Improved separation and analysis of glycolipids by Iatroscan thin–layer chromatography–flame ionization detection

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ABSTRACT

We demonstrate improved power of Iatroscan thin layer chromatography/flame ionization detection (TLC-FID) technique for analysis of complex marine lipid mixture by developing protocol for the separation and analysis of glycolipids including sulfoquinovosyldiacylglycerols (SQDG), monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG). We have modified the common protocol used so far for the analysis of lipid classes by replacing the elution step which uses pure acetone for the elution of acetone mobile polar lipids, with the elution step containing chloroform–acetone (72:28, v:v) for separation of MGDG and DGDG. To separate SQDG from the complex lipid matrix we introduced solvent mixture acetone–chloroform–methanol–formic acid (33:33:33:0.6, v:v:v:v). Quantification of glycolipid classes was performed after calibration with glycolipid standards for the masses between 0.2 and 4 μg. With this new protocol we have successfully separated three glycolipids from the complex particulate lipid mixture of the seawater samples. Such an approach extends the power of existing protocol for the analysis of lipids which altogether ensure detection and quantification of 18 lipid classes what was demonstrated on seawater samples. This enables to gain a very broad system overview of the particularly complex environments as are seas, oceans and freshwaters.

Keywords: sulfoquinovosyldiacylglycerol (SQDG), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), Iatroscan thin–layer chromatography

1. Introduction

The investigations of marine organic matter (OM) are very important since carbon capture and sequestration is a possible way to reduce the atmospheric carbon dioxide level. Among others the investigations of marine lipids take advantage in comparison to other major biochemicals due to their stable nature compared to carbohydrates and proteins [1] leading to their selective accumulation in the water column as the particulate organic matter sinks [2]. Lipids characterized at the molecular level are good biogeochemical markers for the carbon cycling, identification of different sources and processes of organic matter in the sea, for the environmental conditions including nutritional regimes and temperature [3-8]. Different instrumental techniques are employed among marine scientist to analyze lipids and their classes; thin layer chromatography (TLC) [9-11], gas chromatography (GC) [5, 12, 13], HPLC [14, 15], the use of which depend on the final target of the investigation.

In comparison to conventional chromatography techniques the Iatroscan thin layer chromatography with flame ionization detection (TLC-FID) obtains data from a large sample rapidly as the instrument measures up to 10 rods in a single series, where each of these represents an independent chromatographic system. Because of its high sample throughput, it is particularly useful for series quantitative separations of substances that cannot be analysed by GC because of their low volatility or because of the irreversible adsorption of components on the column. Advantages of TLC-FID over more structural sensitive techniques as GC and HPLC lie also in the fact that TLC-FID is capable of measuring all lipid classes from highly non-polar, like hydrocarbons, to polar lipids, like phospholipids without prefractionation, saponification, derivatization, or other treatments, the procedures often used in GC analysis [16]. Methods based on LC coupled with mass spectrometry (LC-MS) have several advantages over other lipidomic techniques, such as more reliable identification of individual lipid species, even at trace levels, separation of isomers and isobars enabled by measuring different retention times (LC part) and by recording different fragmentation spectra (MS part). In addition, current LC instruments permit more effective separation, and reduce analysis time and solvent consumption [17]. The principal drawback of TLC is its inability to resolve individual closely related molecular compounds in complex samples, thus limited information is available on the individual molecular species present. However, this disadvantage can be turn into its strength as TLC-FID gives information of total lipid content close to true gravimetric values [18] which

is highly important for lipid mass balance of different systems. Apart of FID other detection can be combined with TLC including fluorescence spectroscopy [19] and fluorescence image analysis [20]. TLC-FID technique has been evaluated against quality assurance standards originally developed for gas chromatographic analysis at the U.S. Environmental Protection Agency (U.S. EPA, Atlantic Ecology Division). It met the QA criteria prescribed for consistent external calibrations, low blanks, and precise replicate analysis [21-18]. The marine lipid separation by TLC method is continuously improving since the method inauguration in 1960ies [22-24].

Glycoglycerolipids (GL) widely occur in natural products, especially in the marine algae and higher plants [20, 25, 26]. Structurally, GL are characterized by a 1,2-diacyl-*sn*-glycerol moiety with mono- or oligosaccharide attached at the *sn*-3 position of the glycerol backbone. The nutrient availability, high temperatures and sunlight intensities have shown multiple influences on the phytoplankton GL accumulation in the Northern Adriatic Sea [8]. Natural GL have been shown to possess a variety of bioactivities which make them valuable molecular targets for further investigation. However, the low natural abundance coupled with the difficulty of isolation and detection due to the variety of structures represented among the polar components of lipid extracts, by their instability and by the lack of standards hamper their evaluation in reactivity and accumulation under the changing environmental conditions. Up to now glycolipids as monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG) were analysed jointly as the one TLC-FID peak, while the TLC-FID method was lacking in accurate protocol for separation and detection of sulfoquinovosyldiacylglycerols (SQDQ). Parrish et al. [22] described a TLC-FID method approach for the separation of algal GL, which present a number of complications, such as interference with polar pigments and other components and the lack of commercially available standards. Thus, reevaluation and improve separate TLC-FID analysis of MGDG, DGDG and SQDQ is urgently needed. Moreover, Iatroscan summed lipid classes for aquatic samples were routinely 80–95% of those obtained by gravimetry [18, 24]. The discrepancy between the methods for determination of total lipid may reflect the fact that TLC-FID is quantitative only for non-volatile compounds but also could be the consequence of inappropriate choice of calibration standard for the particular lipid class. Thus, to evaluate the total lipid content more properly, there is a need to increase the separation capacity of the TLC-FID to be able to adequately quantify as much as possible individual lipid classes by using stepwise development and unique selective scanning by Iatroscan instrumentation. The often utilised separation scheme in marine studies till now, involves several steps for analysis of 16 lipid classes [27] where particular GL compounds were

not individually separated. Thus, the main focus of the present study is to improve Iatroscan TLC-FID technique for analysis of complex natural lipid mixture by developing protocol for the separation and analysis of MGDG, DGDG and SQDQ. Such an approach extends the power of existing protocol for the analysis of lipids which altogether ensure detection and quantification of 18 lipid classes. Finally, we illustrate applicability of the new approach on seawater samples, having in mind that many research groups that are using TLC-FID in the analysis of aquatic lipids [*i.e*. 28-32], should have benefit of our improved protocol.

2. Materials and methods

2.1. Chemicals

Standards including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) (all isolated from plant leafs) were supplied from Lipid Products (UK), while phosphatidylglycerol (PG, 1,2-Dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt) and solvents including acetone, chloroform, methanol (all HPLC grade) and formic acid (p.a.) were from Sigma–Aldrich (USA) and dichloromethane (HPLC grade) was supplied from Merck (Germany).

2.2. Seawater samples

For the particulate lipid class determination 3.5 L of Northern Adriatic seawater were filtered through GF/F filters (0.7 μm pore size, Whatman, precombusted at 450 °C for 5 h) immediately after sampling. The filters were stored in liquid nitrogen until lipid extraction and analysis. Particulate lipids were extracted twice with 10 ml of one–phase solvent mixture of dichloromethane–methanol–water (1:2:0.8, v:v:v) and once with 10 ml of two-phase mixture of dichloromethane–0.73% NaCl (1:1, v:v) and once with 10 ml of dichloromethane [33]. Before extraction procedure 10 μg of *n*–hexadecanone (KET) was added to each sample as internal standard to evaluate and to compensate for losses and variability throughout the sample preparation (including extraction) and subsequent steps of analytical processes [18]. Mono-functional, saturated, unbranched ketones are minor components of the lipids of marine organisms which have been proposed decades ago as appropriate internal standards for marine lipid work [34, 35]. Percent recoveries (% recovery) are calculated from the ratio of *n*-

hexadecanone mass recovered and the theoretical mass of *n*-hexadecanone added to the sample prior to analysis:

$$\% \text{ recovery} = (m(KET_{recovered})/m(KET_{theoretical}) \times 100 \quad \text{(Eq. 1)}$$

Mass of *n*-hexadecanone recovered is calculated from the area of signal obtained (A_{KET}) and calibration curves made previously for *n*-hexadecanone as follow:

$$m(KET_{recovered}) = (A_{KET}/1041.21)^{(1/1.34)} \quad \text{(Eq. 2)}$$

Theoretical mass of *n*-hexadecanone (KET) was calculated as follows:

$$m(KET_{theoretical}) = (m(\text{added KET}) \times \% \text{ spotted sample}) \quad \text{(Eq. 3)}$$

% spotted sample refers to the percentage of sample spotted on TLC Chromarode.

The extracts were evaporated until dryness under nitrogen atmosphere and redissolved in 20 μL of dichloromethane. Each seawater extract was analyzed two to four times: for the analysis 2 μl aliquots of 14–20 μl solution in dichloromethane were spotted on Chromarods with a semiautomatic sample spotter. The recoveries were 96±14%.

2.3. TLC-FID analysis

Lipid classes were determined by TLC-FID (Iatroscan MK–VI, Iatron, Japan) with a hydrogen flow of 160 mL/min and an air flow of 2000 mL/min. Lipid classes were separated on silica-coated quartz thin-layer chromatography (TLC) rods (Chromarods SIII) (SES–Analysesysteme, Germany) and quantified by external calibration with a standard lipid mixture. Calibrations of MGDG, DGDG, SQDG and PG were performed based on 10–16 concentrations spanning a range from 0.2 to 2.7–5.0 μg. Data (peak area vs. mass (in μg)) are fitted with power functions.

Lipid separation scheme involves subsequent elution steps in the solvent systems of increasing polarity [6, 23, 24, 36]. The details of the separation scheme so far used in our laboratory are given in Gašparović et al. [27]. The complete scheme used so far allowed separation of 16 lipid classes: Hydrocarbons, wax esters and steryl esters, fatty acid methyl esters and ketone (HC, WE, ME, and KET, respectively) were separated with *n*-hexane–diethyl ether–formic acid (97:3:0.2, v:v:v) for 28 min. Triacylglycerols and free fatty acids (TG and FFA, respectively) were separated with *n*-hexane–diethyl ether–formic acid (80:20:0.2, v:v:v) for 30 min. Alcohols, 1,3–diacylglycerols, sterols and 1,2–diacylglycerols (ALC, 1,3DG, ST, and 1,2DG, respectively) were separated with an additional 20 min in the previous solvent

mixture. Pigments and monoacylglycerols (PIG and MG, respectively) were separated with chloroform–acetone–formic acid (95:5:0.6, v:v:v) for 32 min. This was followed by 8 min in acetone (100 %) to elute jointly MGDG and DGDG as a one peak. Finally, chloroform–methanol–ammonium hydroxide (50:50:5, v:v:v) during 40 min allowed separation of mono– and di–phosphatidylglycerols, phosphatidylethanolamines, phosphatidylcholines (PG, PE, PC, respectively). In majority groups glycolipids are analyzed as a part of polar lipids [32], acetone mobile polar lipids [36] or chloroplast lipids [6]. Method limits of detection (LODs) were determined as analyte concentrations that correspond to signal-to-noise ratio (S/N) of 3. Method limits of quantification (LOQs) were determined as concentrations at which S/N is at least 10 and have a repeatability better than 5.0% (relative standard deviation, RSD, n = 5) to ensure highly reliable results for low concentrations of analytes.

Based on the calibration equations, chromatogram peak area (A), sampled seawater volume (V_{sample}), percent of the sample spotted (% spotted sample) and from the lipid recovery (% recovery) (obtained by using the internal standard; *n*-hexadecanone) the concentration of particular lipid (C) in the natural sample was calculated as follows:

$$C=((((m \times 100)/\%\text{spotted sample})/V_{sample}) \times 100)/\%\text{ recovery} \qquad \text{(Eq. 4)}$$

2.4. Mass spectrometry analysis

Lipid mixture extracted from the Northern Adriatic Sea (Station 107, February 2015) was separated into lipid-class bands on silica-coated quartz thin-layer chromatography (TLC) rods (Chromarods). Developed glycolipids (MGDG (1st fraction) and DGDG (2nd fraction) were separately desorbed from the Chromarods using dichloromethane. Two fractions were analyzed by electrospray ionization (ESI) MS and tandem mass spectrometry (MS/MS).

ESI MS and MS/MS mass spectra were recorded on an amazon ETD ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with the standard ESI ion source (nebulizer pressure: 8 psi; drying gas flow rate: 5 dm^3/min; drying gas temperature: 250 ºC; the potential on the capillary: –/+ 4500 V). Immediately before analysis dichloromethane was evaporated and MGDG and DGDG were redissolved in a solution of methanol, chloroform and 50 mM sodium acetate (300:665:35, v:v:v) to produce positively charged sodium adduct ions [M+Na]+ according to Welti et al. [37]. Samples were infused into the ESI source by a syringe pump at a 1 μL/min flow rate. Helium was used as a collision gas. ESI MS/MS was performed using collision energy between 0.4 and 1 eV. The positions of the acyl chains (*sn*-1 or *sn*-2)

were determined by their relative percent composition according to the procedure established by Guella et al. [38].

3. Results and discussion

3.1. Development of solvent systems for separation of MGDG, DGDG and SQDG.

The development of suitable solvent system(s) for separation of MGDG, DGDG and SQDG requires firstly identification of the optimal solvent system between those used so far for the polar lipid classes separation and quantification (described in section 2.3). Firstly, it was revealed that the solvent system chloroform–acetone–formic acid (95:5:0.6, v:v:v) for elution of PIG and MG is not influencing the migration of MGDG and DGDG. Folowing development in acetone during 8 min, used so far, separates MGDG and DGDG within one joint peak (which was often the largest peak among others in the majority of seawater samples [8, 39]). It means that acetone equally affects both, MGDG and DGDG migration from the rode origin, resulting in their uniform scan times.

Curve 1 in Fig. 1a represents chromatogram of MGDG (because of close similarity with MGDG the chromatogram for DGDG is not shown), spotted as a single lipid and developed in acetone during 8 min, at scan time from about 300–350 s/10. Curve 2 represents only slight migration of spotted SQDG after development in acetone for 8 min recorded at scan time 400 to 550 s/10 indicating that SQDG was insufficently affected by acetone. When both MGDG and SQDG were spotted (Fig. 1a, curve 3) on the same rode, partial scan of up to 400 s/10 would not influence SQDG which has to be developed by additional solution system.

Testing the influence of solvent system chloroform–methanol–25% ammonium hydroxide (50:50:5, v:v:v) on the SQDG elution after 40 min resulted in its migration on Chromarod to the position of 120–220 s/10 (Fig. 1b, curve 1). However, the small and irregular SQDG peak is an indication that the applied solvent system is not a good choice for SQDG elution. As there is no material left in the origin, there is considerable probability that the part of SQDG already run out from the rode. Further on, the scan time of SQDG in this elution system coincides with scan time of PG (Fig. 1b, curve 2). Thus, after spotting of both SQDG and PG and elution in chloroform–methanol–ammonium hydroxide (50:50:5, v:v:v) solvent system their peaks overlapped (Fig. 1b, curve 3). This finding pointed to the serious threat that the former application of above elution system in standard protocol of the marine lipid analysis could lead to the overestimation of the PG quantity.

Therefore, a new protocol for SQDG determination was needed. Parrish et al. [22] published on SQDG separation using a solvent system acetone–formic acid (49:1, v/v) during 30 min, where they observed occurrence of small SQDG peak. Following them (Fig. 2, curve 1) SQDG standard was also developed as a small SQDG peak at scan time 200–300 s/10. However, this separation resulted in a huge SQDG peak at the origin region (scan time about 530 s/10) suggesting that above solvent system is not adequately for complete SQDG elution.

With the aim to find a new solvent system appropriate for total SQDG elution, we tested different solvent combinations and different elution and scan times for each of them. General knowledge gained is that chloroform does not influence the SQDG migration from the origin, and that acetone is more effective, but results in SQDG spreading through the whole rod. Therefore a solvent system was needed to constrict SQDG migration resulting in the appearance of a unique and regular separation peak. This was managed by adding methanol and formic acid to the mixture of acetone and chloroform. Formic acid as a buffer component is a common additive of mobile phases when using LC separations when pH must be controlled to achieve separation of target compounds with acid-basic properties. In many cases, real buffering is not as critical as having a generally acidic mobile phase. It is considered that the silica surface of Chromarods is slightly acidic and ionized silanol groups interact through ion-exchange with ionized bases giving rise to a peak tailing. Thus, when the mobile phase pH is ≤3, the ionization of carboxylic acids is suppressed, minimizing the possible ionic interactions with the silica surface and generally increasing retention. On the other hand direct esterification of alcohols with carboxylic acids is a readily reversible process and the removal of the water by-product during the course of the reaction is the approach to maximize the yield of ester [40]. Thus, the addition of low concentration of formic acid to a mobile phase containing methanol in this study does not favour esterification in solvent system niether the reaction of formic acid with the glycolipids as analytes with free hydroxyl groups. The solvent system acetone–chloroform–methanol–formic acid (33:33:33:0.6, v:v:v:v) appeared to be optimal for the efficient SQDG elution (Fig. 2, curve 2). Adding more than 33% of acetone to this solvent system caused again a broadening of the SQDG peak.

Further testing was performed to test the solvent system acetone–chloroform–methanol–formic acid (33:33:33:0.6, v:v:v:v) on simultaneous PG co-elution. Spotting of SQDG and PG, and their development for 40 min in proposed solvent system, lead to the efficient separation of SQDG and PG (Fig. 3). Scan times of SQDG and PG in above conditions are from 20 to 100 s/10 and from 100 to 250 s/10, respectively.

This development has another advantage over the previous commonly used development systems described in Section 2.3. for the separation of polar phospholipids. Namely, PG and PE were often insufficiently separated in chloroform–methanol–ammonium hydroxide (50:50:5, v:v:v) leading to partial peak overlapping and difficulties in obtaining reproducible quantification of two lipid classes. The solvent system acetone–chloroform–methanol–formic acid (33:33:33:0.6, v:v:v:v) do not affect PE migration. Thus, complete PE development is performed by applying the final solvent system *i.e.* chloroform–methanol–ammonium hydroxide (50:50:5, v:v:v) for 30 min. The separated elution of PG and PE in two mentioned solvent systems enables enhanced determination of both PG and PE.

Different solvent systems for the separation and detection of MGDG and DGDG were tested as well. Solvent system chloroform–acetone (72:28, v:v) during 30 min enabled satisfactory elution of these two glycolipids (Fig. 4). Scan times of MGDG and DGDG in these conditions are from 40 to 100 s/10 and from 120 to 200 s/10, respectively. This solvent system does not influence simultaneous migration of SQDG and other more polar lipids from the rode origin (data not shown).

Our results demonstrate a progress in detection of particular glycolipids but also an overall improvement in separation of polar lipids, thus, we propose a new separation protocol for the analysis of 18 lipid classes in natural samples as shown in Fig. 5a. An example of development of 18 lipid classes using new procedure is shown in Fig 5b.

3.2. Calibrations

In above proposed protocol our approach is directly related to the novelty in separation and detection of MGDG, DGDG, SQDG and PG, thus, their calibration was performed following here described chloroform–acetone (72:28, v:v) and acetone–chloroform–methanol–formic acid (33:33:33:0.6, v:v:v:v) development steps. To obtain calibration curves of MGDG, DGDG, SQDG and PG, the mixture of four lipids of increasing masses (0.2 – 4 μg) was spotted and developed in the two successive newly proposed solvent systems. First development lasted 30 min in chloroform–acetone (72:28, v:v). The partial scan enabled the detection of MGDG and DGDG peaks. The redevelopment for 40 min in acetone–chloroform–methanol–formic acid (33:33:33:0.6, v:v:v:v) system allowed successive detection of SQDG and PG.

The lipid quantification was achieved using calibration curves obtained for each standard by plotting peak area against lipid amount spotted. Most of calibration curves reported in the literature for Iatroscan are fitted by a power low equation for loads ranging from 1 to 10

μg of standard lipid as for such large loadings FID responce is not linear [22, 23]. The responses for MGDG, DGDG, SQDG and PG, fitted by power low calibration curves, are shown in Fig. 6. The equations (with high values of coefficient of determination, R^2>0.99) and inverse functions for determination of lipid mass are:

$$A_{SQDG} = 1422.29 \times m_{SQDG}^{1.54} \ (R^2=0.9978) \quad \text{(Eq. 5)}$$

$$m_{SQDG} = (A_{SQDG}/1422.29)^{(1/1.54)} \quad \text{(Eq. 6)}$$

$$A_{MGDG} = 638.77 \times m_{MGDG}^{1.87} \ (R^2=0.9959) \quad \text{(Eq. 7)}$$

$$m_{MGDG} = (A_{MGDG}/638.77)^{(1/1.87)} \quad \text{(Eq. 8)}$$

$$A_{DGDG} = 758.15 \times m_{DGDG}^{1.50} \ (R^2=0.9904) \quad \text{(Eq. 9)}$$

$$m_{DGDG} = (A_{DGDG}/758.15)^{(1/1.50)} \quad \text{(Eq. 10)}$$

$$A_{PG} = 444.28 \times m_{PG}^{2.38} \ (R^2=0.9975) \quad \text{(Eq. 11)}$$

$$m_{PG} = (A_{PG})/444.28)^{(1/2.38)} \quad \text{(Eq. 12)}$$

where „A“ and „m“ represent peak area and mass, respectively. The limit of detection (LOD) values of 0.06, 0.23, 0.10, 0.27 μg and limit of quantification (LOQ) values of 0.14, 0.40, 0.21, 0.46 μg were determined for SQDG, MGDG, DGDG and PG, respectively.

3.3. Determination of glycolipids and PG in seawater

To test the reliability of the improved development of SQDG, MGDG and DGDG, lipid mixtures extracted from the surface seawater samples (5 m depth) collected from different trophic status areas (nutrient richer Station 101 and low nutrient level station RV001 [41]) and seasons were analysed. As a new protocol for GL development directly affects the separation of PG, it was necessary to test PG separation from such complex mixtures as well. As it may be seen in Fig. 7., the TLC-FID chromatograms of real samples show appropriate separation of particular four lipid classes. The scan times for SQDG and PG concur for the real samples and standard compounds proving reliable analysis of particular lipid classes. However, in the case of seawater MGDG and DGDG the scan times are slightly shifted to longer times in comparison to the standards. Because of that there is potential threat that observed peaks in seawater samples are some other natural lipid compounds co-eluted at the scan rates similar to standard glycolipid compounds. On the other hand, in seawater a spectrum of glycolipids co-exist differencing in saturated/unsaturated fatty acid compositions at the glycerol backbone. So, there indeed exists a presumption that dominant seawater glycolipids differ from selected MGDG and DGDG standards.

To prove the assumption on inappropriate standards for MGDG and DGDG Iatroscan detection of marine lipids, we have developed those glycolipids on the rods in chloroform–acetone (72:28, v:v) from the northern Adriatic sample, station 107, 5 m, February 2015. Afterwards, we separately extracted developed lipids from the rode on positions of scan time around 100 s/10 (Fraction I) and 300 s/10 (Fraction II) by dichloromethane and analyzed composition with molecular level sensitive ESI MS and MS/MS. Major peaks observed within two *m/z* ranges, 750-850 (Fraction I) and 900-1000 (Fraction II), are selected for collision induced disociation (CID) fragmentation. The MGDG and DGDG lipids from the two fractions were identified analyzing fragmentation paterns and product ions. The positions of the acyl chains (*sn*-1 or *sn*-2) were determined by their relative percent composition according to the procedure established by Guella *et al*. [38]. Thus, ESI MS and MS/MS analysis of the lipids collected within the Fraction I shows MGDG compounds (Fig. 8a). At the same time lipids from Fraction II contain DGDGs (Fig. 8b). In respect to identify ions with *m/z* 921, 949 and 977 having the highest intensities (Fig. 8b) we have performed MS/MS analysis but recorded fragments could not be assigned to possible DGDG fragment [38] and those MS peaks are not identify as DGDG lipids. Those results confirm that indeed seawater MGDG and DGDG compounds have been developed by our improved TLC approach and that in the same develompment conditions natural marine glycolipid mixture elute a bit later than the used single standard compounds. As there is no marine MGDG and DGDG commercially available standards in this step we may accept that the scan times for the developed natural MGDG and DGDG are 100 and 300 s/10, respectively. The same scan times for the MGDG and DGDG are obtained for the different samples collected in the north Atlantic and middle Adriatic. Next step should involve development of the semi-preparative HPLC protocol for isolation and concentration of particular glycolipids from natural seawater samples and their additional analysis with the aim to be used as authentic standards in marine lipid chromatographic analyses.

The evaluated concentrations (percentage of total lipid) of the SQDG, MGDG, DGDG and PG (Eq. 4) in the sample from station 101, from May 2013 were 3.7 μg L^{-1} (11.5%), 3.0 μg L^{-1} (9.5%), 1.0 μg L^{-1} (3.2%), and 7.4 μg L^{-1} (23.0%), respectively. The evaluated concentrations in the sample from station RV 001, August 2013, are 2.4 μg L^{-1} (9.0%), 2.6 μg L^{-1} (9.5%), 0.3 μg L^{-1} (1.2%), and 4.5 μg L^{-1} (16.5%), respectively. The standard deviation determined from duplicate runs for the sample from station 101 accounted for 4.9%, 4.1%, 0.5%, and 4.9% of the relative abundance of SQDG, MGDG, DGDG and PG, respectively,

while for the sample from station RV001 accounted for 4.4%, 14.6%, 3.7%, and 1.0% of the relative abundance of SQDG, MGDG, DGDG and PG, respectively.

Investigation of marine lipids is important on many aspects. Characterization of marine lipids on a molecular level enables their use as good geochemical markers for the identification of different sources and processes of organic matter in the sea [7, 29, 36]. Recently it was shown that marine plankton developed adaptation mechanisms to environmental stress by glycolipid accumulation [42].

4. Conclusions

Our results introduce improved TLC protocol reliable for the separation of particular glycolipids i.e. SQDG, MGDG and DGDG, together with PG as shown on the example of complex lipid mixture from the real seawater samples. Such an approach extends the power of existing TLC-FID protocol for the analysis of lipids which altogether ensure detection and quantification of 18 lipid classes. Although improved separation scheme requires an additional 1h of working time extension in comparison to the previous protocol, it directly enables gaining a broad marine/freshwater system overview as a possible start for more detail investigations of the system of interest by using molecular level sensitive techniques (e.g. LC/MS).

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Figure captions

Fig. 1. a) Chromatograms of MGDG standard (1.33 μg) (full line 1), of SQDG standard (2 μg) (dash line 2), and mixture of MGDG and SQDG standards (1.33 μg and 2 μg, respectively) (dot line 3) developed during 8 min in acetone. b) Chromatograms of SQDG standard (1 μg) (full line 1), of PG standard (1.2 μg) (dash line 2), and mixture of SQDG and PG standards (1 μg and 1.2 μg, respectively) (dot line 3) developed during 40 min in chloroform–methanol–ammonium hydroxide (50:50:5, v:v:v).

Fig. 2. Chromatograms of SQDG standard developed during 30 min in acetone–formic acid (49:1, v/v) (2.0 μg) (full line 1) and during 40 min in acetone–chloroform–methanol–formic acid (33:33:33:0.6, v:v:v:v) (0.84 μg) (dash line 2).

Fig. 3. Chromatograms of 0.3 μg and 0.8 μg SQDG standard (peaks 1 and 1′), and 0.3 μg and 0.8 μg PG standard (peaks 2 and 2′) developed during 40 min in acetone–chloroform–methanol–formic acid (33:33:33:0.6, v:v:v:v).

Fig. 4. Chromatograms of 0.8 μg and 1.8 μg MGDG standard (peaks 1 and 1′), and 0.8 μg and 1.8 μg DGDG standard (peaks 2 and 2′) developed during 30 min in chloroform–acetone (72:28, v:v) solvent systems.

Fig. 5. a) Scheme representing new separation protocol for the analysis of 18 lipid classes; b) chromatograms of 18 lipid classes developed with the total procedure obtained for the northern Adriatic Sea sample, station 101, 5 m, May 2013. Abreviations: 1-HC, 2-WE, wax esters and steryl esters; 3-ME, fatty acid methyl esters (not detected in the sample); 5-KET, ketone; 6-TG, triacylglycerols; 7-FFA, free fatty acids; 8-ALC, alcohols; 9-1,3 DG, 1,3–diacylglycerols; 10-ST, sterols; 11-1,2 DG, 1,2–diacylglycerols; 12-PIG, pigments; 13-MG, monoacylglycerols; 14-MGDG, monogalactosyldiacylglycerols; 15-DGDG, digalactosyldiacylglycerols, 16-SQDG, sulfoquinovosyldiacylglycerols; 17-PG, mono– and di–phosphatidylglycerols; 18-PE, phosphatidylethanolamines and 19-PC, phosphatidylcholines.

Fig. 6. Calibration curves for SQDG (triangles), MGDG (squares), DGDG (circles) and PG (stars).

Fig. 7. (a) Fractionation of MGDG and DGDG and (b) SQDG and PG from the lipid extracts of the northern Adriatic Sea samples following new separation scheme; station 101, 5 m, May 2013 (dash lines, peaks 1–4, respectively) and station RV001, 5 m, August 2013 (full lines, peaks 1′–4′, respectively). Inset in (a) represent magnified peak 2′.

Fig. 8. MGDG and DGDG fractions of the lipid mixture extracted from the Northern Adriatic Sea (station 107, 5 m, February 2015): a) Positive-ion ESI mass spectra of MGDG and b)

Positive-ion ESI mass spectra of DGDG. For both fractions mass spectra are recorded in the range of *m/z* 100-1200. Here are presented parts of the spectra where peaks of the interest are detected.

Fig. 1

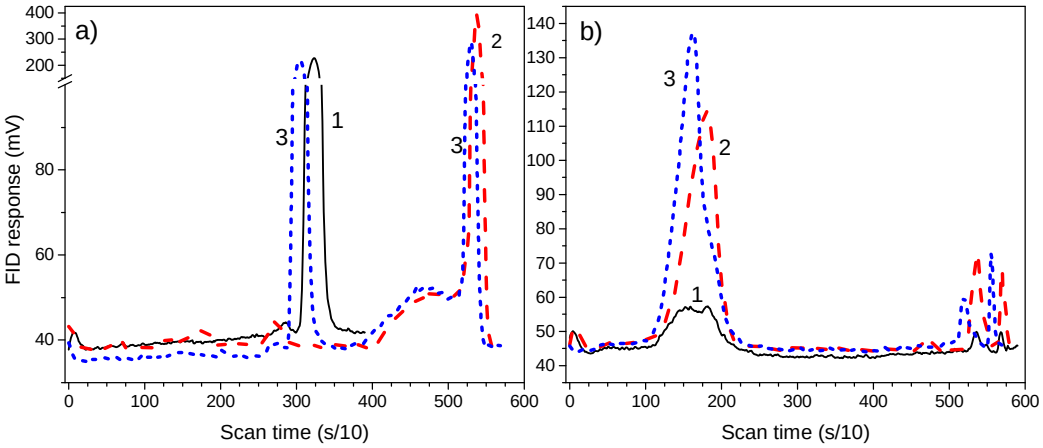
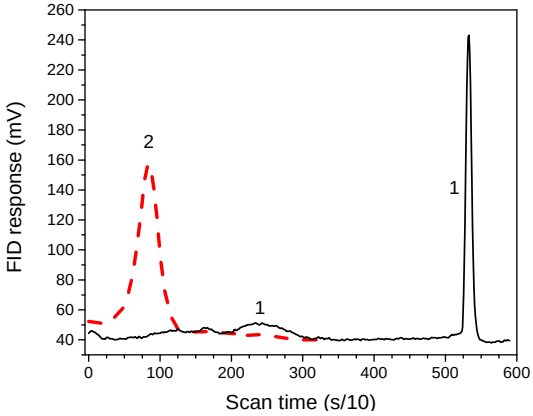

a)
b)
FID response (mV)
Scan time (s/10)
1
2
3

Fig. 2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Scan time (s/10)
1
2

Fig. 3

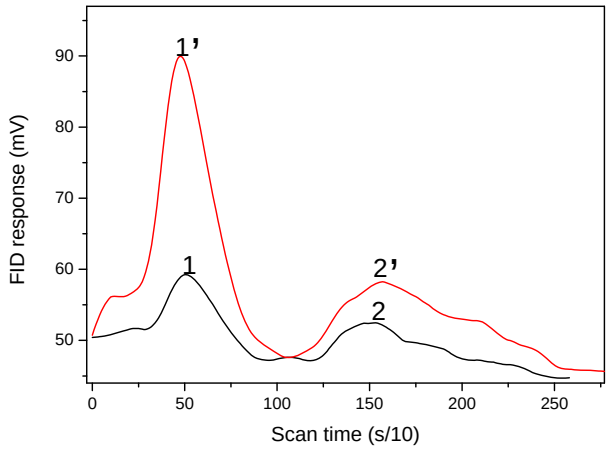

FID response (mV)
Scan time (s/10)
1'
2'
1
2

Fig. 4

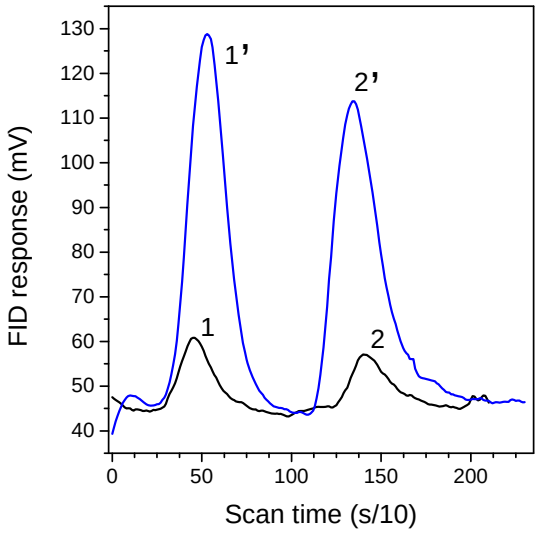

FID response (mV)
Scan time (s/10)
1'
2'
1
2

Fig. 5

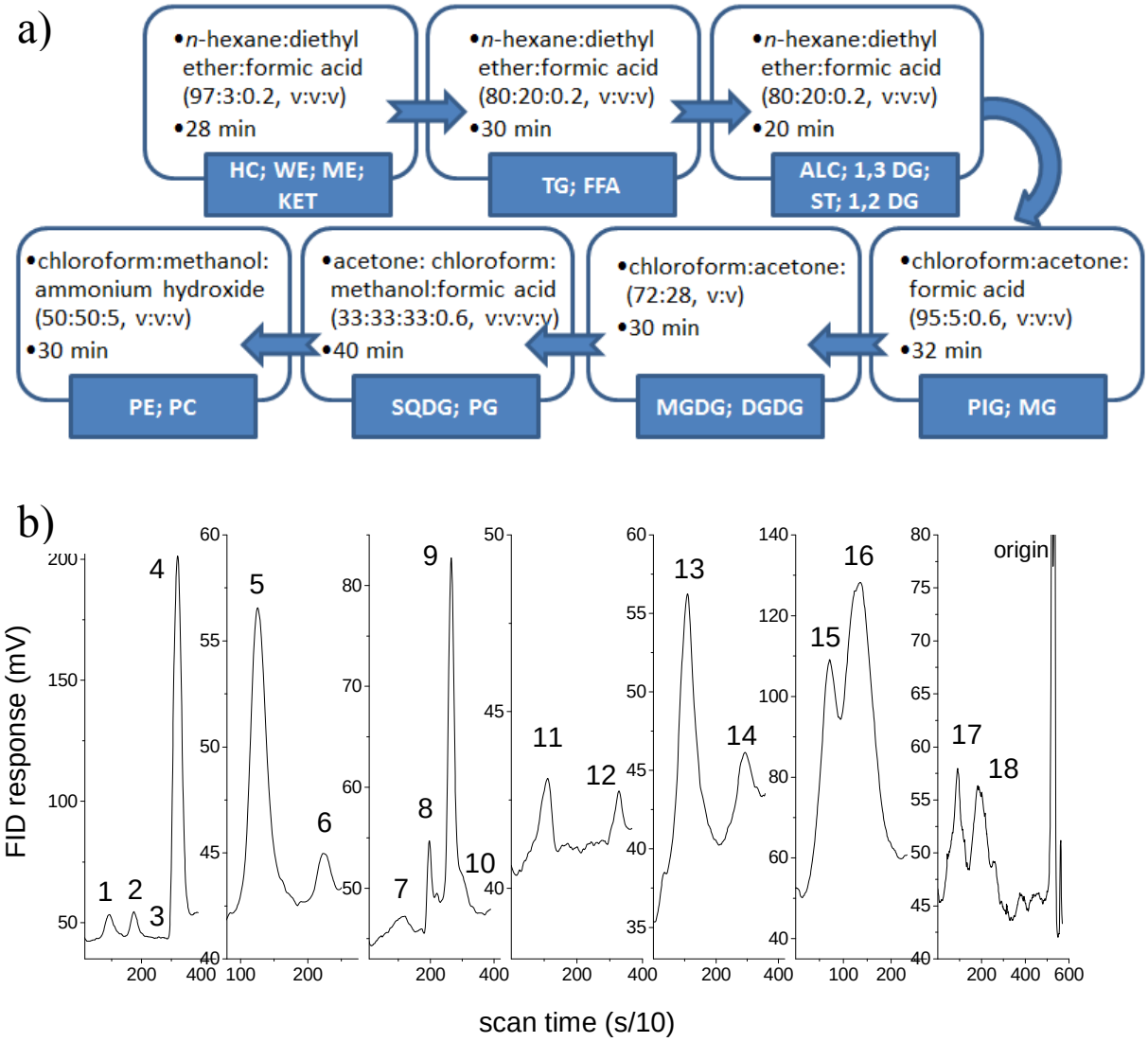

a)
•n-hexane:diethyl ether:formic acid (97:3:0.2, v:v:v)
•28 min
HC; WE; ME; KET
•n-hexane:diethyl ether:formic acid (80:20:0.2, v:v:v)
•30 min
TG; FFA
•n-hexane:diethyl ether:formic acid (80:20:0.2, v:v:v)
•20 min
ALC; 1,3 DG; ST; 1,2 DG
•chloroform:acetone: formic acid (95:5:0.6, v:v:v)
•32 min
PIG; MG
•chloroform:acetone: (72:28, v:v)
•30 min
MGDG; DGDG
•acetone: chloroform: methanol:formic acid (33:33:33:0.6, v:v:v:v)
•40 min
SQDG; PG
•chloroform:methanol: ammonium hydroxide (50:50:5, v:v:v)
•30 min
PE; PC
b)
FID response (mV)
scan time (s/10)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
origin

Fig. 6

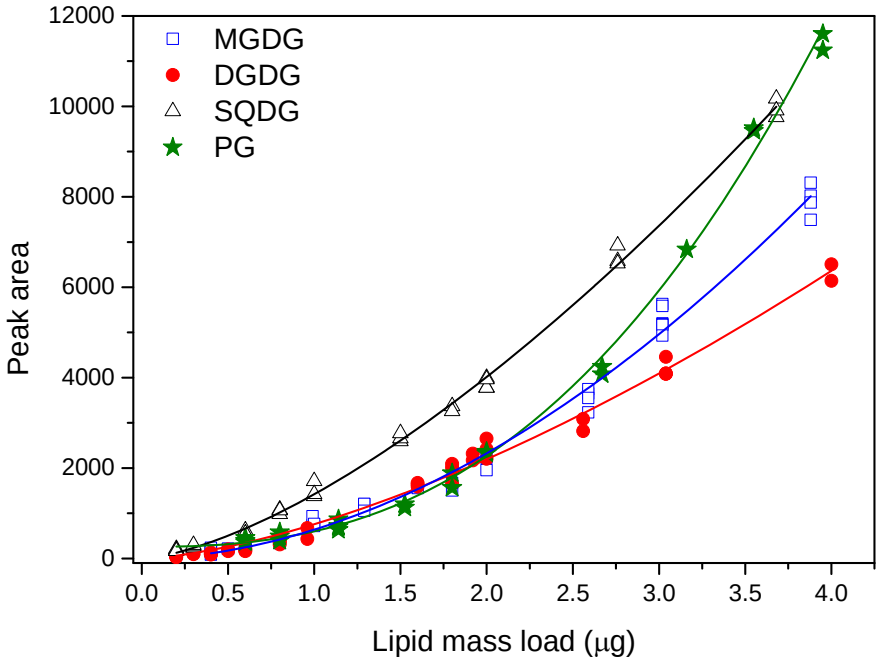

MGDG
DGDG
SQDG
PG
Peak area
Lipid mass load (μg)

Fig. 7

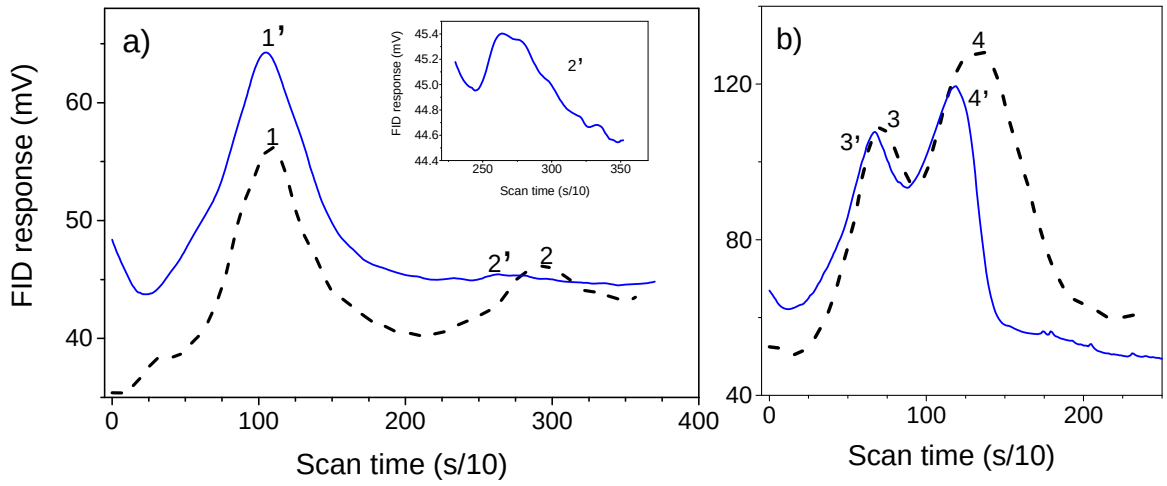
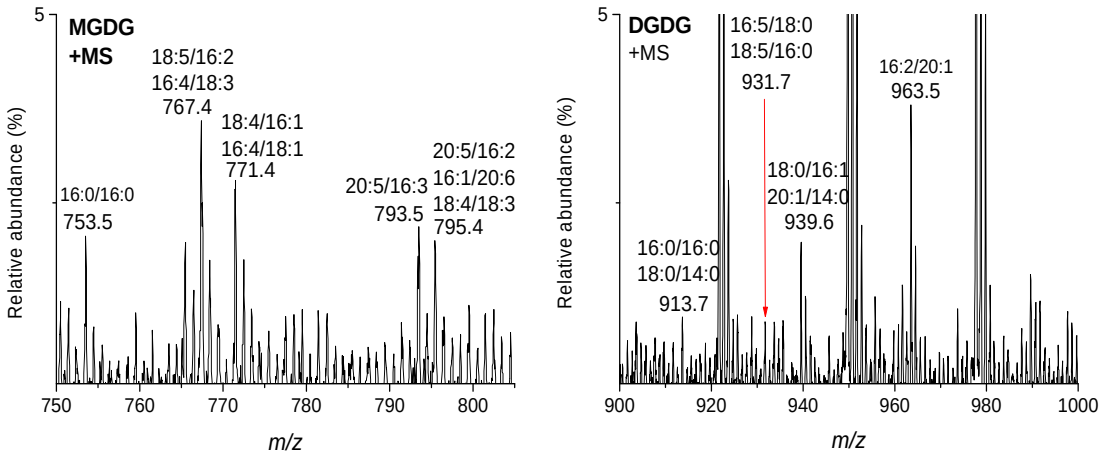

a)
b)
FID response (mV)
Scan time (s/10)
1'
1
2'
2
3'
3
4'
4

Fig. 8


MGDG
+MS
18:5/16:2
16:4/18:3
767.4
18:4/16:1
16:4/18:1
771.4
16:0/16:0
753.5
20:5/16:3
793.5
20:5/16:2
16:1/20:6
18:4/18:3
795.4
Relative abundance (%)
m/z
DGDG
+MS
16:5/18:0
18:5/16:0
931.7
16:2/20:1
963.5
18:0/16:1
20:1/14:0
939.6
16:0/16:0
18:0/14:0
913.7
Relative abundance (%)
m/z