic background at twofold shorter run time compared with the previous method based on a diol column. The HILIC method offers separation according to lipid class similar to a diol column, and can thus be recommended for lipid fingerprinting. The method based on RP separation offers the unique possibility of analyzing intact polar lipids and core lipids in the same chromatographic run and an alternative mode of lipid separation based mainly on side chain structure. This method is especially suitable for separation of compounds based on side chain length, degree of saturation and/or presence of acyl/ether bonds. The combination of both newly developed chromatographic methods provides a powerful tool for the analysis of lipid distributions in environmental samples at ultra-low concentration.

Keywords: intact polar lipid, reversed phase, HILIC, biomarker, complex matrix

1. Introduction

Phospho- and glycolipids are the main constituents of the lipid bilayer of cell membranes (e.g. Finean et al., 1984; Madigan and Martinko, 2006). The majority of membrane lipids are glycerol-based, with two hydrophobic chains connected to the glycerol backbone via ester or ether bonds and a polar head group. Bacterial and eukaryotic cell membranes typically comprise lipid bilayers consisting of two fatty acids (FAs) esterified to the glycerol backbone (diacylglycerol, DAG) and a phosphate- or glyco-based polar head group. Some bacteria, such as sulfate reducing bacteria contain mixed ether-acyl (acyletherglycerol, AEG) or diether (dietherglycerol, DEG) membrane core lipids (e.g. Rütters et al., 2001; Sturt et al., 2004). In contrast, archaeal membranes consist of either bilayer or monolayer structures with isoprenoid alcohols connected via ether bonds to the glycerol and polar head groups generally resembling those of their bacterial and eukaryotic counterparts. The isoprenoid core lipid structures exist either as dialkyl diethers or as membrane-spanning tetraethers with four ether bonds and varying number of rings in the two biphytanyl chains (e.g. De Rosa et al., 1986; Koga et al., 1993). The specialization of microorganisms with respect to the wide range of processes in which membranes are involved has resulted in large lipid diversity (Dowhan, 1997). This diversity of core lipids and polar head groups has been successfully employed to correlate lipid structure with source organisms, biological processes and environmental conditions (e.g. Nichols and Wood, 1968; Imhoff, 1991; Murata and Siegenthaler, 1998; Rütters et al., 2001; Koga and Morii., 2005; van Mooy et al., 2009; Schubotz et al., 2009; Lanekoff and Karlsson 2010) and qualifies intact polar lipids (IPLs) as excellent biomarkers. In the marine sciences, such biomarker analysis has been widely and successfully used. Water column analysis, for example, made use of IPLs to study microbial biomass composition in the Black Sea (Schubotz et al., 2009) or to evidence a correlation with phosphate availability in the Mediterranean Sea (Popendorf et al., 2011). IPL analysis of sediments is hindered by

the complex matrix from which lipids are extracted but nevertheless has become a valuable tool for the study of the deep subseafloor biosphere. IPLs have contributed to the analysis of microbial communities in shallow sediments (Rütters et al., 2002), shown evidence for microbial biomass in deep subsurface sediments (Zink et al., 2003; Sturt et al., 2004; Biddle et al., 2006; Lipp et al., 2008), and characterized the microbial community associated with petroleum degradation within asphalts and surrounding oil-impregnated sediments (Schubotz et al., 2011).

IPLs have traditionally been analyzed using thin layer chromatography (TLC; Myher and Kuksis, 1995) or by analysis of their apolar derivatives as breakdown products in the environment or as products artificially prepared in the laboratory via chemical degradation [e.g. phospholipid-derived FAs (PLFAs); White and Findlay, 1988; Asselineau and Asselineau, 1990]. Recent advances now allow analysis of the membrane lipids in their intact form as intact polar lipids. Using high-performance liquid chromatography-mass spectrometry (HPLC-MS) we can now explore the full potential of the lipid inventory, including the valuable taxonomic information within the polar head group.

Reversed phase (RP) chromatography is the most widely employed method for HPLC, but most analyses of intact polar lipids in complex environmental samples are still carried out using normal phase (NP) chromatography with pure or diol-modified silica gel (Lutzke and Braughler, 1990; Olsson et al., 1996). NP chromatography has the advantage of a separation pattern according to polar head group polarity into lipid classes, similar to TLC. In the case of microbial lipids used as biomarkers in environmental samples, possibly the most common methods are based on diol columns with eluents consisting of hexane, isopropanol (IPA) and water (Karlsson et al., 1998; Rütters et al., 2001). These chromatographic protocols have only been slightly modified during the last decade. Modifications include different buffer composition (e.g., Sturt et al., 2004), longer columns (Boumann et al., 2006), higher flow rate

(Logemann et al., 2011), and polyvinyl alcohol (PVA) column packing material (Van Mooy and Fredricks, 2010), or combinations thereof.

Even though method development for RP separation began early, with Kaufmann and Olsson (1993) for example optimizing phosphatidylcholine (PC) and phosphatidylethanolamine (PE) separation, RP has usually remained in use only when very specific goals are pursued. In contrast to NP chromatography, RP separation provides orthogonal selectivity based on chain length and hydrophobicity and thereby complementary separation, allowing more precise characterization of compounds inside a lipid class (e.g., Sommer et al., 2006; Lanekoff and Karlsson, 2010; Willmann et al., 2011).

Another chromatographic system that has been used for lipid analysis is based on hydrophilic interaction liquid chromatography (HILIC). This is a relatively recent development, and is based on liquid-liquid partitioning between an organic solvent (typically acetonitrile, MeCN) and a polar solvent associated with the polar stationary phase (Alpert, 1990). An advantage of HILIC is the use of polar solvents that are more adequate for electrospray ionization (ESI) than the apolar solvents typically used for NP and should lead to more stable ES conditions and higher ionization efficiency. Initial HILIC attempts, mainly in biomedical sciences, were performed to detect IPLs: Fischbeck et al. (2009) were able to analyze sphingolipids in meat, Schwalbe-Herrmann et al. (2010) achieved separation of five lipid classes in human blood plasma, Zheng et al. (2010) employed HILIC to allow lipid profiling in the parasite *Leishmania donovani* and Zhu et al. (2012) were able to separate phospholipid classes in plasma.

The availability of ultra-high pressure liquid chromatography (UHPLC) with columns filled with particles < 2µm, combined with fast MS scanning, has recently enabled new possibilities for chromatographic separation. Smaller particles are designed to deliver increased resolving power, even at high flow rate. Thus, better peak resolution, sensitivity and analysis time may

be expected in comparison with the common 3 or 5 µm particle size (e.g., Churchwell et al., 2005). In both the HILIC and RP phase methods we tested stationary phases based on ethylene-bridged hybrid particles (BEH). In BEH columns, ethylene-bridged groups in the silica matrix are responsible for reducing the number of silanol groups and thus modify retention behavior. Furthermore, these columns have proven increased stability towards pH and temperature (O´Gara and Wyndham, 2006; Grumbach et al. 2008). We tested 1.7 µm particle size BEH columns that can be used at high pressure of up to 1000 bar.

Although important analytical improvements have been achieved for lipid analysis, for example in life sciences, these developments have not been transferred to environmental samples with their highly complex sample matrices in which only trace amounts of lipids are present. Also, the applicability of such methods for the detection of the wide range of lipid structures covering small to large molecules with a wide spectrum of polarity has not been tested systematically. The present study concentrated on development and evaluation of new state-of-the-art methods employing both RP and HILIC separation with high efficiency columns, taking into account the typical range of lipid structures found in marine sediments and their special sample matrices. The methods had to provide good chromatographic separation, proper peak shape and good response factor for a wide range of IPLs of different origin, as well as chain and head group characteristics.

2. Material and methods

2.1. Analytical equipment and reagents

Optimization of chromatographic separation was carried out with a Dionex Ultimate 3000RS UHPLC instrument connected via an ESI source to a Bruker maXis high resolution quadrupole time-of-flight mass spectrometer (Q-TOF). Five different RP columns were tested: RP1 (Agilent Eclipse XDB-C_{18}; Agilent Technologies, Böblingen, Germany), RP2

(Nucleodur C_{18} ISIS; Macherey and Nagel, Düren, Germany), RP3 (ACE3 C_{18}; MZ Analysentechnik, Mainz, Germany), RP4 (Waters XBridge C_{18}) and RP5 (Waters Acquity UPLC BEH C_{18}), both Waters Corporation, Eschborn, Germany. For HILIC separation two columns were tested: H1 (Waters Acquity UPLC BEH HILIC) and H2 (Waters Acquity UPLC BEH Amide), both Waters Corporation, Eschborn, Germany. The H1 column is specifically designed for HILIC separations and offers high chemical stability. The H2 column shares the same principles, but the amide groups bound to the BEH substrate supply additional retention mechanisms. The H2 column is also characterized by a wide pH and temperature range. All columns were 2.1 x 150 mm and particle size was 3.5 µm for RP1 and RP4, 3 µm for RP2 and RP3, and 1.7 µm for RP5, H1 and H2. Optimized methods were compared to the method of Sturt et al. (2004) using a LiChrospher 100 Diol column (5 µm, 2.1 x 150 mm, Grace Davison Discovery Sciences-Alltech Grom GmbH, Worms, Germany).

Standards for IPLs were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). For the optimization of chromatographic methods, a mixture of six compounds, including different polar head groups, side chain length and unsaturation, and core structure type was chosen. In the diacylglycerolipid (DAG) group, chain length ranged from C_{16} [combined with (N)-methylphosphatidylethanolamine; C_{16}-PME] to C_{21} (in combination with a phosphocholine head group; C_{21}-PC). Additionally two archaeal lipids were used: a dietherglycerolipid (DEG) with a phosphatidylethanolamine head group (PE-AR; archaeol, AR) and a glycerol dialkyl glycerol tetraether (GDGT) with a phosphatidylglycerol (PG) on one side and a monoglycosidic (1G) head group on the other side of the core lipid (1G-GDGT-PG; glycerol dialkyl glyceroltetraether, GDGT). A sphingolipid, a C_{18} monoglucosyl ceramide (1Glc-Cer), and C_{16}-PAF (platelet activation factor), a phospholipid with an ether-linked C_{16} alkyl, a C_2 acyl chain and a PC head group were chosen to complete the mixture. For evaluation of the final methods, 1,2-dihexadecanoyl-sn-glycero-3-phosphatidic acid (C_{16}-

PA) and 1,2-dihexadecanoyl-sn-glycero-3-phosphoserine (C_{16}-PS) were also included (see appendix 1 for details)

LC-MS grade methanol (MeOH), dichloromethane (DCM) and MeCN were obtained from Merck Chemicals (Darmstadt, Germany). IPA, HCO_2H (98%) and NH_4OH (>25% NH_3) were purchased from Sigma Aldrich (Steinheim, Germany). Tetrahydrofuran (THF) was obtained from Carl Roth (Karlsruhe, Germany). Both IPA and deionized water were additionally purified via distillation.

2.2. RP chromatography

Following results from Lanekoff and Karlsson (2010) and Lin et al. (2012), RP separation was based on a gradient from 100% MeOH to 100% IPA, with addition of HCO_2H and NH_3 buffer. The initial buffer concentration was as described in Sturt et al. (2004) for the diol method (0.12% HCO_2H, 0.04% NH_3). A 45-min gradient with a flow rate of 200 µl min^{-1} was chosen for the columns with 3 or 3.5 µm particle size, and a 20-min gradient with a flow rate of 200 µl min^{-1} for RP5. A first step was column selection with respect to peak shape of injected standards, paying special attention to tailing of amine-bearing compounds. Tailing factor (T) at 5% height was calculated according to the US Pharmacopeia (USP) as T = (a+b)/2a, where a is the distance from the peak leading edge to the midpoint and b the distance from the point at peak midpoint to the trailing edge. Tailing is thus expressed by T > 1.

Once an optimal column was chosen, change in solvent composition was evaluated. In order to change retention behavior of the compounds of interest, addition of water to MeOH as eluent A (1, 5, 10, 15, 20 and 30%) and of MeOH and MeCN to IPA as eluent B (10, 25, 50%) was tested. Method development further included optimizing buffer strength and composition and choosing proper injection solvents. Ionization and MS response were tested

for different ratios of HCO_2H to NH_3 (1:2.5, 1:1, 2:1, 3:1, 10:1) and buffer strength (0.02% HCO_2H, 0.05% NH_3 and 2x and 5x increased concentration).

Environmental samples demand strong solvents for complete dissolution. Samples are therefore often extracted and/or re-suspended in DCM or similar solvent. However, if the injection solvent is much stronger than the eluent into which it is injected, chromatographic issues such as split peaks may occur. Therefore, different combinations of DCM and MeOH (5:1, 1:1, 1:5, 1:10, pure MeOH) were tested to define the optimal injection solvent. Finally, the impact of temperature in the range 20 to 65°C was also evaluated.

2.3. HILIC

A preliminary gradient was developed to compare the retention capacity of two different columns: H1 and H2. Comparison focused mainly on the retention of lipids with less polar head groups. For eluent selection, the initial setup was based on the findings of Schwalbe-Herrmann et al. (2010), who proposed isocratic elution with an eluent of MeCN, MeOH and ammonium acetate buffer (55:35:10). As results were not satisfactory with this method, implementation of gradient methods was assessed, which would allow us to start with higher amounts of MeCN and to gradually replace the aqueous phase with IPA and MeOH to improve retention. Additionally, partial substitution of MeCN with other aprotic solvents (THF and DCM) was tested to improve chromatographic and ionization behavior.

Optimization of buffer strength and pH was performed by testing the effects of different buffer combinations added to the eluent on separation of IPL standard mixtures. The first set of experiments tested HCO_2H:NH_3 ratio (v:v) of 10:1, 3:1, 2:1, 1:1 and 1:2.5 added to both eluent A and B with constant ionic strength. After the most suitable buffer ratio was selected, the ionic strength was optimized (0.02% HCO_2H, 0.02% NH_3 and 2x, 3x, 4x and 5x increased buffer concentration). A second set of experiments dealt with asymmetric buffer

ratio and concentration in eluent A and B. Buffer concentration was chosen such that it was much higher in the more polar, protic eluent B. Four buffer combinations were chosen, which at 10% of eluent B would result in HCO_2H:NH_3 ratio values (v:v) of ca. 4:1, 1:1, 1:2.5 and 1:10.

Making use of the wide temperature tolerance of the amide column, we also compared chromatographic performance at 20, 30, 40 60 and 90 °C. Also, different injection solvents were tested, including DCM:MeOH (5:1) and (9:1) and mobile phase A.

Fig. 1 shows an overview of the method development, including most important results and relevant criteria applied.

2.4. ESI and MS optimization

Once chromatographic methods were developed, conditions for ionization and MS detection were optimized by infusion of IPL standards with a syringe pump via T piece into the flow from the UHPLC. The MS response for single and multiple charged ions, as well as fragment ions, was monitored. Nebulizing and drying gas flow were set lower than recommended by the manufacturer in order to minimize loss in response and generation of multiply charged ions. The large structural diversity of the compounds demanded different fragmentation energy, so a mass dependent fragmentation energy table was designed. Additionally, we made use of the possibility of changing between two collision energy values during each MS^2 event (sweeping), thereby widening the fragmentation potential of the method.

Detection of IPLs was performed in positive ionization mode while scanning a mass-to-charge (m/z) range from 100 to 2000. MS^2 scans were obtained in data-dependent mode. For each MS full scan up to three MS^2 experiments targeted the most abundant ions. Active exclusion limited how often a given ion was selected for fragmentation and thus allowed to also obtain MS^2 data of less abundant ions. Identification of IPLs was based on exact mass of

parent ions (present as either H^+ or NH_4^+ adducts) in combination with characteristic fragmentation patterns.

2.5. Method evaluation with standard mixtures and environmental samples

Once proper methods for RP and HILIC chromatography were set up, their usefulness in analyzing mixtures of standard compounds and environmental samples was tested. First, calibration curves consisting of triplicate measurements at twelve calibration levels in the range 0.001 to 100 ng applied on column were constructed to test linearity and detection limit. For comparison, a calibration curve was also constructed for the standard diol column method applied in our laboratory (Sturt et al., 2004).

Additionally, a sediment sample from the Eastern Mediterranean Sea (EMS-S-SOB, cf. Rossel et al., 2011) was analyzed, in which a large diversity of bacterial and archaeal lipids has been detected. This complex sample provided a realistic test of the separation efficiency and retention order of the new methods vs. the previously method based on a diol column.

3. Results and discussion

3.1. RP chromatography

3.1.1. Column selection

Concerning RP columns, the main concern was the excessive peak tailing, especially for compounds with amine groups, such as PE or PC. Such tailing is directly related to secondary interaction with free silanol groups and, in this case, could not be corrected by pH modification. Lowest tailing factor was obtained with the BEH column (Fig. 2A). Such ethyl-bridged hybrid columns are based on organic and inorganic building blocks and have been described to reduce silanol activity (O´Gara and Wyndham, 2006). Based on best tailing behavior, combined with shorter chromatographic run time, and the expectation that small

particle size is associated with greater peak resolution, this column was chosen for further development.

3.1.2. Solvent and buffer selection

As a second step the selection of solvent and solvent gradient with the BEH column was evaluated. Investigation was based on the method proposed by Lanekoff and Karlsson (2010) for bacterial ladderane lipid separation with a 3 µm hybrid silica gel column for RP-HPLC and Lin et al. (2012), which we adapted to the specific column, particle size and compounds n the present study. A gradient from MeOH to IPA, with an initial 1 min isocratic phase of 100% A, resulted in very rapid elution, with poor resolution in the initial part of the run. Short chain and relatively polar compounds like PAF eluted together with the column void volume. In order to increase retention in this part, we opted for addition of increasing amounts of water to eluent A. Even a very low concentration of water significantly improved resolution. An optimal compromise between analysis time and resolution was achieved by adding 15% water (Fig. 3). Addition of MeCN to mobile phase B was disregarded, as resolution did not improve and response for the intact GDGT standard was significantly lowered at high MeCN concentration. Addition of up to 50% MeOH to eluent B, on the other hand, improved peak shape in the later part of the run (Fig. 3).

The effect of changing buffer ratio and strength was evaluated next. First, injection of IPL standards was performed under different buffer ratio values (Fig. 2B). It was observed that peak shape benefited from extreme values, i.e. eluent strongly dominated by either HCO_2H or NH_3. Optimal conditions were achieved with the most basic condition. Increasing the buffer strength while maintaining this ratio led to an improvement in peak shape; however, buffer strength should not be increased too strongly, as this leads to formation of salt crystals in the ion source.

3.1.3. Column temperature and injection solvent

Modification of column temperature did not significantly impact on chromatographic efficiency. Still, increased column temperature has the advantage of lowering back pressure, especially for the highly viscous IPA. Thus, and thanks to the temperature tolerance of the RP5 column, chromatography was performed at 65 °C.

A last issue to be considered was the choice of injection solvent. When the usual combination of DCM:MeOH (5:1) was used with the RP method, peak fronting and even peak splitting was observed (Fig. 4). This issue was only solved after lowering the proportion of DCM, making injection solvent more similar to eluent A. Good results were observed for DCM:MeOH 1:5 or 1:10 and also for pure MeOH. Still, before choosing injection solvents, their capacity to completely dissolve the analytes of interest had to be confirmed.

The favored method therefore consisted of eluent A as MeOH:water (85:15) and eluent B as IPA:MeOH (50:50), both with addition of 0.04% HCO_2H and 0.1% NH_3. Column temperature was kept at 65 °C, flow rate was 400 µl min^{-1} and the run started with 100% A (held 2 min), followed by a rapid increase to 15% B in 0.1 min and an 18 min gradient to 85% B. The column was then washed with 100% B (8 min). After the return to initial conditions and column equilibration, the complete run was completed in 34 min. Pressure during the run remained < 600 bar, so and thus the method should be suitable for many current LC systems. For systems limited to lower pressure, we would recommend replacing the BEH C_{18} column with a similar column with larger particle size, as for example the Waters XBridge C_{18}.

3.2. HILIC chromatography

3.2.1. Column selection

For systematic comparison of the two HILIC columns, a preliminary method as starting point was needed. The isocratic method proposed by Schwalbe-Herrmann et al. (2010) was not

suitable under our test conditions, as broad and very early eluting peaks were obtained, the last being observed after only 3 min. Such reduced retention may be related to the BEH filling that limits certain retention mechanisms by reducing the availability of silanol groups. As a higher amount of MeCN might improve retention (Grumbach et al. 2008), gradient methods were developed which maximized the amount of MeCN at the beginning of the run. To further increase retention, water was gradually replaced with weaker HILIC solvents (IPA in eluent A and MeOH in eluent B). Chromatography was thereby improved and column comparison was carried out with a gradient from MeCN:IPA (75:25) to pure MeOH. Buffer concentration for these initial tests was 0.04% HCO_2H and 0.1% NH_3. With column H1, the more polar compounds exhibited good chromatographic separation, but glycosidic lipids eluted close to the column void volume, making correct detection and quantification impossible (Fig. 3). However, this lipid type is of major importance in environmental samples, e.g. as monoglycosidic GDGTs, monogalactosyldiacylglyceride typical for photosynthetic membranes or cyanobacterial heterocyst glycolipids. Thus the H1 was therefore substituted with an HILIC amide phase (H2), which shares the same basic principles of HILIC columns, but may increase retention due to the attached amide groups. Initial experiments with this column showed improved retention of glycolipids (Fig. 3), so it was chosen for further method development.

3.2.2. Solvent selection

Eluent optimization with H2 confirmed MeCN:IPA (75:25) as eluent A; further substitution of MeCN with IPA led to peak broadening, especially with MeCN proportion < 50%, when a change in retention mechanism seemed to take place. A higher proportion of MeCN in eluent A led to reduced MS response. Eluent B was modified by addition of water, which generally resulted in better peak shape and peak resolution in the later part of the run. Optimal results were obtained with a mixture of 50% water and 50% MeOH for eluent B.

Although good separation and response were achieved with this setting, a chromatographic problem was observed during the initial few min of the run, evident as a drastic increase in discharge current in the ion source and a simultaneous chromatographic peak. Just before the peak in discharge current, ion intensity was dramatically reduced. This phenomenon was attributed to an interaction of eluent buffer and the chromatographic column because it did not occur when buffer was infused post-column or the gradient was run without a chromatographic column. A possible explanation is that buffer initially accumulated on the column, not reaching the ESI source and thereby making proper ionization impossible. When aqueous eluent B increased, buffer was released, causing the strong discharge current and corresponding chromatographic peak. Mitigation of this issue was achieved by (i) the presence of aqueous eluent B during the initial few min of the run and (ii) reduced buffer concentration in eluent A (addressed in Section 3.2.3).

The presence of eluent B at the beginning of the run is problematic because early eluting compounds will be even less retained. This loss of retention capacity could not be compensated for by increasing MeCN proportion in eluent A as compound response decreased significantly. Therefore, partially replacing IPA in eluent A with a less polar, aprotic solvent was tested. Such a solvent increases retention as it is not expected to compete for hydrogen bonding and at the same time guarantees full solubility and detection of compounds of interest. Gradual substitution of IPA with THF or DCM led to increased retention, with highest values for 1Glc-Cer observed with 5% THF or 25% DCM. However, like increasing buffer strength, these substitutions resulted in poorer peak separation at the central part of the chromatographic run, with C_{21}-PC and PE-AR eluting close to each other. DCM was chosen for the final method vs. THF because of a greater increase in retention. An optimal eluent A composition was established at MeCN:DCM 75:25 (Fig. 3).

3.2.3. Buffer selection

Five different symmetric buffer ratio values with constant ionic strength were tested. The results showed that a higher concentration of NH_3 produced narrower peaks and better retention. The use of extremely acidic solutions (HCO_2H:NH_3, v:v, 10:1) also increased retention of the tested compounds. Both observations might be related to ion exchange occurring under these conditions. The effect of changing buffer ratio was not only evident in improved chromatographic behavior, but also in ionization efficiency, which was strongly variable depending on compound class. The buffer combinations with highest NH_3 concentration (1:2.5, HCO_2H:NH_3, v:v) or most acidic conditions (10:1, HCO_2H:NH_3, v:v) generally decreased response for all compounds and were therefore not used. Some compounds, such as PAF or 1Glc-Cer were found to ionize more effectively under acidic conditions, whereas ionization of other compounds benefited from higher NH_3 amount. In the case of the archaeal 1G-GDGT-PG, the response was strongly dependent on solvent pH. Optimal response for 1G-GDGT-PG was obtained with a HCO_2H:NH_3 ratio of 1:1; this ratio also resulted in good response for all the other compounds tested and was among the methods with narrowest peaks and highest retention. Increasing buffer concentration had a positive impact on peak shape, but negatively affected separation of lipid classes with phospho head groups, which tended to elute closer to each other.

As mentioned above, chromatographic issues associated with buffer precipitation on the column could be avoided by reducing buffer concentration in eluent A. Therefore four asymmetric buffer combinations were tested. At 10% eluent B these combinations resulted in HCO_2H:NH_3 values (v:v) of ca. 4:1, 1:1, 1:2.5 and 1:10. As observed for symmetric buffer composition, a ratio of 1:1 offered best peak shape and response. To achieve this value, while providing enough NH_3 to guarantee effective ionization, and avoiding excessively high buffer concentration in eluent B, the following asymmetric buffer composition is recommended: 0.01% HCO_2H: 0.01% NH_3 in eluent A and 0.4% HCO_2H: 0.4% NH_3 in eluent B.

3.2.4. Column temperature and injection solvent

Further method optimization dealt with column temperature and its effect on separation. Under our test conditions, increasing temperature translated into decreased retention capacity, with the greatest retention at 30 °C. The effect of temperature on HILIC retention was discussed by Hao et al. (2008), who compared different combinations of columns, analytes and solvent. In most cases, and especially with HILIC columns with neutral active sites where ion exchange is minimized, they observed that temperature negatively correlated with retention. Such behavior is explained via modified analyte transferring enthalpy between eluent and column (or column-associated layer) and increased diffusion coefficient. Peak shape on the other hand is positively affected by higher temperature, and in this case an optimum was found at 40 °C. Possibly, the effects responsible for slightly increased retention of the compounds at low temperature resulted in a more gradual release of the analyte, leading to peak broadening. The usual injection solvent, DCM:MeOH (5:1, v:v), resulted in peak fronting for PE and PC compounds, which disappeared by lowering the MeOH concentration to a ratio of 9:1 (v:v).

Thus the final method includes MeCN:DCM (75:25) with 0.01% HCO_2H and NH_3 as eluent A and MeOH:water (50:50) with 0.4% HCO_2H and NH_3 as eluent B. The column is maintained at 40 °C, with a flow rate of 400 µL min^{-1}. The gradient starts with 1%B (2.5 min), increasing to 5% B at 4 min, to 25% B at 22.5 min and to 40% B at 26.5 min. A 1 min washing step with 40% B is followed by return to initial conditions and column equilibration (8 min). If optimal separation of more polar lipids is prioritized over improved retention of early eluting compounds, a gradient starting with 5% B is recommended. The entire chromatographic run is completed in 35.5 min and back pressure remains <600 bar. Samples should be dissolved in DCM:MeOH (9:1) prior to injection.

3.3 Standard and environmental sample analysis

3.3.1. Analysis of standard solutions

Calibration curves offered an initial impression of the capabilities of the newly developed methods regarding sensitivity and the linearity of response. The widest linear range was obtained with the diol and HILIC amide columns, with linearity in some cases extending to four orders of magnitude (e.g. 0.01-100 ng on-column). Still, response curves generally showed some degree of saturation at higher concentration. Such curve flattening may in the first place be attributed to detector saturation, especially evident in RP analysis, where peaks are very narrow and thus reach critical levels of peak intensity more easily. However, in some cases this phenomenon cannot be explained with detector saturation. Fig. 5 shows calibration curves for C_{16}-PME. While not achieving detector saturation intensity, the response of the monomer no longer linearly increased at high concentration. The reason for this behavior is probably dimer formation. Indeed, by plotting calibration curves for the sum of mono- and dimers of these compounds, extended linearity was observed. Thus, for proper calibration it is beneficial to consider both monomer and dimer ions.

Table 1 shows the detection limit for the different compounds. The RP method achieves very low detection limit, including a dramatic improvement in the detection of 1G-GDGT-PG and acidic phospholipids such as PA or PS. The sensitivity for other phospho- and glycolipids is also excellent in RP analysis. Diol and HILIC amide methods struggle with the detection of acidic phospholipids and the detection limit of 1G-GDGT-PG is at least an order of magnitude higher than in the case of RP. For the other compounds, the HILIC amide method offers the lowest detection limit, as low as 5 pg on column. Another interesting aspect is the large difference in response according to characteristics such as head group or chain length. Similar results have been described for example by Koivusalo et al. (2001); future studies should more precisely evaluate this influence of head group in the proposed methods to provide optimal choices of internal or external standards for quantitative analysis.

3.3.2. Environmental samples

In order to test the actual capabilities of the methods under more realistic conditions including a multitude of IPLs and a more complex analytical matrix, we analyzed a sediment sample from the Eastern Mediterranean that is rich in bacterial and archaeal IPLs (Rossel et al., 2011), with the reference diol method and the two new methods. Fig. 6 shows density maps obtained by the three methods tested. Density maps show retention time on the x-axis and the m/z values of the IPL parent ions on the y-axis, darker colors denote higher relative intensities in the third dimension. Such representation thus supplies important additional information in comparison to the standard 2D representations. The diol and HILIC amide methods share a similar separation pattern according to lipid class. However, the HILIC amide column offers a higher resolution and much shorter analysis time than the diol column. All series of lipids with different polar head groups are better separated from each other than with the reference diol column, examples are the separation of GDGTs and ARs with 2G and PG head groups or the different DAG lipids with phospho head groups. The more complex separation mechanisms involved in HILIC amide chromatography separate compounds inseparable on a diol column. An additional advantage of the HILIC amide method is a lower chromatographic background. Moreover, contaminants (e.g., polymers introduced during sample storage or preparation) that coelute on the diol column with the lipids of interest are not retained on the column, and thus do not interfere with analytes.

As expected, the RP method led to a completely different separation pattern. Compounds were mainly separated by chain hydrophobicity and most bacterial phospholipids eluted closely together. While such separation according to chain length is a considerable disadvantage when screening bacterial lipids in a complex sample, it also offers some benefits. Fig. 7 shows how increasing chain length, decreasing degree of saturation or the presence of ether bonds instead of ester bonds affects elution of the compounds,

demonstrating the capacity for separation of compounds with the same head group and only slight differences in FA structure. This should for example contribute to cleaner MS^2 spectra, as parent ions with different degree of unsaturation are separated and should no longer be fragmented together in the mass spectrometer when wider isolation windows must be used. Also, long chain compounds like archaeols or GDGTs are nicely separated from all other compounds at the end of the run, where less coelution and thus more effective ionization are expected. Interestingly, not only the intact polar GDGTs, but also the core lipids (isoprenoid and branched GDGTs, other novel core lipids; cf. Liu et al., 2011, 2012a, b) may be analyzed with the method. The lack of a polar head group leads to the elution of core GDGTs in the injection peak in diol and HILIC amide methods, while in RP methods they elute at the very end of the chromatographic run, thereby being easily detected. This behavior opens interesting future avenues for quantification of both intact and core GDGTs in one analysis. On the negative side, it should also be noted that RP measurements are affected by polymeric contamination. While such contamination is not retained on HILIC amide columns, it typically stretches over a longer elution range in RP runs.

Fig. 8 shows a quantitative comparison of different measurements. As occurred with establishing detection limits for pure compounds, both peak area and signal-to-noise ratio (SNR) are consistently higher with the two new methods than in the usual diol method. Specifically, monoglycosidic AR and GDGT are best detected with the RP method, while the HILIC amide method achieves < 40% of the response. In the case of the diol column, monoglycosidic AR and GDGT are actually not detected, which means that some of the key components of the lipid signature of this sample have previously been overlooked. This is possibly related to the elution properties of these compounds close to the column void volume. In RP separation, increased chain length translates to stronger retention, while on diol and to some degree HILIC amide columns, the relatively low polarity of a monoglycosidic head group leads to weak retention and thus coelution with other non-retained compounds.

Such behavior is altered when GDGTs or ARs incorporate more polar phospho head groups or combinations of phospho and glycosidic head groups. In such a case, retention behavior on HILIC amide columns is increased and excellent response obtained.

Bacterial DAG and AEG lipids achieve the highest peak areas with the RP method. Two groups of ornithine lipids (OL) occur in the sample, the greatest response for both being achieved after separation on the HILIC amide column. Interestingly, while both OL groups co-elute on the RP column, they are well separated with the diol and amide columns, suggesting differences in head group distribution.

Signal-to-noise ratio is generally best with the RP column, thanks to extremely narrow peaks. Detection of GDGTs benefits especially from excellent peak shape and thus high SNR. With the present sample, RP is beaten by the HILIC amide method only in the case of more polar archaeols, OLs and one GDGT (PG-GDGT-PG). This feature makes the proposed RP method an ideal choice for the analysis of components at very low concentration.

4. Conclusions

Two methods were developed and thoroughly tested. They widen the range of analytical techniques available for the study of IPLs. Both offer strongly reduced analysis time (around 35 min) compared with the previous method (60 min). Although our study was carried out with UHPLC equipment, pressure during the run remained < 600 bar; so these rapid methods may be suitable for most modern HPLC equipment.

The HILIC amide method maintains the advantage of established methods concerning separation order and clustering of lipid classes, while the detection limit for standards in clean solvents is significantly improved. During analysis of environmental samples, cleaner chromatograms are obtained thanks to non-retained background. Improved peak separation is achieved via the more complex retention mechanisms involved in HILIC. HILIC amide

separation is optimal for DAGs and for many ARs and GDGTs. In contrast to the diol method, monoglycosidic lipids are properly detected. Therefore, we recommend replacing the usual diol-based method for marine extracts, especially if the purpose is lipid fingerprinting.

RP methods offer very good peak shape, with very narrow peaks and thus excellent detection limit and SNR. Chromatographic separation of lipids is different from that with HILIC amide or diol columns, as it is based on chain hydrophobicity. This at first makes interpretation of results more difficult, but the separation is advantageous in some cases. For example, GDGTs are easily separated from all other lipid classes and show excellent response thanks to higher sensitivity and less ion suppression, due to the absence of coeluting compounds. In particular for isotope analysis or structural identification of archaeal IPLs (e.g. Biddle et al., 2006; Liu et al., 2012a, b), the technique should show promise when transferred to the preparative scale. The possibility of characterizing IPLs and core lipids in the same chromatographic run is another promising feature of the method. The detection of bacterial phosphoglycerolipids, especially the acidic ones, may benefit from improved response with RP chromatography. An additional advantage is the ability to separate lipids with the same head group and only slight variation in the hydrophobic chains. The different separation mechanisms involved in RP and HILIC amide separation may also be beneficial for multidimensional chromatography.

Acknowledgements

This work was funded by the Deutsche Forschungsgemeinschaft (DFG) through grants Inst 144/300-1 (LC Q-TOF system), HI616/10-1 (K.-U. H.), LI1901/1-1 (J.S.L.), the DFG-Research Center and Excellence Cluster 'The Ocean in the Earth System', and by the European Research Council under the European Union's Seventh Framework Programme– "Ideas" Specific Programme, ERC grant agreement No. 247153 (PI: K.-U.H., funding for: J.S.L. and L.W. We thank Dr. Harald Koefeler and one anonymous reviewer for helpful comments and corrections that significantly improved the quality of the manuscript.

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Figure Captions

Fig. 1. Overview of method development for RP and HILIC amide methods. Optimized conditions and most relevant criteria are shown.

Fig. 2. Tailing factor (T) at 5% of peak height, calculated according to US Pharmacopeia (USP) as T = (a+b)/2a (where a is the distance from the leading edge of the peak to the midpoint and b the distance from the point at peak midpoint to the tailing edge). **(A)** C_{21} PC on the five RP columns tested with HCO_2H:NH_3 ratio 0.12:0.04 (v:v) and **(B)** on the BEH C_{18} column with different ratio values of HCO_2H:NH_3 (v:v) and different buffer strength (separated by axis break).

Fig. 3. Improved chromatographic separation for RP (left) and HILIC (right) methods achieved by column and/or solvent selection compared to standard diol method (top). Early eluting compounds, PAF (A) on RP and 1Glc-Cer (B) on HILIC, are shown together with C_{16}-PME (C) and PE-AR (D).

Fig. 4. Comparison of injection solvents in RP chromatography (Waters Acquity UPLC BEH C_{18}). Extracted ion chromatograms of (peaks from left to right, 25 ng on column) PAF, 1Glc-Cer, PE-AR and C_{21}-PC injected in DCM:MeOH **(A)** 5:1 (v:v) and **(B)** 1:5 (v:v)**.**

Fig. 5. Calibration curve for C_{16}-PME considering monomer (filled symbols) or the sum of monomer and dimer (open symbols) measured with (A) diol, (B) HILIC amide and (C) RP method. Insert shows zoom of the calibration curve in the lower range.

Fig. 6. Density maps for environmental sample EMS-S-SOB (cf. Rossel et al., 2011) obtained with (A) Diol, (B) HILIC amide and (C) RP methods. Number code: 1: core GDGT, 2: 1G-GDGT, 3: 2G-GDGT, 4: PG-GDGT, 5: PG-GDGT-PG, 6: 2G-GDGT-PG, 7: core AR, 8: core OH AR, 9: 1G-AR, 10: 1G-OH-AR, 11: 2G-AR, 12: PG-AR, 13: PE-AR, 14: PI-AR, 15: PI-OH-AR, 16: PI-ext-AR, 17: PI-ext-OH-AR, 18: PI-ext-AR (unsaturated), 19: DPG, 20: PG-

DAG, 21: PG-AEG, 22: PE-DAG, 23: PME-DAG, 24: PC-DAG, 25: OL1, 26: OL 2. Blue solid lines and circle identify bacterial lipids, red dashed circles identify archaeal lipids. For abbreviations, see text.

Fig. 7. Extracted ion chromatograms for (A) PE and (B) PG compounds measured with the RP method (total number of carbons and double bonds in both side chains in annotation). AEG identifies acyl/etherglycerophospholipid; remaining structures are diacylglycerophospholipids.

Fig. 8. Peak area (left) and signal to noise ratio (right) for 26 lipid classes in sample EMS-S-SOB (cf. Rossel et al., 2011) measured with the three methods: diol (black bars), HILIC amide (grey bars) and RP (white bars with pattern). Values for each lipid class are normalized to the highest value obtained with any of the three methods.

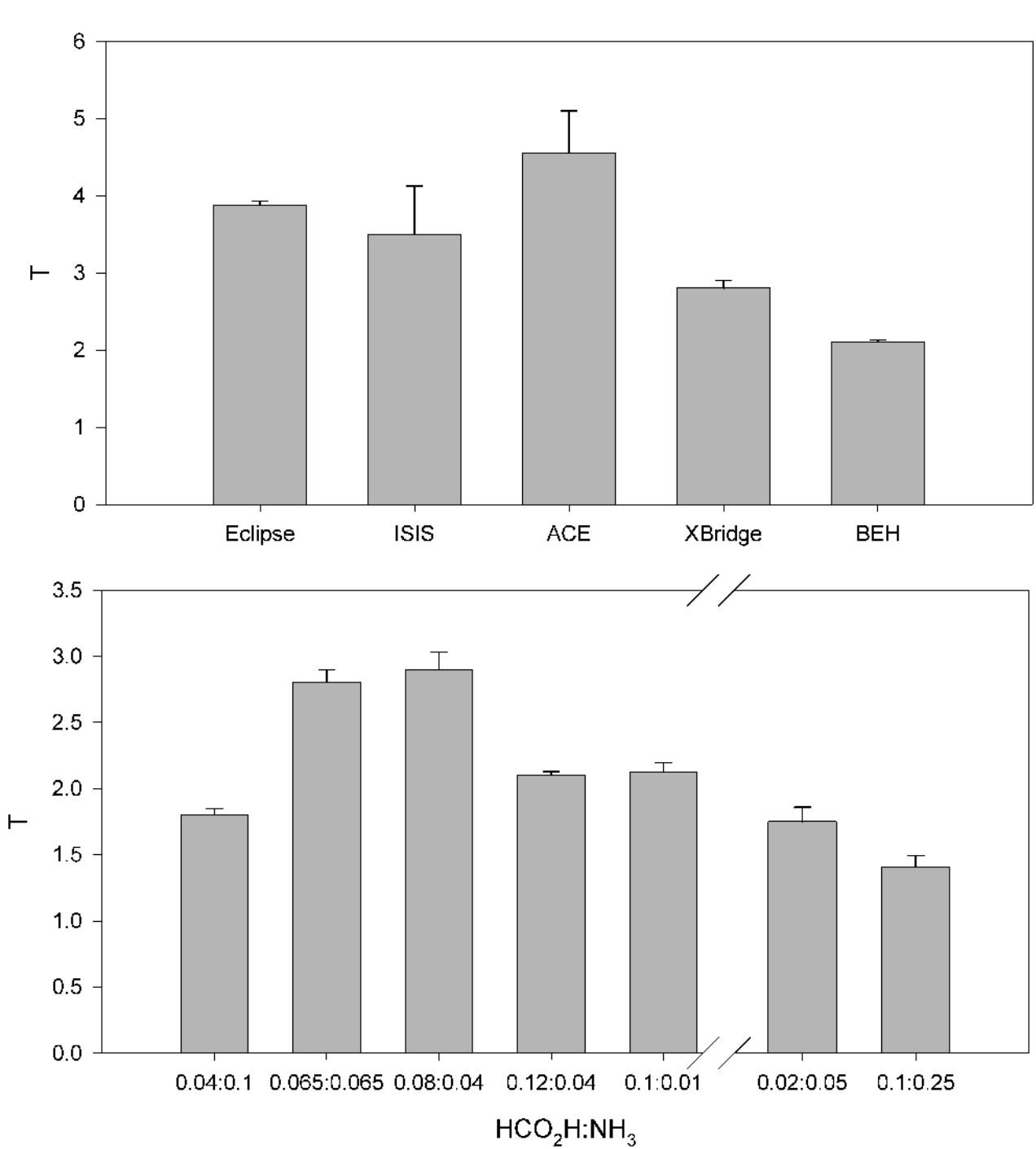
Table 1

Detection limit (ng on column; pmol on column in parentheses) (SNR>3[a]) for the newly developed methods and the diol method currently used in our laboratory.

Compound[b]	Diol	HILIC amide	RP
PAF	0.01 (0.019)	0.005 (0.010)	0.005 (0.010)
C_{16}-PA	5 (7.5)	10 (15)	1 (1.5)
C_{16}-PME	0.05 (0.071)	0.005 (0.007)	0.01 (0.014)
1Glc-Cer	0.1 (0.14)	0.01 (0.014)	0.1 (0.14)
C_{16}-PS	2 (2.6)	2 (2.6)	0.1 (0.13)
C_{21}-PC	0.01 (0.011)	0.01 (0.011)	0.005 (0.006)
PE-AR	0.01 (0.013)	0.005 (0.006)	0.01 (0.013)
1G-GDGT-PG	1 (0.62)	1 (0.62)	0.1 (0.062)

[a] Signal-to-noise ratio; [b] for details see Section 2.1


RP
Selection of column: 5 RP columns tested
Avoid tailing of basic lipids
Acquity UPLC BEH C18
Solvent selection: MeCN; MeOH; IPA; H2O
Retention of less polar compounds
Peak resolution
eluent A: 15% H2O 85% MeOH
eluent B: 50% MeOH 50% IPA
Buffer selection: HCO2H:NH3 ratio and strength
Response and peak shape
Response and peak shape
eluent A: 0.04% HCO2H 0.1% NH3
eluent B: 0.04% HCO2H 0.1% NH3
Injection solvent
Avoid peak fronting
MeOH:DCM 9:1
Column temperature
Reduce backpressure
65°C
HILIC
Selection of column: Acquity HILIC Acquity Amide
Retention of less polar compounds
Acquity UPLC BEH Amide
Solvent selection: MeCN; DCM; THF; MeOH; IPA; H2O
Retention of less polar compounds Better column loading
Peak resolution
eluent A: 75% MeCN 25% DCM
eluent B: 50% H2O 50% MeOH
Buffer selection: HCO2H:NH3 ratio and strength
Avoid buffer precipitation
asymmetric buffer
Response and peak shape
Response and peak shape
eluent A: 0.01% HCO2H 0.01% NH3
eluent B: 0.4% HCO2H 0.4% NH3
Injection solvent
Avoid peak broadening
DCM:MeOH 9:1
Column temperature
Retention and peak shape
40°C


T
6
5
4
3
2
1
0
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ISIS
ACE
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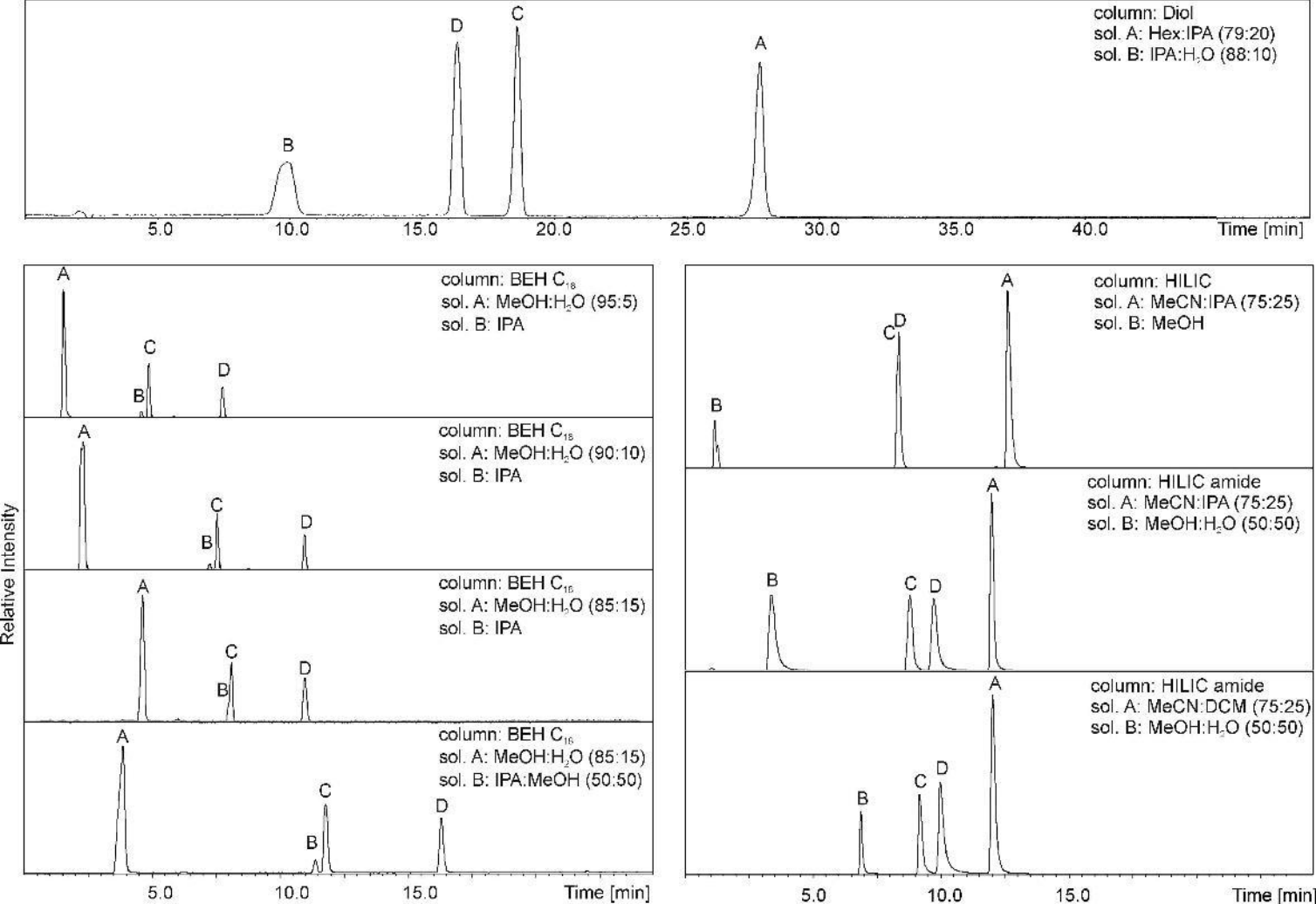
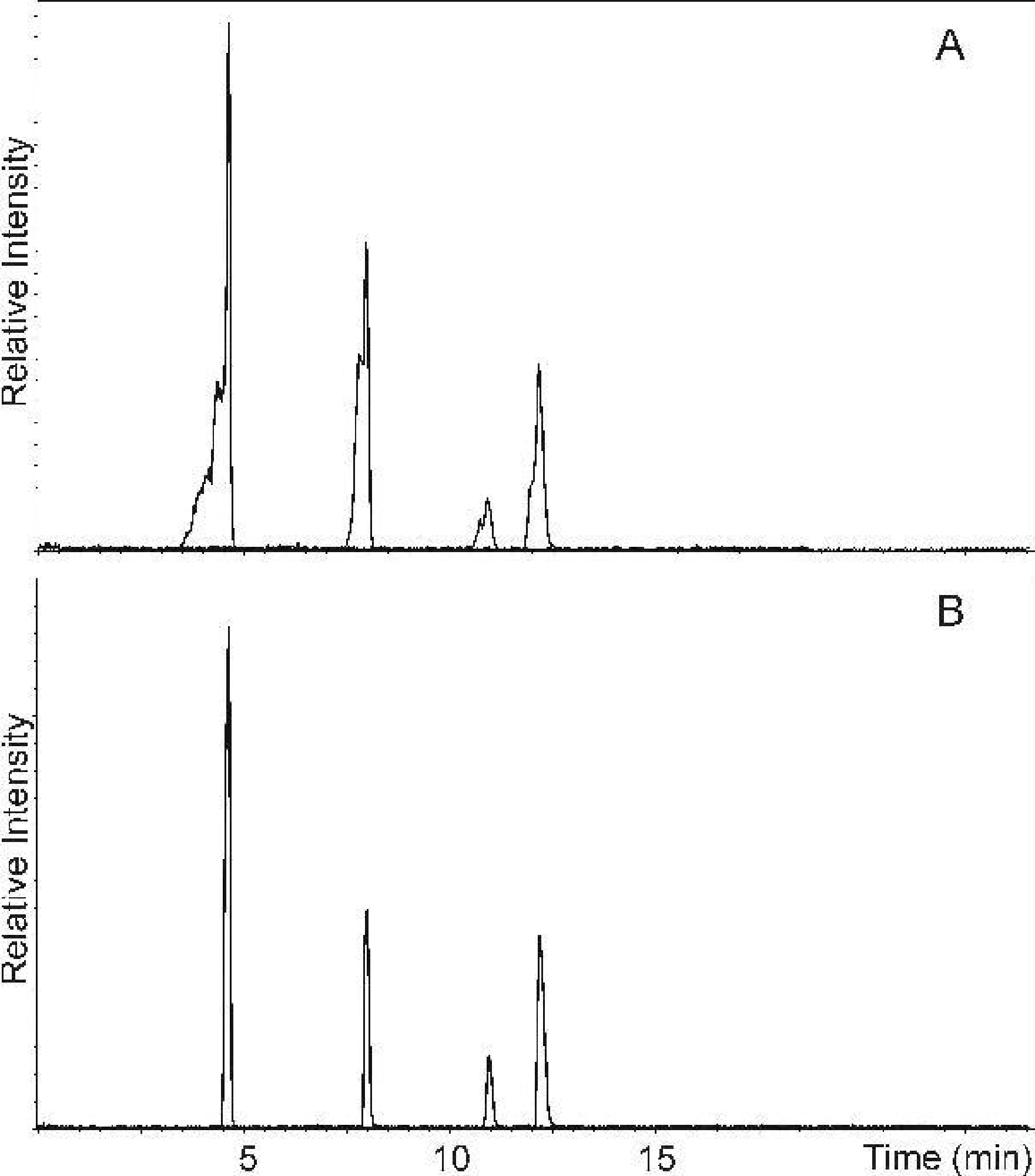
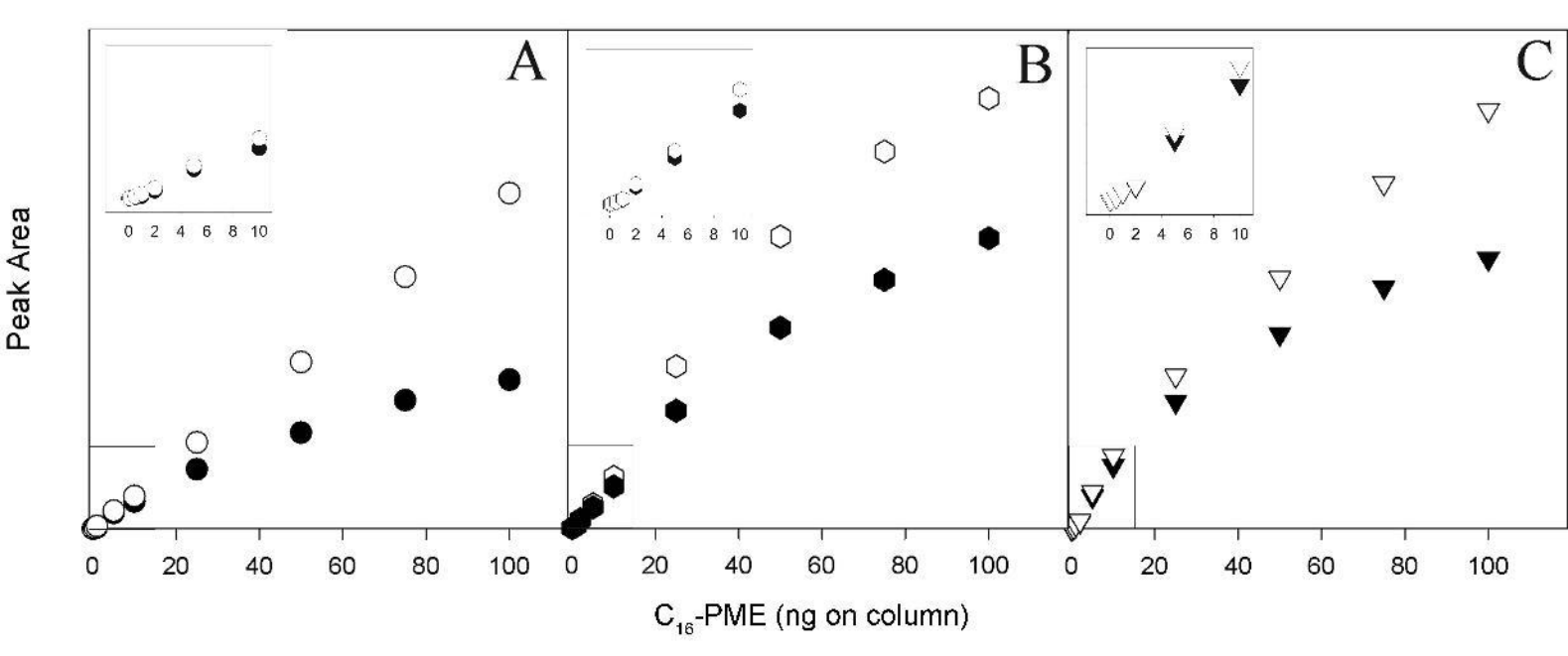
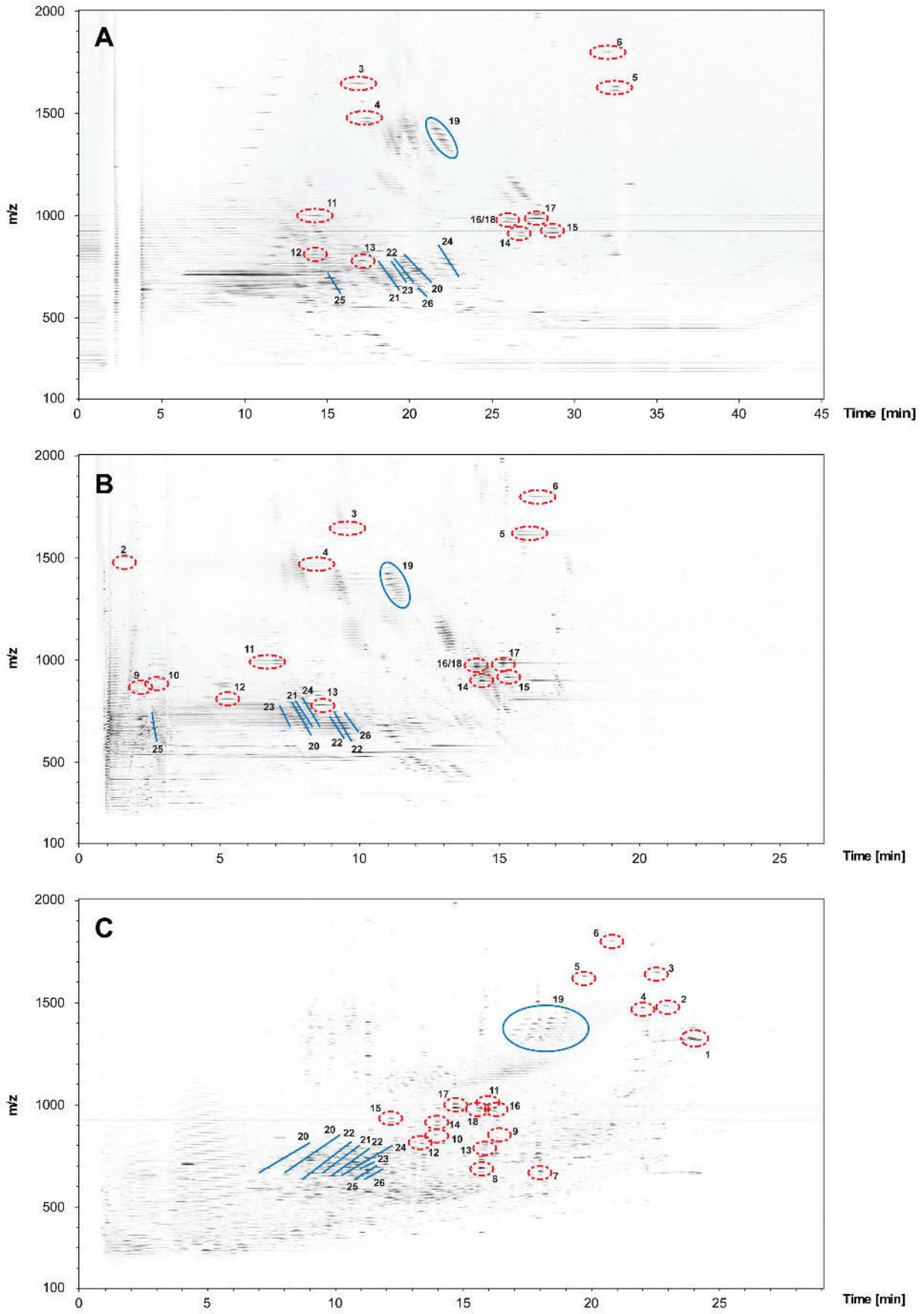
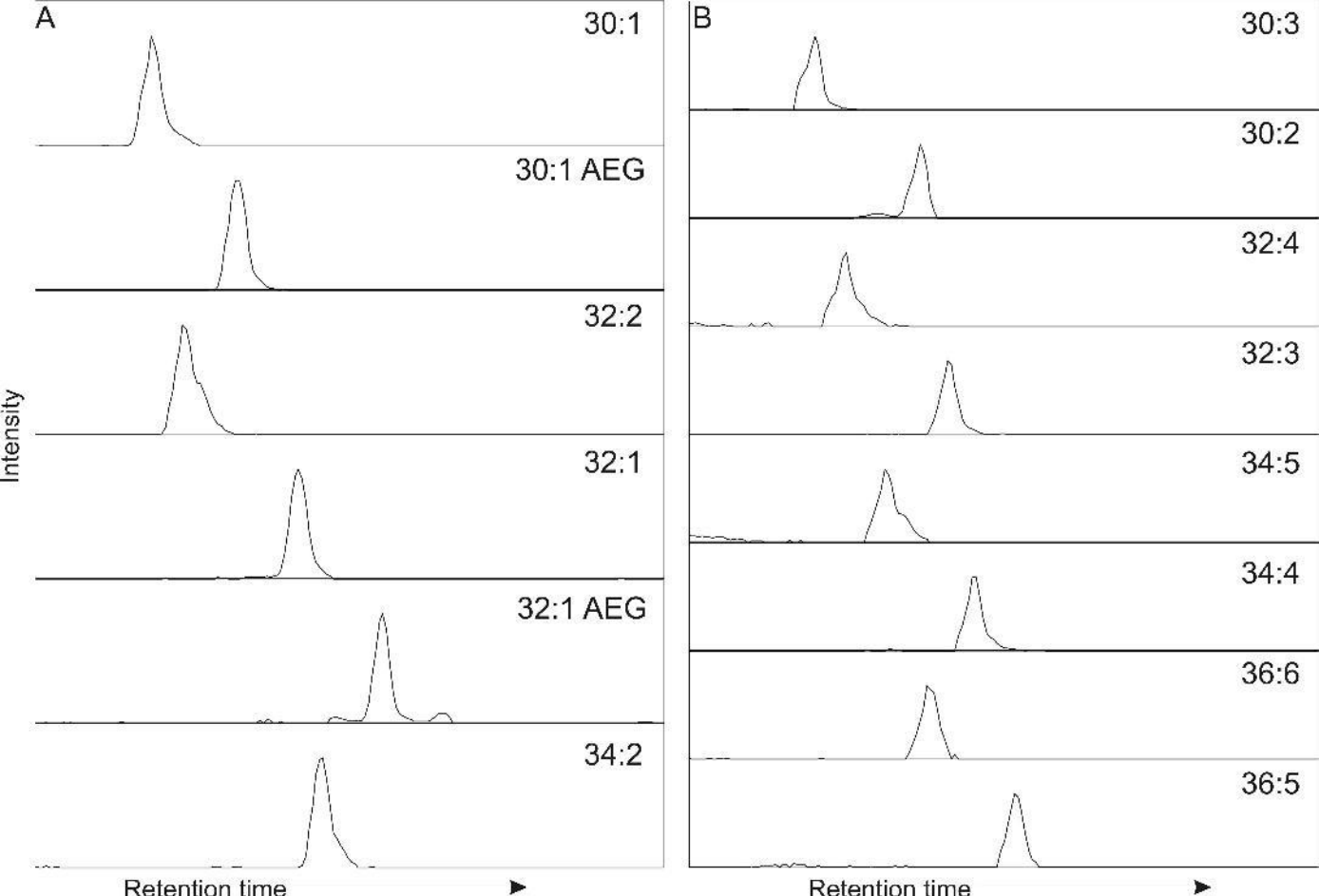
T
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.04:0.1
0.065:0.065
0.08:0.04
0.12:0.04
0.1:0.01
0.02:0.05
0.1:0.25
HCO2H:NH3

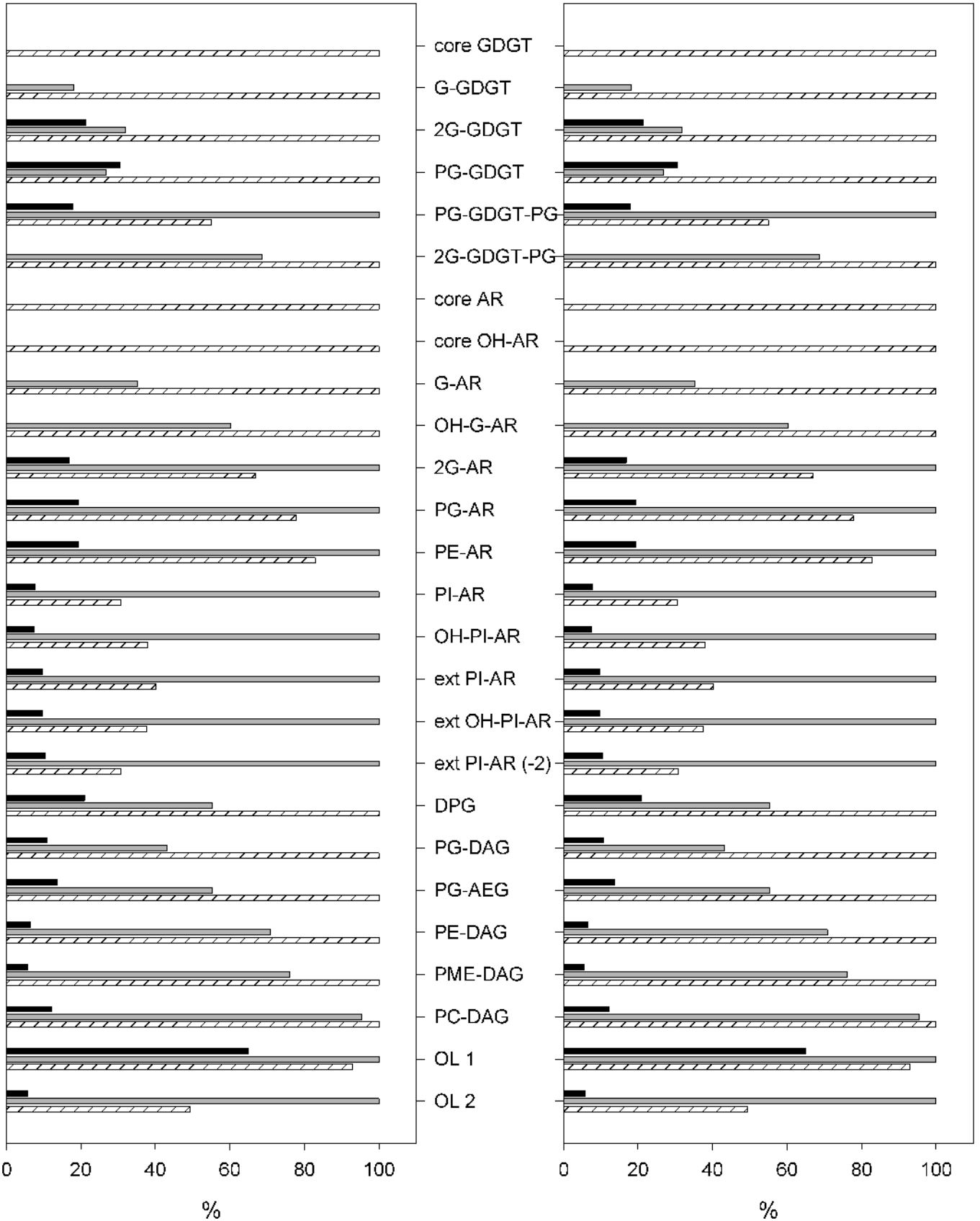

column: Diol
sol. A: Hex:IPA (79:20)
sol. B: IPA:H2O (88:10)
A
B
C
D
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
Time [min]
column: BEH C18
sol. A: MeOH:H2O (95:5)
sol. B: IPA
column: BEH C18
sol. A: MeOH:H2O (90:10)
sol. B: IPA
column: BEH C18
sol. A: MeOH:H2O (85:15)
sol. B: IPA
column: BEH C18
sol. A: MeOH:H2O (85:15)
sol. B: IPA:MeOH (50:50)
Relative Intensity
5.0
10.0
15.0
Time [min]
column: HILIC
sol. A: MeCN:IPA (75:25)
sol. B: MeOH
column: HILIC amide
sol. A: MeCN:IPA (75:25)
sol. B: MeOH:H2O (50:50)
column: HILIC amide
sol. A: MeCN:DCM (75:25)
sol. B: MeOH:H2O (50:50)
5.0
10.0
15.0
Time [min]


A
Relative Intensity
B
Relative Intensity
5
10
15
Time (min)


A
B
C
Peak Area
0 2 4 6 8 10
0 20 40 60 80 100
C_{16}-PME (ng on column)


A
B
C
m/z
Time [min]
2000
1500
1000
500
100
0
5
10
15
20
25
30
35
40
45


A
30:1
30:1 AEG
32:2
32:1
32:1 AEG
34:2
Intensity
Retention time
B
30:3
30:2
32:4
32:3
34:5
34:4
36:6
36:5
Retention time


core GDGT
G-GDGT
2G-GDGT
PG-GDGT
PG-GDGT-PG
2G-GDGT-PG
core AR
core OH-AR
G-AR
OH-G-AR
2G-AR
PG-AR
PE-AR
PI-AR
OH-PI-AR
ext PI-AR
ext OH-PI-AR
ext PI-AR (-2)
DPG
PG-DAG
PG-AEG
PE-DAG
PME-DAG
PC-DAG
OL 1
OL 2
0
20
40
60
80
100
%
0
20
40
60
80
100
%

Appendix 1: Overview of compounds used for method development and evaluation

Name	Chemical Formula	Abbreviation	m/z
1-hexadecanoyl-2-acetyl-*sn*-glycero-3-phosphocholine	$C_{26}H_{54}NO_7P$	C_{16}-PAF	524.371 (H^+)
1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidic acid	$C_{35}H_{69}O_8P$	C_{16}-PA	666.507 (NH_4^+)
1,2-dihexadecanoyl-*sn*-glycero-3-phospho-*N*-methylethanolamine	$C_{38}H_{76}NO_8P$	C_{16}-PME	706.538 (H^+)
1,2-dihexadecanoyl-*sn*-glycero-3-phosphoserine	$C_{38}H_{74}NO_{10}P$	C_{16}-PS	736.512 (H^+)
N-(octadecanoyl)-1-ß-glucosyl-sphing-4-enine	$C_{42}H_{81}NO_8$	1Glc-Cer	728.603 (H^+)
1,2-di-*O*-phytanyl-*sn*-glycero-3-phosphoethanolamine	$C_{45}H_{94}NO_6P$	PE-AR	776.689 (H^+)
1,2-diheneicosanoyl-*sn*-glycero-3-phosphocholine	$C_{50}H_{100}NO_8P$	C_{21}-PC	874.726 (H^+)
2,3,2',3'-tetra-*O*-dibiphytanyl-di-*sn*-glycero-l'-gulosyl-l-phosphoglycerol	$C_{95}H_{189}O_{16}P$	1G-GDGT-PG	1635.405 (NH_4^+)