Rapid sample preparation for ganglioside analysis by liquid chromatography mass spectrometry

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Abstract

Gangliosides (GG) are glycosphingolipids, composed of a ceramide moiety (fatty acid and long chain base) linked to an oligosaccharide chain containing one (or more) molecule of sialic acid. After lipid extraction from biological matrices, quantification of GG by liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI/MS) can be impacted by ion suppression effects due to co-elution with more abundant lipids in the matrix. In this study, a simple, rapid and efficient method to purify GG from biological samples by Phree columns is proposed. This approach proved to be useful in eliminating phospholipids (PL) from the matrix and thus increasing the signal of GG classes and molecular species in rat brain samples during LC-ESI/MS analysis.

Keywords:

Lipids, Gangliosides, Purification, Liquid chromatography-mass spectrometry, Quantitative analysis, Ion suppression.

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1. Introduction

Efficient sample preparation is a key step in molecular analysis of biological matrices. The procedure must maximize the yield of extraction and minimize the losses, while offering a good purification. It is especially crucial when the molecules of interest are present in small amounts in the sample.

Gangliosides, glycosphingolipids of the cell membrane, fulfil this criteria since they represent at the most 10 % of all lipids [1]. They are mostly expressed in the nervous system of which they contribute to the development and maintenance [2]. They also play major roles in tumorigenesis and are increasingly studied as markers or targets for cancer therapy [3]. GG make a wide and heterogeneous family of compounds due to variability in the oligosaccharide chain and the ceramide moiety they comprise [4]. The main challenge of GG analysis is to get rid of PL that are much more abundant in biological samples and largely interfere with GG detection. Indeed, both compounds are amphiphatic and have a similar polarity resulting in co-elution during chromatographic separation. This leads to a large signal reduction due to ion suppression phenomenon when using LC-ESI/MS, which has developed over the past years as a relevant and powerful technique to analyze GG [5]. PL removal is therefore critical for an accurate analysis of GG using LC-ESI/MS. The historical preparation technique of GG relies on phase partition of a lipid extract using a mixture of organic solvents (chloroform and methanol) and water [6]. PL are eliminated in the chloroformic phase while GG are collected in the methanol/water phase. They can be further purified using solid phase extraction to get rid of free sugars, salts etc… This multi-step protocol requiring large volumes of solvents is cumbersome and time-consuming and increases the risk of sample losses. Alternatively, PL can be eliminated using a base treatment [7] but it leads to degradation of acetylated GGs that can account for a significant proportion of GGs in some tissues. Here we propose a novel approach based on the use of Phree cartridges (Phree phospholipid removal columns) originally designed to get rid of phosphatidylcholines (PC) in biological samples for MS purposes, especially proteomics and metabolomics. We found out that these columns are very efficient to get rid of PL, especially PC and sphingomyelins, in lipid extracts and therefore to purify GG in only one easy step. The present results clearly show that this straightforward approach enables a significant improvement in GG detection using LC-ESI/MS.

2. Materials and methods

2.1. Lipids and other material

Chloroform ($CHCl_3$) was obtained from SDS (France). Ammonium acetate, acetonitrile (CH_3CN), methanol (CH_3OH) and water (H_2O) of Optima LC/MS grade were all from Fisher Scientific (France). Commercially available GG standards from natural sources (bovine or human) were obtained from Matreya (LLC, USA). PL standards were purchased from Avanti Polar Lipids INC-Coger (Paris, France).

2.2. Tissue sample preparation

Eleven-week-old male Wistar Han rats were obtained from colonies established in the animal quarters of our laboratory (CSGA). Samples of 100 mg of brain were thoroughly homogenized in 1. mL distilled H_2O with tungsten microbeads using a tissue lyser (Qiagen, The Netherlands, 1 min 30 s at 30 Hz speed).

2.3. Lipid extraction

First, total lipids were extracted from rat brain samples homogenized in H_2O, overnight at 4°C, with 10 volumes of $CHCl_3/CH_3OH$ (1:1, v/v). The residual pellets obtained after centrifugation (1500g, 5 min)

were then re-extracted with 3 mL $CHCl_3/CH_3OH$ (1:1, v/v), 3 mL $CHCl_3/CH_3OH$ (2:1, v/v) and 3 mL $CHCl_3/CH_3OH/H_2O$ (48:35:10, v/v/v). The four lipid extracts were combined, and evaporated to dryness under a stream of nitrogen.

2.4. Purification of GG by phase partition

Each total lipid extract was re-dissolved in 3 mL $CHCl_3/CH_3OH$ (1:1, v/v) and GG were separated from other lipids by phase partition by adding 1 mL H_2O. After centrifugation (1500g, 3 min), the upper aqueous phase was collected while lower organic phase was re-extracted twice with 2 mL CH_3OH/H_2O (1:1, v/v). The three upper phases containing GG were combined, dried under a stream of nitrogen and re-dissolved in 2 mL CH_3OH/PBS 10mM (1:1, v/v). Then, the GG extracts were purified on a C18 silica gel column (Sep-Pak Vac 6cc, 500 mg, Waters, USA) first washed with 7 mL CH_3OH and pre-equilibrated with 7 mL CH_3OH/PBS 10mM (1:1, v/v). After washing with 10 mL H_2O, purified GGs were eluted with 6 mL CH_3OH and 4 mL $CHCl_3/CH_3OH$ (2:1, v/v). GG extracts were evaporated to dryness under a stream of nitrogen, re-dissolved in 1 mL $CHCl_3/CH_3OH$ (2:1, v/v) and stored at -20°C until further analyses.

2.5. Purification of GG using Phree cartridges

The total lipid extracts were re-dissolved in 200 µL $CHCl_3/CH_3OH$ (2:1, v/v) and loaded into the Phree cartridges (Phenomenex, USA). 1 mL CH_3OH was added to each Phree cartridge to elute the purified GG from the cartridges. The GG extracts were evaporated to dryness under a stream of nitrogen, re-dissolved in $CHCl_3/CH_3OH$ (2:1, v/v) and stored at -20°C for further analysis. Phree cartridges were also applied to check whether the use of this method has an impact on GG enrichment. For this purpose, a mixture of GG standards (GM3, GM2, GD3, GD1a, GD1b, GT1b and GQ1b) was used as a model and prepared at 10 pmol in 200 µl of CHCl3/CH3OH (2:1, v/v). This mixture is loaded into Phree column and GGs were then eluted with CH3OH, evaporated and dissolved in 200 µl CHCl3/CH3OH (2:1, v/v) for further MS analysis. The result of this experiment is compared to that of the same mixture without any treatment.

2.6. Ganglioside analysis by liquid chromatography - mass spectrometry

GG were first separated by liquid chromatography under Hydrophilic Interaction Liquid Chromatography (HILIC) conditions using a silica Kinetex column (150 mm × 2.1 mm inner diameter, 2.6 µm, HILIC; Phenomenex). The mobile phase consisted of (A) ACN/water (90:10, v/v) containing 10 mM ammonium acetate and (B) ACN/water (50:50, v/v) containing 10 mM ammonium acetate. The solvent-gradient system was as follows: 0-1 min 100% A, 4 min 79% A, 9 min 78% A, 14-18 min 50% A, and 19-48 min 100% A. The flow rate was 400 µl*min^{-1}, the injection volume was10 µl, and the column was maintained at 30°C. GG were then analyzed by a triple quadrupole mass spectrometer (QqQ-MS) equipped with a heated electrospray ionization source. For MS experiments, GG were analyzed in Selected Reaction Monitoring (SRM) in negative ion mode. Sialic acid with m/z 290 was a characteristic and abundant fragment for all GG species in negative ion mode. The signal of a GG class was calculated as the sum of the peak areas of all molecular species detected in this class. The proportion of each GG species of a specific GG class was calculated as the ratio of its peak area to

the sum of all detected peak areas in this class. For more information about the developed analytical methods, see [8].

2.7. Separation and quantification of PL classes by liquid chromatography coupled to charged aerosol detector (Corona-CAD).

The phosphorus content of the total lipid extracts was determined according to the method developed by Bartlett and Lewis [9]. The samples were then diluted to the appropriate concentration of 500µg/mL of PL in $CHCl_3$/CH_3OH (1:1, v/v).

PL classes were first separated by liquid chromatography under HILIC conditions using a Thermo Accucore column (150 x 2.1 mm, 2.6 µm, Hilic, Thermo Scientific, USA); the column was maintained at 40°C. The mobile phase consisted of (A) ACN/H2O (95:5, v/v) containing 5 mM ammonium acetate and (B) ACN/H2O (50:50, v/v) containing 10 mM ammonium acetate. The solvent gradient system was as follows: 0 min 100% A, 1 min 95% A, 20 min 80% A, 23 min 65% A, 24 min 100% A and 24–39 min 100% A. The flow rate was 800 µl*min^{-1} and the injection volume was 5 µL. Detection of PL classes was performed using a Corona™ Ultra RS Charged Aerosol Detector™ (CCAD, Thermo Scientific, USA). More information about the analysis method including detector parameters and other conditions could be found in the work published recently by Le Bon *et al.*[10].

3. Results and discussion

For this study, the lipid extract of rat brain was chosen as model. Indeed, the brain is a neural tissue containing significant amounts of GG, and presenting a large diversity of GG classes, tetraosylGG such as GD1a, GD1b and GT1b being the most abundant ones [11]. Moreover, acetylated GG forms are also present in the brain [11].

Our monophasic extraction method enabled a fast and efficient extraction of GG along with total lipids from biological matrices [8] Moreover, the previously developed HILIC methods allowed a good separation of PL classes on one hand and GG classes, including acetylated forms, on the other hand, according to their increased polarity [8,10,11]. However, the more abundant PL in the lipid sample, of similar polarity, result in co-elution with GG under these chromatographic conditions leading to ion suppression phenomenon when chromatographic system is coupled to ESI-MS. The Fig. 1 clearly shows the co-elution of PC, sphingomyelins (SM) and lysophosphatidylcholines (LPC) with the less polar GG (GM3 and GM2) under the HILIC conditions. Removal of PL, prior to analysis, is thus essential for an accurate analysis of GG by HILIC-ESI-MS.

The phase partition method followed by C18 cartridges used in our previous works [8,10–12], is known to be a powerful tool to remove PL from lipid extracts. However, this multi-step method is very time consuming. In the present work, we propose a faster method to purify GG from biological matrices. This approach is based on the use of Phree cartridges originally designed to eliminate PC from biological samples during metabolomic or proteomic development using MS. In order to compare the efficiency of the different purification methods, non-purified and purified lipid extracts were analyzed in HILIC-Corona. As seen in Fig. 2, Phree columns allowed a specific elimination of choline-containing

PL while phase partition method allowed the elimination of both PC and phosphatidylethanolamine (PE) classes but not of SM.

To evaluate the impact of the different purification methods on GG quantitative analysis, we compared the GG class signals obtained in MS in the non-purified brain lipid extract and in the same lipid extract after purification of GG by phase partition followed by C18 cartridge or using Phree cartridge (Fig. 3). Results clearly demonstrate that the highest signals are obtained using Phree cartridges. The signal of GM3 class is increased 5 times compared to the signal of GM3 in non-purified extract. The signal of more complex and polar GG classes such as GD1a, GD1b and GQ1b is increased about 2 times, even if they do not co-elute with PL classes. This might be due to the elimination by Phree purification of other molecules with ion-suppressing effects. A complementary experiment was conducted on a mixture of GG standards to check whether the use of Phree cartridges contributes to enrich GGs. Fig. 4 obviously shows that Phree treatment does not preferentially enrich any GG class. On the contrary, Phree treatment, in the absence of PLs from the matrix, results in a slight decrease in the less polar GG (GM3, GM2 and GD3) signal, and a higher decrease in MS signal of the most polar GGs (GD1a, GD1b, GT1b and GQ1b) as shown in Fig. 4. This decrease in the signal of GG classes is not observed when analyzing the rat brain sample (rich in choline-containing PLs) after Phree treatment, due to the selectivity of Phree cartridges to PLs (Fig. 3).

Especially for GM3, no purification of the rat brain lipid extract leads to a very low signal, due to co-elution with the highly abundant PC. The signal of some other GG classes like GD1a and GD1b was also reduced, as compared to purified samples, after co-elution with other compounds of similar polarity. It appears that non-purified lipid extract could be essentially used for the quantification of GM4, GT1b and for some minor GG classes, for which a purification does not seem to improve detection, probably because ionization efficiency was not impacted by other molecules from the total lipids.

Unexpectedly, results indicate that GG purification by phase partition does not bring any benefit for the quantification of GG by LC-MS in the matrix used in this work (Fig.3). We indeed noted that there is a lower signal for all GG classes compared to the purification with Phree cartridge. Except for GM3 which is the most impacted by the presence of PL, the GG signal is also lower when compared with non-purified lipid extract, while we have seen above that phase partition is efficient to get rid of PL (Fig. 2). This could be explained by the loss of a significant amount of GG during the purification process by phase partition followed by C18 purification due to the numerous steps of GG transfer. Moreover, it is striking that GM4 class is hardly detectable in rat brain sample following phase partition. This is certainly due to the fact that GM4 class, and probably some GM3 species, GG with the shortest oligosaccharide chain, were eliminated in the chloroformic phase with the bulk of PL while more polar GG were collected in the methanol/water phase. This was not the case when using Phree cartridges.

In conclusion, while the phase partition is an efficient method to purify GG in complex biological matrices and reduce ion suppression effects during ESI-MS analysis, its low extraction and purification yields do not allow to improve the MS signals as compared to non-purified extracts. On the contrary,

eliminating PL using the powerful Phree cartridges enables a large increase in the MS signal of most GG classes in the brain extract, especially the major ones, by reducing ion suppression effects.

The analysis method by ESI-MS (QqQ) allowed the quantification of a great diversity of GG molecular species (for a review, see reference [5]). GG species identified in this work are listed in the supplementary table with their MS signal and their percentage according to purification methods. As shown in this table, Phree treatment appears to generally improve MS signal of detectable GG species by reducing ion suppression caused by PLs. The distribution of the major molecular species in each GG class was not influenced by the purification method (for example, 36:1 remains the major species in GM3, GM2, GD3, GD2, GD1a, GD1b, GT1b and GQ1b whatever the purification method used). However, the distribution of minor species showed a slight variation for some GG classes depending on the purification method as shown in Fig. 5 giving the molecular species profile of GM3 and GD1a as examples. In addition, some minor GG species, which are absent from non-purified lipid extract profile such as GM3(d36:5), GM3(d36:4) and GD3(d36:5), or from the profile of lipid extract purified by phase partition like GD3(d40:2) become detectable with Phree purification (see the supplementary table).

4. **Conclusion**

Ion suppression in ESI-MS could influence quantitative analysis of the molecules of interest, such as GG, in a complex lipid extract. To address this issue, we chose to purify GG from lipid extract prior to ESI-MS analysis. A novel method, the Phree cartridges, was evaluated to purify GG and compared to the usual phase partition coupled to C18 cartridge purification protocol. Phree Phospholipid Removal Column showed to be a powerful and fast tool to remove PL from lipid extract, especially choline-containing PL. This purification method resulted in an increase of the signal of GG classes and species when analyzed by HILIC-ESI/MS. It thus appeared as a better alternative to phase partition which is also an efficient method to eliminate PL classes but is tedious and induces losses of some GG.

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Figures captions

Fig.1. Overlay of the total ion chromatogram of a mixture of ganglioside standards and of choline-containing PL as obtained by HILIC-QqQ-MS (under the same chromatographic conditions). Ganglioside standards (GM3, GM2, GD3, GD1a, GD1b, GT1b and GQ1b) were analyzed by SRM mode in negative ionization. Choline phospholipid standards (PC, LPC and SM) were analyzed by precursor ion scanning of 184 in positive ion mode. The chromatographic conditions allowed obtaining this result are found in the Materials and Methods section.

Fig. 2. Representative chromatograms showing the separation of PL classes by HILIC-CAD from rat brain lipid extract without purification (A), after purification by phase partition and C18 cartridge (B) or using Phree cartridge (C).

Fig. 3. Comparison of the signal observed in QqQ-MS for the different GG classes in rat brain lipid extract without any purification of GG (white), or after purification of GG by phase partition and C18 cartridge (grey), or using Phree cartridge (black). Data are presented as means ± sd, n=3.

Fig 4. Comparison of the signal observed in QqQ-MS for the different GG classes in a mixture of GG standards with no treatment (white), and after Phree treatment (black). Data are presented as means ± sd, n=5.

Fig 5. Molecular species composition of GM3 (A) and GD1a (B), without any purification of GG (white), or after purification of GG by phase partition and C18 cartridge (grey), or using Phree cartridge (black). Data are presented as means ± sd, n=3.

Fig.1.

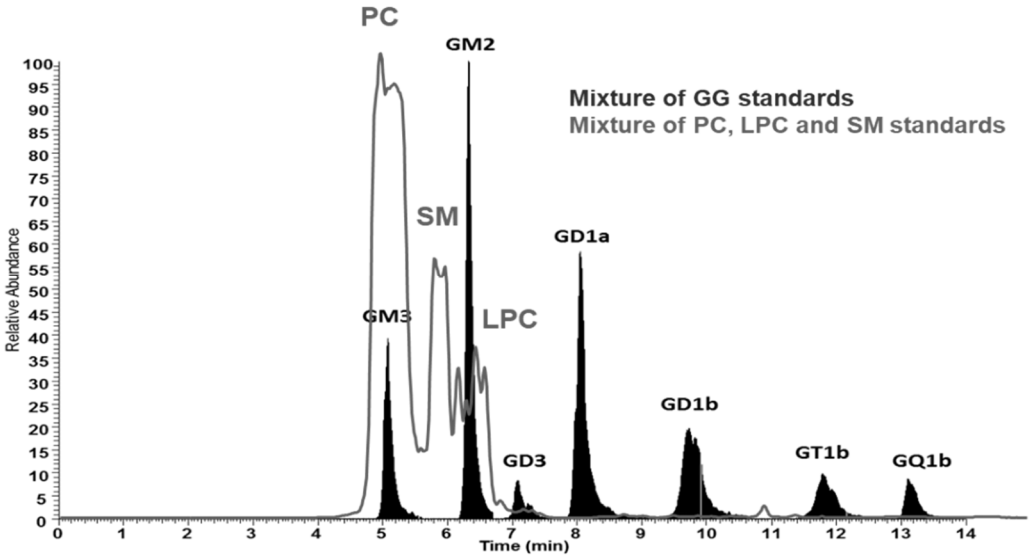

PC
GM2
Mixture of GG standards
Mixture of PC, LPC and SM standards
SM
GD1a
GM3
LPC
GD1b
GD3
GT1b
GQ1b
Relative Abundance
Time (min)

Fig. 2.

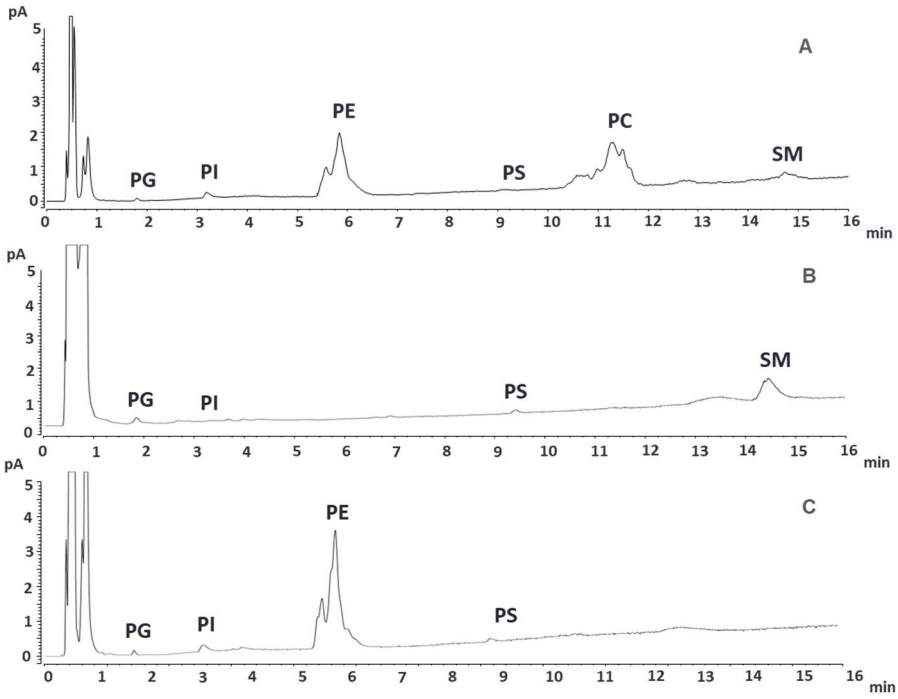
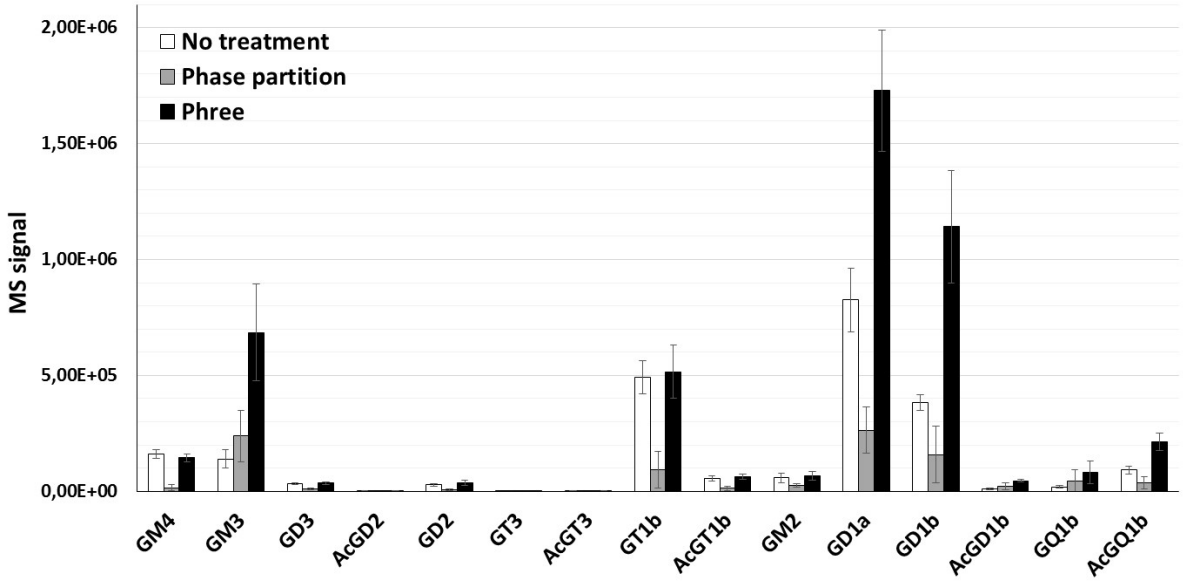


Fig. 3.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GD2
GT3
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GM2
GD1a
GD1b
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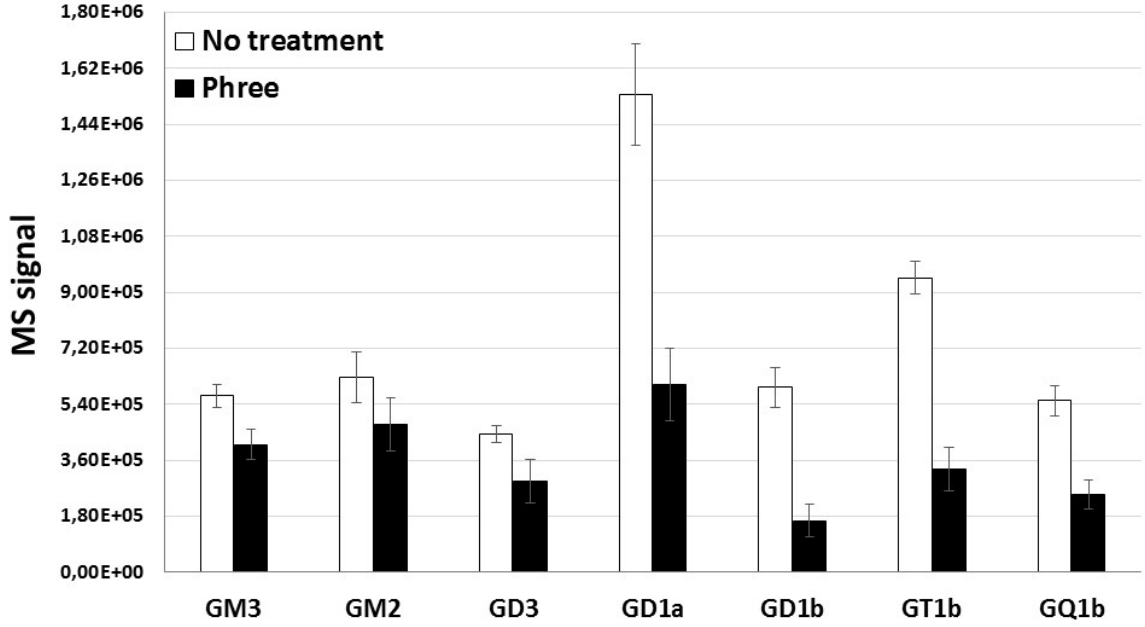
GQ1b
AcGQ1b

Fig 4.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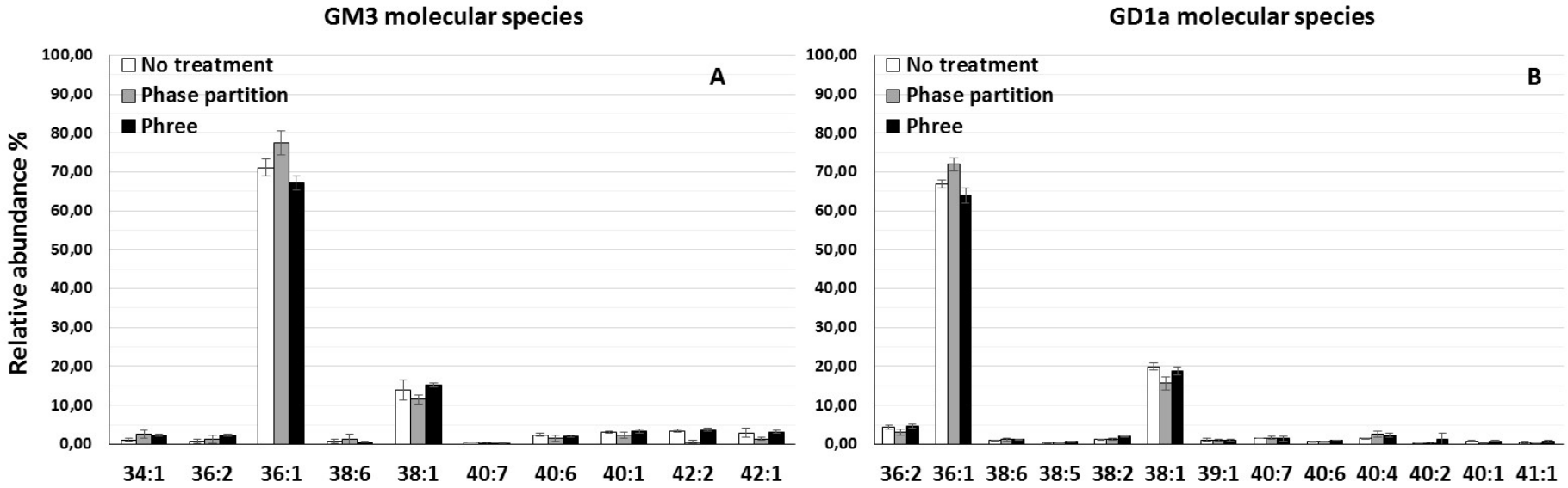
Phree
GM3
GM2
GD3
GD1a
GD1b
GT1b
GQ1b

Fig 5.


GM3 molecular species
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No treatment
Phase partition
Phree
Relative abundance %
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90,00
80,00
70,00
60,00
50,00
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20,00
10,00
0,00
34:1
36:2
36:1
38:6
38:1
40:7
40:6
40:1
42:2
42:1
GD1a molecular species
B
No treatment
Phase partition
Phree
36:2
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38:5
38:2
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