Chapter 2

A simple and universal method for the separation and identification of phospholipid molecular species

Kim Retra[1,2], Onno B. Bleijerveld[1], Renske A. van Gestel[1], Aloysius G.M. Tielens[1], Jaap J. van Hellemond[1], Jos F. Brouwers[1]

[1] Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
[2] Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands

submitted

Abstract

One of the major challenges in lipidomics is to obtain as much information about the lipidome as possible. Here, we present a novel HPLC-MS/MS method that separates molecular species of all phospholipid classes in one single run. The method is sensitive, robust and allows lipid fingerprinting using full scan mass spectrometry, as well as lipid class specific scanning. Excellent separation of isobaric- and even isomeric species is achieved, and original levels of lysolipids can be determined without interference from lysolipids formed from diacyl species by source fragmentation. Using this method, more than 400 phospholipid species were identified and quantified in crude lipid extracts from rat liver and the parasitic helminth *Schistosoma mansoni*.

Introduction

Phospholipids play a multitude of roles in biological systems. Phospholipids are involved in cell signaling, either directly (phosphoinositides, platelet activating factor, lysophosphatidic acid (monoacylglycerophosphatidic acid, lysoGPA), lysophosphatidylserine (monoacylglycerophosphoserine, lysoGPSer), etc.) or indirectly, by supplying precursors for active compounds like prostaglandins and diacylglycerols. Phospholipids are main constituents of biological membranes and are thus involved in intracellular and extracellular compartmentalization. Furthermore, phospholipids play important roles in the maintenance of lipid homeostasis, in host-pathogen interactions and in (membrane-) protein function [1-5].

Phospholipids can be classified according to their polar head group, their backbone, the fatty acids that are linked to this backbone and the type of linkage at the *sn*-1 and *sn*-2 position of the glycerol backbone. Many hundreds of molecular species have been identified in cellular lipidomes [5-8] and this number only increases as analytical methods are gaining power and sensitivity.

Functions of phospholipids do, however, not only depend on the presence or absence of certain molecular species, but more on their concentration and the total lipid context. For instance, clustering of specific membrane proteins is supposedly driven by organization of (phospho-)lipids into micro-domains, with the phospholipids in these domains sharing a low level of unsaturation [9]. Lipidomics research is, therefore, more and more focusing at the systems-level, rather than investigating the function of just one lipid at a time.

To enable systems-level lipidomics, the lipidome should be defined in as much detail as possible. Hence, not only the lipid class, but also the exact molecular species composition within each class should be defined to allow an unbiased search for biomarkers. In the past years, it has become clear that both HPLC and (tandem) mass spectrometry are indispensable tools in lipidomics [10,11].

Here, we describe and apply a universal method for the separation of molecular species of phospholipids, their lysoforms and free fatty acids (FFA), and their subsequent identification by on-line, data-driven tandem mass spectrometry. The method exploits the possibilities of state-of-the-art mass spectrometry, and relies on HPLC to separate lipid species that can not be identified by mass spectrometry alone.

Materials and Methods

Chemicals and materials

All solvents used were from Lab-Scan, Dublin, Ireland and were of HPLC grade.
GPGro(18:1/18:1) and di-oleoyl lysobisphosphatidic acid (LBPA, also named bis-monoacylphosphatidic acid (BMP)) were purchased from Avanti polar lipids (Alabaster, AL, USA). Ammonium acetate, serine and silica-G for column chromatography were purchased from Merck (Darmstadt, Germany). Hydrochloric acid was purchased from Baker (St. Louis, MO, USA).

Preparation of samples

Schistosoma mansoni parasites were isolated from ether anesthetized hamsters, 45-48 days after infection, by perfusion of the heart at 37°C with 0.9% NaCl. Schistosomes were washed and homogenized in 0.9% NaCl (approx. 20% v/v). Livers isolated from male Wistar rats were homogenized in 0.9% NaCl (approx. 20% v/v). Lipids were extracted from both samples according to the method of Bligh and Dyer [12], with the minor modification that 0.5% 6M HCl was added to the second chloroform wash to increase recovery of acidic phospholipids. The phospholipids and free fatty acids were separated from neutral lipids (cholesterol, cholesterol esters and triacylglycerols) by fractionation on a 1 ml silica column prepared from 0.063-0.200mm silica 60 (Merck, Darmstadt, Germany). Lipid extracts were dissolved in chloroform and loaded on top of the silica column and eluted successively with acetone (4 volumes) and methanol (4 volumes). The last fraction, which contained the purified phospholipids, was dried under nitrogen and stored at -20°C until HPLC-MS analysis. A GPSer containing fraction was made from *S. mansoni* adult worms as described before [13].

High Performance Liquid Chromatography

The purified phospholipids were dissolved in methanol : acetonitrile : chloroform : water (46 : 20 : 17 : 17, v/v/v/v). Separation of molecular species was performed on a Synergi 4 µm MAX-RP 18 A column (250 x 3 mm) (Phenomenex, CA, USA). Elution was performed with a linear gradient of water in methanol/acetonitrile (60 : 40, v/v) decreasing from 12.5% to 0% in 25 min, followed by further isocratic elution for another 25 minutes. The flow rate was kept constant at 0.425 ml·min^{-1}

and 1 µM serine and 2.5 mM ammonium acetate were used in all solvents as additives.

Mass spectrometry

Mass spectrometry of lipids was performed using electrospray ionization, on a 4000QTRAP system (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Source temperature was set to 450°C and nitrogen was used as curtain gas. The declustering potential was optimized using lipid standards. The optimal collision energy was dependent on the type of experiment performed and was set to +45V (precursor scanning *m/z* 184), -45V (precursor scanning *m/z* -196), +35V (neutral loss 141), -30V (precursor scanning *m/z* -241), and -40V (neutral loss scanning 87 Da) respectively. For quantification of molecular species, samples were measured in multiple reaction monitoring mode (MRM), monitoring for 95 head-group specific mass transitions with a total dwell time of 1 s, using the same settings as above.

Data analysis

Data analysis was performed with Analyst^{tm} v 1.4.1 software (Sciex, Toronto, Canada) including the LipidProfiler v1.0 script and the metabolomics export script (Sciex, Toronto, Canada).

Results and Discussion

Chromatography

Current chromatographic methods for the separation of lipids can be classified in two categories based on the stationary phase that is used (recently reviewed in [14]). The first category uses polar (normal phase) column material such as silica or diol modified-silica [6,15,16]. With these methods, phospholipids are separated primarily on the interaction of the polar head group with the column material, and the (crude) lipid extracts are thus separated into lipid classes. With the most modern column materials, even some sub-fractionation of a lipid class into molecular species may be observed, but resolution of isobaric species (such as 16:0/22:4; 18:0/20:4 and 18:1/20:3) cannot be achieved by these methods [6].

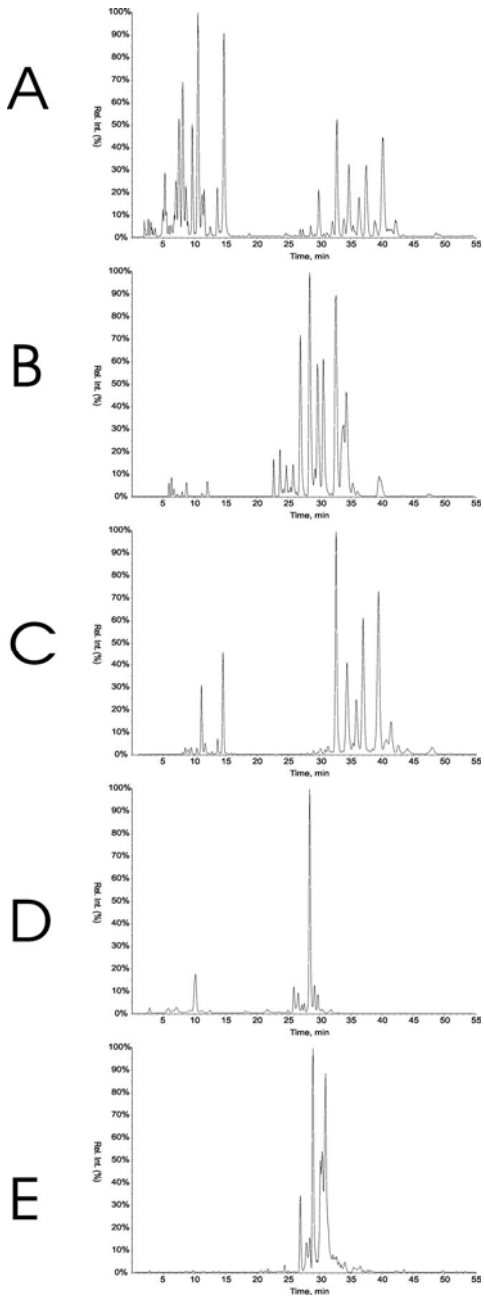

Figure 1: Analysis of a crude phospholipid extract of rat liver cells by HPLC-MS/MS. A: Base peak chromatogram as recorded by negative ionisation mass spectrometry. During the first 20 minutes, free fatty acids, lyso-lipids and other polar lipids elute. After 20 minutes, diradyl species elute. B: Precursor scanning for *m/z* +184, identifying GPCho and SM species. C: Neutral loss of +141 amu, identifying GPEtn species. D: Precursor scanning of *m/z* -241, identifying GPIns species. E: Neutral loss scanning of -87 amu, identifying GPSer species.

The second category, reversed-phase chromatography, typically uses a C18-modified stationary phase and separates lipids on the hydrophobic interaction between radyl chains and stationary phase. Advantage of reversed-phase chromatography is the high resolving power, and two lipid species that only differ in the intra-molecular position of a single double bond can be separated [17]. However, most reversed-phase methods are optimized for a single or just a few lipid classes [18-20] due to the need for class-specific additives to block aspecific interaction between headgroup and stationary phase, which makes these methods less suitable for the analysis of crude lipid extracts.
Our goal was to design a method that is compatible with crude lipid extracts, but also provides a high resolution species separation. In Figure 1A, the base peak chromatogram is shown as recorded during the separation of a crude lipid extract from rat liver with this newly developed reversed-phase method. This method combines the main advantages of the two methods described above: it is applicable to crude lipid extracts and benefits from the high resolving power of a reversed-phase stationary phase. The combination of latest generation reversed-phase material and the use of ammonium acetate and serine as additives in the mobile phase, made this method lipid class independent, and therefore, universally applicable for the separation of molecular species of phospholipids.

Using the freely available Metabolomics Export Script (Sciex, Toronto, Canada), more than 400 compounds having a signal to noise ratio exceeding 9, were identified in the sample of Figure 1A. Depending on the amount and origin of a lipid extract, the number of detected compounds may come close to a thousand (data not shown).

Lipid fingerprinting by full scan HPLC-MS

The negative charge on the phosphate(di-)ester of phospholipids has made negative ionization common practice in mass spectrometry of phospholipids. Phosphatidylglycerol (GPGro), phosphatidylinoositol (GPIns), GPSer, GPA and phosphoinositides are nearly exclusively measured in negative mode [21]. Phosphatidyletanolamine (GPEtn) may be measured both in positive- or in negative mode. In the positive mode, a higher sensitivity can be achieved (with the exception of ether-linked species) but the negative ionisation mode gives more informative ions upon fragmentation [22]. Phosphatidylcholine (GPCho) and

sphingomyeline are most often measured in positive mode, which is related to the positively charged quaternary nitrogen in the head group. Nevertheless, also these lipid classes can, and were, detected in negative mode ionization (see below) [17].

Lipid class specific scanning

Full lipid fingerprinting by HPLC-MS/MS generates large amounts of data, and depending on the experiments at hand, there will often be a significant amount of redundancy in the data. For instance, if one is interested only in the GPCho lipid class, the presence of ions from other lipid classes is often confusing. Obviously, this may be prevented by isolation of GPCho preceding HPLC analysis, but tandem mass spectrometry offers a more efficient and attractive alternative in the exploitation of head group specific fragmentation.

Head group specific fragmentation has been described in detail elsewhere [10,23]. In brief, each phospholipid class shows fragmentation at the phospho(di-)ester linkage, resulting in a diagnostic fragment ion. For GPCho, this diagnostic ion is phosphorylcholine ($(HO)_2POOCH_2CH_2N(CH_3)_3^+$, *m/z* +184) and scanning for precursor ions that produce a fragment of *m/z* +184 will visualize GPCho and SM molecules only. Similar scans can be programmed to specifically visualize GPEtn, GPIns, GPSer, GPGro and even phosphoinositides. This head group specific scanning can be done on-line, and Figure 1B-E shows phospholipid-class specific scanning during the chromatography of a crude lipid extract of rat liver. Thus, head group-specific scanning reduced the complexity of the data.

Lysolipids and preparative HPLC

Assessment of lysolipids and FFA in crude lipid extracts by MS has been troublesome due to the fact that lysolipids and FFA may be artificially produced from diacyllipids during the ionization process (so called source fragmentation). In fact, the difference in efficiencies in which the *sn*-1 and *sn*-2 fatty acyls are lost from the glycerol backbone has successfully been used to assign positional isomers of phospholipids [24-26]. However, when FFA, lysolipids and diacyllipids are simultaneously ionized, it is not possible to discriminate between endogenous lysolipid (or FFA) and lysolipid (or FFA) resulting from source fragmentation. Notably, during the first 20 min of chromatography, the FFA and lysoforms of phospholipids elute, followed

by elution of the diacyl species (Figure 1A-E). In our method, FFA, lysolipids and diacyl species are now separated by HPLC prior to MS analysis, which allows accurate assessment of endogenous lysolipids and FFA in crude lipid extracts.

The high resolving power of reversed-phase HPLC enables isolation of highly purified lipid species. With the exception of the low amount of serine, the mobile phase is fully volatile, so concentration of purified compounds is easily achieved. As an example, Figure 2 shows the separation of (lyso)GPSer species, isolated from the parasitic helminth *Schistosoma mansoni*, and partially purified by anion exchange chromatography. In contrast to rat liver GPSer (Figure 1E), *S. mansoni* contained relative high amounts of lysoGPSer and it has been suggested that these lyso species are responsible for the T-cell hypo-responsiveness observed during chronic schistosome infections [4]. It is also known that this biological activity is not shared among all lysoGPSer species [4]. Using a post-column flow splitter, this method now enabled us to individually isolate all molecular species of lysoGPSer, and test these for their biological activity.

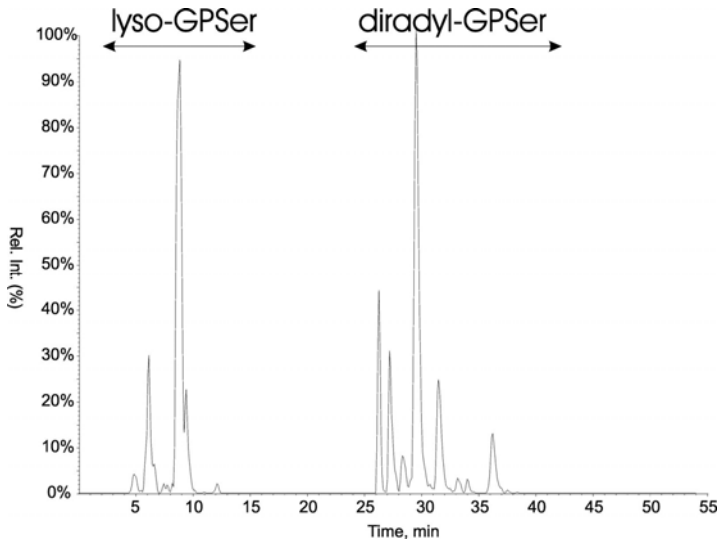

Figure 2: Neutral loss scan of a purified GPSer fraction from *S. mansoni*, showing clear separation between lysophospholipids and diradylphospholipids. With a simple post-column flow splitter the biological active lysolipids can be isolated without contamination of diradyl species.

Isobaric and isomeric species resolved by HPLC

One of the biggest challenges in lipidomics is the correct assignment of isobaric species. Isobaric lipid species have the same (nominal) mass, but contain different fatty acids and/or head groups, and therefore, these phospholipids will have distinct biological and biophysical properties.

In Figure 3A, a small fraction of the contour plot (plotting measured *m/z* values against elution times) shows several lipids, of which four (marked b-e) have the identical *m/z* ratio (766.7 amu). These isobaric lipids elute well separated within a six-minute time window. In Figure 3B-E the on-line recorded product spectra of the respective lipids are shown. Based on the observed headgroup fragments and the carboxylate ions, the lipid species were identified as *N,N*-dimethyl GPEtn(16:0/20:4) (B); GPEtn(20:2/18:2) (C); GPEtn(16:0/22:4) (D) and GPEtn(18:0/20:4) (E) respectively.

It should be noted that the detected *N,N*-dimethyl GPEtn(16:0/20:4) is derived from GPCho(16:0/20:4) after loss of CH_3^+ from the choline headgroup by source fragmentation. This is a well-known fragmentation occurring during negative mode ionization of phosphatidylcholine and illustrates an additional advantage of this method. In positive mode, the abundant, and easily ionized GPCho, often dominates the mass spectrum, leading to ion suppression and reduced sensitivity for other lipid classes such as GPEtn. Due to the reduced ion formation from GPCho in the negative mode and the reduced lipid concentration during ionization in HPLC-MS, this problem does not occur in the method described here. Of course, careful calibration is needed to correct for differences in ionization efficiencies between phospholipid classes in negative mode when quantitative analysis is required.

As an alternative to HPLC-MS, others have resolved isobaric species using only tandem mass spectrometry [8,27]. These "MS-only" methods however, require extensive computational processing to correct for the contributions of the ^{13}C isotopes of more unsaturated species. For example, to correctly quantify the component (*m/z* 768.7) eluting at 41 minutes in Figure 3A without HPLC separation, corrections have to be made for the abundance of the isobaric component eluting approximately 1 minute later, but one also has to correct for the ^{13}C contributions of components B-E.

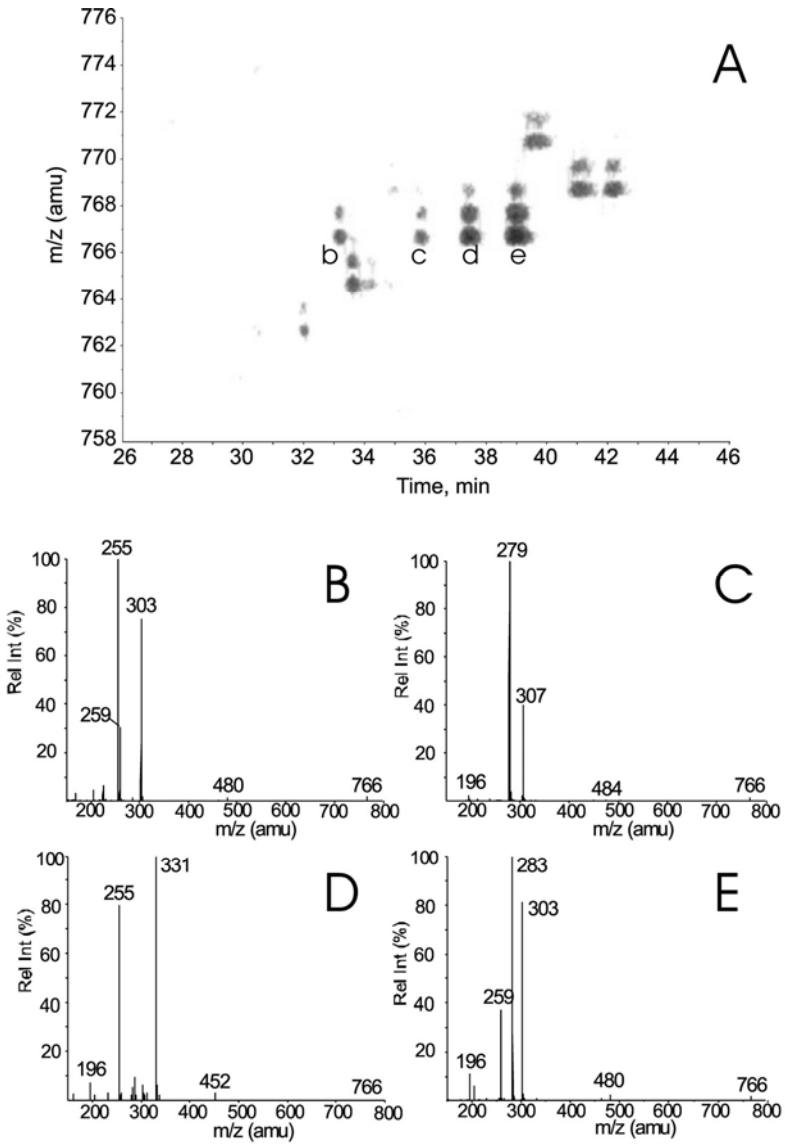

Figure 3: Detail of a contour plot (A) representing a part of the data from Figure 1. The separation of four isobaric species (labelled b-e) is shown in panel A. Product spectra identify these compounds as B: *N,N* dimethyl-GPEtn(16:0/20:4); C: GPEtn(18:2/20:2); D: GPEtn(16:0/22:4); E: GPEtn(18:0/20:4).

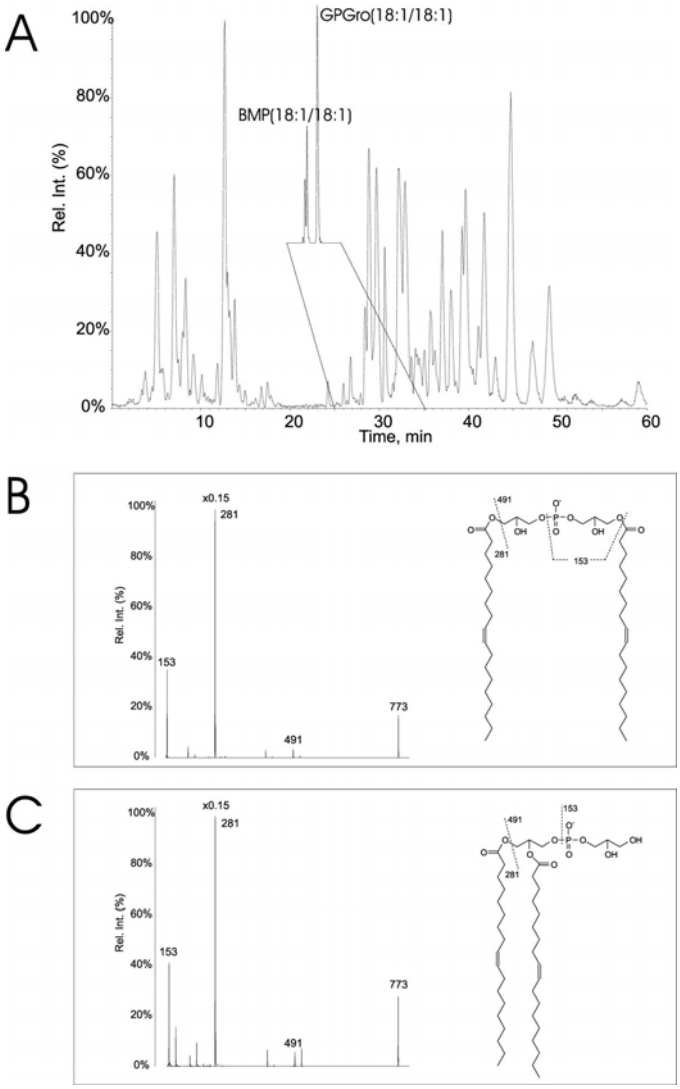

Figure 4: Analysis of a crude phospholipid extract of *S. mansoni,* spiked with GPGro(18:1/18:1) and 18:1/18:1 *bis*-monoacyl phosphatidic acid (BMP, also known as lysobisphosphatidic acid, LBPA). In panel A the base peak chromatogram is shown, and the insert shows the baseline separation of the two isobaric compounds. B: Fragmentation spectrum of BMP(18:1/18:1). C: Fragmentation spectrum of GPGro(18:1/18:1).

In Figure 4A the base peak chromatogram is shown recorded during chromatography of a total lipid extract of the parasitic helminth *Schistosoma mansoni*, that had been spiked with the isobaric compounds GPGro(18:1/18:1) and bis(mono-oleoylglycero)phosphate (BMP(18:1/18:1) also called lysobisphosphatidic acid (LBPA)). Despite the fact that these two compounds are structurally very similar (being build from two oleic acids, two glycerol and a phosphate group), their biological activity is very different. BMP is a marker for the late endocytic pathway, and it has been shown that this particular lipid plays a role in intra-membrane cholesterol transport [28,29] and endosome structure and function [30-32]. These functions are not shared between BMP(18:1/18:1) and GPGro(18:1/18:1). The insert in Figure 4A shows the extracted ion chromatogram of *m/z* 773.7 (the molecular ion of the compounds), demonstrating the clear base-line separation of the two compounds. The BMP even subfractionates into two peaks, likely to represent positional isomers of BMP [33,34]. The fact that HPLC is a prerequisite to discriminate between BMP and GPGro is further illustrated in Figure 4B-C, which show the fragmentation spectra of BMP and GPGro, respectively. These two fragmentation spectra are nearly identical, differing slightly in relative abundances of fragment ions only. This precludes accurate measurement of these two compounds in a mixture based on fragmentation spectra only.

Lipid species in rat liver and schistosomes

When using mass spectrometry for quantitative purposes, one has to consider the fact that the response factor of the machine (i.e. the amount of head group specific fragments produced at a given lipid species concentration) depends on the type of machine, the unsaturation of the species, the lipid concentration and the molecular weight (acyl length) of that species [35]. As a result, accurate molar quantification is only possible using a broad set of internal standards, closely resembling the species one wishes to quantify. In case of samples with an unknown composition, or when the sample contains unusual lipids such as in *S. mansoni*, this may prove problematic [6,17]. However, under the conditions described in this method, light (approximately 750 Da) and heavy (approximately 850 Da) molecular species had response factors that differed by less than 5% from the response factor of an average weight (approximately 800 Da) species, which is in good agreement with published results by others [23,35]. To

demonstrate the general applicability, we used the method above for the quantification of molecular species in rat liver and *Schistoma mansoni*, organisms with large differences in the relative abundance of various phospholipid species. By using on-line HPLC-MS/MS we identified over 400 compounds and quantified those contributing more than 0.5% to the total area under the curve (Table 1-4)*. Clear differences are seen between the two samples, reflecting the characteristic elongation of fatty acids obtained from the host by *S. mansoni* [36]. This chain elongation results in the relatively unusual fatty acid eicosenoic acid (20:1) being present in up to 20% in GPCho species of schistosomes (Table 1) and 22% in schistosomal GPEtn species (Table 2). The amount of 20:1 in the GPSer class was 14.5%, but the most notable characteristic of the molecular species of schistosome GPSer is the abundant presence of very long chain fatty acids: over 50% of the molecular species contained a fatty radyl of 22 carbon atoms or more, where, in rat liver, this was less than 10% (Table 3). GPIns contained only a trace amount of molecular species containing 20:1 (1.5%, Table 4), which is remarkable considering the high amounts of this schistosome-specific fatty acid in the other lipid classes. These data suggest that schistosomes either have highly specific (trans-)acylases responsible for the distinct acyl composition of the various phospholipid classes, or use very distinctive pools of diacylglycerol for the synthesis of the various glycerophospholipid classes. Considering the fact schistosomes are reported to have a high rate of acyl turnover [37], it is unlikely that lipid class species specificity obtained during phospholipid synthesis will be maintained. We are currently investigating the substrate specificity of schistosomal (trans-)acylases and exploring the likeliness that this parasitic characteristic may offer a candidate for drug development.

In conclusion, we have described a simple, rapid and broadly applicable technique for the separation and identification of phospholipid molecular species. Applying this method, allowed us to identify over 400 molecular species, and resulted in the identification of lipid class and parasite-specific characteristics.

* Tables can be found at the pages 48 to 53.

Acknowledgements

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Table 1: Molecular species of phosphatidylcholine detected in rat liver and *S. mansoni* adult worms. Species that comprise more than 0.5% of the total GPCho are shown. Percentages shown are the percentages of the total GPCho species mentioned in the table. Species are quantified by MRM with $[M+H]^+$ for MS1 and *m/z* 184 for MS2. Shown is the average ± standard deviation of a single lipid extract injected and analyzed in triplicate. Only species contributing more than 0.5% are indicated. * Less than 0.5%. # Unidentified.

m/z	**component**		**Abundance in rat liver (mole %)**	**Abundance in *S. mansoni* (mole %)**
720	16:0 / 16:0	1-alkyl, 2-acylGPCho	4.0 ± 0.30	2.8 ± 0.12
732	16:0 / 16:1	diacylGPCho	1.1 ± 0.08	1.6 ± 0.10
732	14:0 / 18:1	diacylGPCho	0.5 ± 0.07	*
734	16:0 / 16:0	diacylGPCho	9.0 ± 0.72	2.6 ± 0.27
746	16:0 / 18:0	1-*Z*-alkenyl, 2-acylGPCho	4.8 ± 0.12	*
758	16:0 / 18:2	diacylGPCho	5.8 ± 0.07	7.2 ± 0.32
760	18:0 / 16:1	diacylGPCho	*	4.6 ± 0.22
760	16:0 / 18:1	diacylGPCho	13.5 ± 0.29	2.8 ± 0.28
760	18:1 / 16:0	diacylGPCho	3.0 ± 0.20	*
762	16:0 / 18:0	diacylGPCho	9.8 ± 0.40	3.2 ± 0.10
774	16:0 / 19:1	diacylGPCho	*	2.2 ± 0.16
782	16:0 / 20:4	diacylGPCho	*	5.1 ± 0.09
784	18:1 / 18:2	diacylGPCho	*	3.5 ± 0.07
786	16:0 / 20:2 & 18:1 / 18:1	diacylGPCho diacylGPCho	*	6.6 ± 0.05
786	18:0 / 18:2	diacylGPCho	8.0 ± 0.31	*
786	18:1 / 18:1	diacylGPCho	1.0 ± 0.17	2.1 ± 0.12
788	16:0 / 20:1	diacylGPCho	*	10.0 ± 0.17
788	18:0 / 18:1	diacylGPCho	22.2 ± 0.46	4.3 ± 0.07
790	18:0 / 18:0	diacylGPCho	*	3.1 ± 0.17
790	16:0 / 20:0	diacylGPCho	1.5 ± 0.18	*
802	18:0 / 20:1	1-alkyl, 2-acylGPCho	*	0.9 ± 0.06
802	17:0 / 20:1	diacylGPCho	*	1.0 ± 0.03
806	16:0 / 22:6	diacylGPCho	1.6 ± 0.18	3.3 ± 0.16
808	18:1 / 20:4	diacylGPCho	*	2.5 ± 0.08
810	16:0 / 22:4	diacylGPCho	*	3.3 ± 0.20
810	18:0 / 20:4	diacylGPCho	4.4 ± 0.33	4.3 ± 0.13

812	20:1 / 18:2 & 18:1 / 20:2	diacylGPCho diacylGPCho	*	1.4 ± 0.04
812	18:0 / 20:3	diacylGPCho	0.8 ± 0.05	1.0 ± 0.02
814	18:1 / 20:1	diacylGPCho	*	3.1 ± 0.13
814	18:0 / 20:2	diacylGPCho	0.9 ± 0.11	*
816	16:0 / 22:1	diacylGPCho	1.6 ± 0.15	1.1 ± 0.08
830	18:2 / 22:6	diacylGPCho	0.6 ± 0.08	*
832	18:1 / 22:6	diacylGPCho	*	1.0 ± 0.02
834	20:2 / 20:4	diacylGPCho	*	1.0 ± 0.10
834	18:0 / 22:6	diacylGPCho	1.3 ± 0.09	2.6 ± 0.07
838	18:0 / 22:4	diacylGPCho	*	4.2 ± 0.33
840	18:0 / 22:3	diacylGPCho	*	0.9 ± 0.14
842	20:1 / 20:1	diacylGPCho	2.0 ± 0.23	2.3 ± 0.12
846	24:0 / 16:0	diacylGPCho	2.6 ± 0.22	1.4 ± 0.09
846	20:0 / 20:0	diacylGPCho	*	1.1 ± 0.09
848	#	diacylGPCho	*	1.1 ± 0.06
864	20:1 / 22:4	diacylGPCho	*	0.8 ± 0.14

Table 2: Molecular species of phosphatidylethanolamine detected in rat liver and in *S. mansoni* adult worms. Species that comprise more than 0.5% of the total GPEtn are shown. Percentages shown are the percentages of the total GPEtn species mentioned in the table. Species are quantified by MRM with $[M+H]^+$ for MS1 and $[M+H-141]^+$ for MS2. Shown is the average ± standard deviation of a single lipid extract injected and analyzed in triplicate. Only species contributing more than 0.5% are indicated. * Less than 0.5%. # Unidentified.

m/z	**component**		**Abundance in rat liver (mole %)**	**Abundance in *S. mansoni* (mole %)**
692	16:0 / 16:0	diacylGPEtn	0.7 ± 0.06	*
716	16:0 / 18:2	diacylGPEtn	7.1 ± 0.12	3.0 ± 0.05
718	16:0 / 18:1	diacylGPEtn	5.5 ± 0.11	2.3 ± 0.17
720	16:0 / 18:0	diacylGPEtn	4.0 ± 0.19	*
730	17:0 / 18:2	diacylGPEtn	0.7 ± 0.03	*
732	18:0 / 18:1	1-alkyl, 2-acylGPEtn	*	0.5 ± 0.08
732	17:0 / 18:1	diacylGPEtn	0.7 ± 0.05	*
734	17:0 / 18:0	diacylGPEtn	0.5 ± 0.01	*
740	16:0 / 20:4	diacylGPEtn	4.0 ± 0.10	0.7 ± 0.04
742	18:1 / 18:2 & 16:0 / 20:3	diacylGPEtn diacylGPEtn	*	3.1 ± 0,32
742	16:0 / 20:3	diacylGPEtn	2.2 ± 0.14	*
744	18:0 / 18:2	diacylGPEtn	*	9.2 ± 0.49
744	18:0 / 18:2 & 16:0 / 20:2	diacylGPEtn diacylGPEtn	12.1 ± 0.39	*
746	18:0 / 18:1 & 16:0 / 20:1	diacylGPEtn diacylGPEtn	8.4 ± 0.40	14.5 ± 0.55
748	18:0 / 18:0	diacylGPEtn	3.3 ± 0.35	*
760	19:0 / 18:1	diacylGPEtn	*	0.6 ± 0.13
760	17:0 / 20:1	diacylGPEtn	0.5 ± 0.09	1.1 ± 0.08
764	16:0 / 22:6	diacylGPEtn	12.3 ± 0.51	1.5 ± 0.12
766	18:1 / 20:4	diacylGPEtn	3.3 ± 0.10	1.5 ± 0.04
766	18:0 / 20.5	diacylGPEtn	0.9 ± 0.02	*
768	18:1 / 20:3	diacylGPEtn	*	1.0 ± 0.03
768	16:0 / 22:4	diacylGPEtn	1.0 ± 0.10	1.5 ± 0.04
768	18:0 / 20:4	diacylGPEtn	9.5 ± 0.69	6.1 ± 0.35
770	18:0 / 20:3	diacylGPEtn	3.5 ± 0.68	2.0 ± 0.12

770	16:0 / 22:3	diacylGPEtn	1.5 ± 1.39	2.5 ± 0.11
772	18:1 / 20:1	diacylGPEtn	*	6.0 ± 0.18
772	18:0 / 20:2	diacylGPEtn	0.9 ± 0.13	*
774	18:0 / 20:1	diacylGPEtn	0.8 ± 0.02	11.4 ± 0.35
776	#		*	1.1 ± 0.04
786	#		0.6 ± 0.16	*
788	20:4 / 20:4	diacylGPEtn	0.6 ± 0.06	*
790	18:1 / 22:6	diacylGPEtn	1.1 ± 0.05	*
790	18:2 / 22:5	diacylGPEtn	0.7 ± 0.02	*
792	18:0 / 22:6	diacylGPEtn	9.1 ± 0.82	4.6 ± 0.26
794	18:0 / 22:5	diacylGPEtn	1.6 ± 0.11	2.7 ± 0.19
794	20:1 / 20:4	diacylGPEtn	0.5 ± 0.04	*
796	18:0 / 22:4	diacylGPEtn	1.9 ± 0.08	13.8 ± 1.03
798	20:1 / 20:2	diacylGPEtn	*	2.2 ± 0.10
798	18:0 / 22:3	diacylGPEtn	*	0.9 ± 0.11
800	#		*	2.1 ± 0.18
814	20:3 / 22:6	diacylGPEtn	0.5 ± 0.12	*
818	20:1 / 22:6	diacylGPEtn	*	0.6 ± 0.08
822	18:0 / 24:5 & 20:1 / 22:4	diacylGPEtn diacylGPEtn	1.6 ± 0.11	0.9 ± 0.07
824	18:0 / 24:4	diacylGPEtn	*	1.6 ± 0.08
824	20:0 / 22:4	diacylGPEtn	*	1.1 ± 0.14

Table 3: Molecular species of phosphatidylserine found in rat liver and in *S. mansoni* adult worms. Species are quantified by MRM with $[M-H]^-$ for MS1 and $[M-H-87]^-$ for MS2. Shown is the average ± standard deviation of a single lipid extract injected and analyzed in triplicate. Only species contributing more than 0.5% are indicated. * Less than 0.5%. # Unidentified.

m/z	**component**		**Abundance in rat liver (mole %)**	**Abundance in *S. mansoni* (mole %)**
734	16:0 / 16:0	diacylGPSer	0.8 ± 0.10	0.8 ± 0.10
760	16:0 / 18:1	diacylGPSer	1.9 ± 0.05	1.4 ± 0.13
762	#		7.7 ± 0.28	*
786	18:0 /18:2 & 18:1 / 18:1	diacylGPSer diacylGPSer	*	6.6 ± 0.21
786	18:0 / 18:2	diacylGPSer	7.1 ± 0.18	*
788	18:0 / 18:1	diacylGPSer	33.8 ± 1.14	8.0 ± 0.07
790	18:0 / 18:0	diacylGPSer	6.4 ± 0.57	*
810	18:0 / 20:4	diacylGPSer	17.1 ± 1.24	8.4 ± 0.20
812	18:0 / 20:3	diacylGPSer	5.4 ± 1.25	3.1 ± 0.37
814	#		*	2.8 ± 0.22
814	18:0 / 20:2	diacylGPSer	1.1 ± 0.58	*
814	18:1 / 20:1	diacylGPSer	0.9 ± 0.62	*
816	18:0 / 20:1	diacylGPSer	*	5.9 ± 0.83
816	20:0 / 18:1	diacylGPSer	4.2 ± 0.50	*
834	18:0 / 22:6	diacylGPSer	3.9 ± 0.82	10.3 ± 0.54
836	20:1 / 20:4	diacylGPSer	*	8.6 ± 0.38
836	18:0 / 22:5	diacylGPSer	*	2.3 ± 0.21
836	20:2 / 20:3	diacylGPSer	3.5 ± 0.66	*
838	18:0 / 22:4	diacylGPSer	5.8 ± 1.11	27.7 ± 0.85
840	18:0 / 22:3	diacylGPSer	*	2.4 ± 0.18
852	19:0 / 22:4	diacylGPSer	*	0.6 ± 0.14
852	20:0 / 22:4	1-alkyl, 2-acylGPSer	*	0.5 ± 0.07
864	20:0 / 22:5	diacylGPSer	*	1.7 ± 0.11
864	18:0 / 24:5	diacylGPSer	*	0.9 ± 0.12
866	20:0 / 22:4	diacylGPSer	*	2.2 ± 0.17
866	18:0 / 24:4	diacylGPSer	*	2.9 ± 0.32

Table 4: Molecular species of phosphatidylinositol detected in rat liver and *S. mansoni* adult worms. Species that comprise more than 0.5% of the total GPIns are shown. Percentages shown are the percentages of the total GPIns species mentioned in the table. Species are quantified by MRM with $[M-H]^-$ for MS1 and *m/z* 241 for MS2. Shown is the average ± standard deviation of a single lipid extract injected and analyzed in triplicate. Only species contributing more than 0.5% are indicated. * Less than 0.5%. # Unidentified.

m/z	**component**		**Abundance in rat liver (mole%)**	**Abundance in *S. mansoni* (mole%)**
795	16:0 / 15:0	diacylGPIns	*	1.3 ± 0.20
809	16:0 / 16:0	diacylGPIns	0.8 ± 0.03	0.7 ± 0.32
821	15:0 / 18:1	diacylGPIns	*	1.4 ± 0.11
833	16:0 / 18:2	diacylGPIns	3.3 ± 0.05	4.0 ± 0.24
835	16:0 / 18:1	diacylGPIns	1.3 ± 0.02	6.6 ± 0.63
837	16:0 / 18:0	diacylGPIns	4.7 ± 0.09	1.9 ± 0.05
847	#	diacylGPIns	*	0.5 ± 0.17
847	18:0 / 18:2 & 18:0 / 18:1	1-alkyl, 2-acylGPIns 1-*Z*-alkenyl, 2-acylGPIns	*	1.3 ± 0.04
849	17:0 / 18:1	diacylGPIns	*	1.2 ± 0.20
857	16:0 / 20:4	diacylGPIns	7.8 ± 0.09	2.0 ± 0.19
859	16:0 / 20:3	diacylGPIns	2.1 ± 0.01	2.0 ± 0.36
861	18:0 / 18:2	diacylGPIns	6.2 ± 0.05	19.1 ± 0.44
863	18:0 / 18:1	diacylGPIns	2.5 ± 0.06	17.6 ± 1.44
865	18:0 / 18:0	diacylGPIns	2.7 ± 0.04	1.0 ± 0.34
871	17:0 / 20:4	diacylGPIns	1.0 ± 0.02	*
881	16:0 / 22:6	diacylGPIns	1.5 ± 0.04	*
883	18:1 / 20:4	diacylGPIns	2.5 ± 0.03	*
885	18:0 / 20:4	diacylGPIns	38.7 ± 0.12	25.8 ± 0.91
887	18:0 / 20:3	diacylGPIns	13.9 ± 0.06	5.8 ± 0.06
889	18:0 / 20:2	diacylGPIns	1.8 ± 0.08	3.2 ± 0.39
891	18:0 / 20:1	diacylGPIns	*	1.5 ± 0.29
909	18:0 / 22:6	diacylGPIns	4.9 ± 0.02	1.4 ± 0.39
911	18:0 / 22:5	diacylGPIns	1.1 ± 0.06	*
911	18:1 / 22:4	diacylGPIns	0.8 ± 0.14	*
913	18:0 / 22:4	diacylGPIns	2.4 ± 0.17	1.7 ± 0.30