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1 **Rapid sample preparation for ganglioside analysis by liquid chromatography mass** 2 **spectrometry**

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19

20 **Abstract**

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

29 **Keywords:**

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

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34 work is supported by the Regional Council of Burgundy and the European Regional Development
35 Fund (FEDER).

36 **1. Introduction**

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

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

63 **2. Materials and methods**

64 ***2.1. Lipids and other material***

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

70 ***2.2. Tissue sample preparation***

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

75 ***2.3. Lipid extraction***

76 First, total lipids were extracted from rat brain samples homogenized in H_2O , overnight at 4°C , with 10
77 volumes of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1, v/v). The residual pellets obtained after centrifugation (1500g, 5 min)

78 were then re-extracted with 3 mL CHCl₃/CH₃OH (1:1, v/v), 3 mL CHCl₃/CH₃OH (2:1, v/v) and 3 mL
79 CHCl₃/CH₃OH/H₂O (48:35:10, v/v/v). The four lipid extracts were combined, and evaporated to
80 dryness under a stream of nitrogen.

81 **2.4. Purification of GG by phase partition**

82 Each total lipid extract was re-dissolved in 3 mL CHCl₃/CH₃OH (1:1, v/v) and GG were separated from
83 other lipids by phase partition by adding 1 mL H₂O. After centrifugation (1500g, 3 min), the upper
84 aqueous phase was collected while lower organic phase was re-extracted twice with 2 mL
85 CH₃OH/H₂O (1:1, v/v). The three upper phases containing GG were combined, dried under a stream
86 of nitrogen and re-dissolved in 2 mL CH₃OH/PBS 10mM (1:1, v/v). Then, the GG extracts were
87 purified on a C18 silica gel column (Sep-Pak Vac 6cc, 500 mg, Waters, USA) first washed with 7 mL
88 CH₃OH and pre-equilibrated with 7 mL CH₃OH/PBS 10mM (1:1, v/v). After washing with 10 mL H₂O,
89 purified GGs were eluted with 6 mL CH₃OH and 4 mL CHCl₃/CH₃OH (2:1, v/v). GG extracts were
90 evaporated to dryness under a stream of nitrogen, re-dissolved in 1 mL CHCl₃/CH₃OH (2:1, v/v) and
91 stored at -20°C until further analyses.

92 **2.5. Purification of GG using Phree cartridges**

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

103 **2.6. Ganglioside analysis by liquid chromatography - mass spectrometry**

104 GG were first separated by liquid chromatography under Hydrophilic Interaction Liquid
105 Chromatography (HILIC) conditions using a silica Kinetex column (150 mm × 2.1 mm inner diameter,
106 2.6 µm, HILIC; Phenomenex). The mobile phase consisted of (A) ACN/water (90:10, v/v) containing 10
107 mM ammonium acetate and (B) ACN/water (50:50, v/v) containing 10 mM ammonium acetate. The
108 solvent-gradient system was as follows: 0-1 min 100% A, 4 min 79% A, 9 min 78% A, 14-18 min 50%
109 A, and 19-48 min 100% A. The flow rate was 400 µl*min⁻¹, the injection volume was 10 µl, and the
110 column was maintained at 30°C. GG were then analyzed by a triple quadrupole mass spectrometer
111 (QqQ-MS) equipped with a heated electrospray ionization source. For MS experiments, GG were
112 analyzed in Selected Reaction Monitoring (SRM) in negative ion mode. Sialic acid with m/z 290 was a
113 characteristic and abundant fragment for all GG species in negative ion mode. The signal of a GG
114 class was calculated as the sum of the peak areas of all molecular species detected in this class. The
115 proportion of each GG species of a specific GG class was calculated as the ratio of its peak area to

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

118 **2.7. Separation and quantification of PL classes by liquid chromatography coupled to charged** 119 **aerosol detector (Corona-CAD).**

120 The phosphorus content of the total lipid extracts was determined according to the method developed
121 by Bartlett and Lewis [9]. The samples were then diluted to the appropriate concentration of 500µg/mL
122 of PL in CHCl₃/CH₃OH (1:1, v/v).

123 PL classes were first separated by liquid chromatography under HILIC conditions using a Thermo
124 Accucore column (150 x 2.1 mm, 2.6 µm, Hilic, Thermo Scientific, USA); the column was maintained
125 at 40°C. The mobile phase consisted of (A) ACN/H₂O (95:5, v/v) containing 5 mM ammonium acetate
126 and (B) ACN/H₂O (50:50, v/v) containing 10 mM ammonium acetate. The solvent gradient system was
127 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
128 100% A. The flow rate was 800 µl*min⁻¹ and the injection volume was 5 µL. Detection of PL classes
129 was performed using a Corona™ Ultra RS Charged Aerosol Detector™ (CCAD, Thermo Scientific,
130 USA). More information about the analysis method including detector parameters and other conditions
131 could be found in the work published recently by Le Bon *et al.*[10].

132 **3. Results and discussion**

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

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

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

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

155 To evaluate the impact of the different purification methods on GG quantitative analysis, we compared
156 the GG class signals obtained in MS in the non-purified brain lipid extract and in the same lipid extract
157 after purification of GG by phase partition followed by C18 cartridge or using Phree cartridge (Fig. 3).
158 Results clearly demonstrate that the highest signals are obtained using Phree cartridges. The signal of
159 GM3 class is increased 5 times compared to the signal of GM3 in non-purified extract. The signal of
160 more complex and polar GG classes such as GD1a, GD1b and GQ1b is increased about 2 times,
161 even if they do not co-elute with PL classes. This might be due to the elimination by Phree purification
162 of other molecules with ion-suppressing effects. A complementary experiment was conducted on a
163 mixture of GG standards to check whether the use of Phree cartridges contributes to enrich GGs. Fig.
164 4 obviously shows that Phree treatment does not preferentially enrich any GG class. On the contrary,
165 Phree treatment, in the absence of PLs from the matrix, results in a slight decrease in the less polar
166 GG (GM3, GM2 and GD3) signal, and a higher decrease in MS signal of the most polar GGs (GD1a,
167 GD1b, GT1b and GQ1b) as shown in Fig. 4. This decrease in the signal of GG classes is not observed
168 when analyzing the rat brain sample (rich in choline-containing PLs) after Phree treatment, due to the
169 selectivity of Phree cartridges to PLs (Fig. 3).

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

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

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

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

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

206 4. Conclusion

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

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- 247
- 248

249 **Figures captions**

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

256
257 Fig. 2. Representative chromatograms showing the separation of PL classes by HILIC-CAD from rat
258 brain lipid extract without purification (A), after purification by phase partition and C18 cartridge (B) or
259 using Phree cartridge (C).

260
261 Fig. 3. Comparison of the signal observed in QqQ-MS for the different GG classes in rat brain lipid
262 extract without any purification of GG (white), or after purification of GG by phase partition and C18
263 cartridge (grey), or using Phree cartridge (black). Data are presented as means \pm sd, n=3.

264
265 Fig 4. Comparison of the signal observed in QqQ-MS for the different GG classes in a mixture of GG
266 standards with no treatment (white), and after Phree treatment (black). Data are presented as means
267 \pm sd, n=5.

268
269 Fig 5. Molecular species composition of GM3 (A) and GD1a (B), without any purification of GG (white),
270 or after purification of GG by phase partition and C18 cartridge (grey), or using Phree cartridge (black).
271 Data are presented as means \pm sd, n=3.

Fig.1.

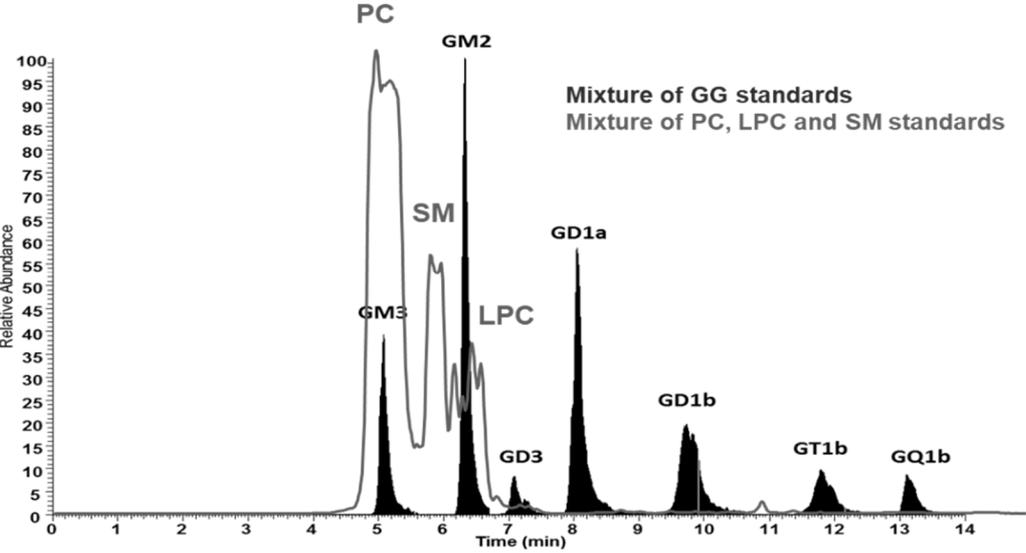


Fig. 2.

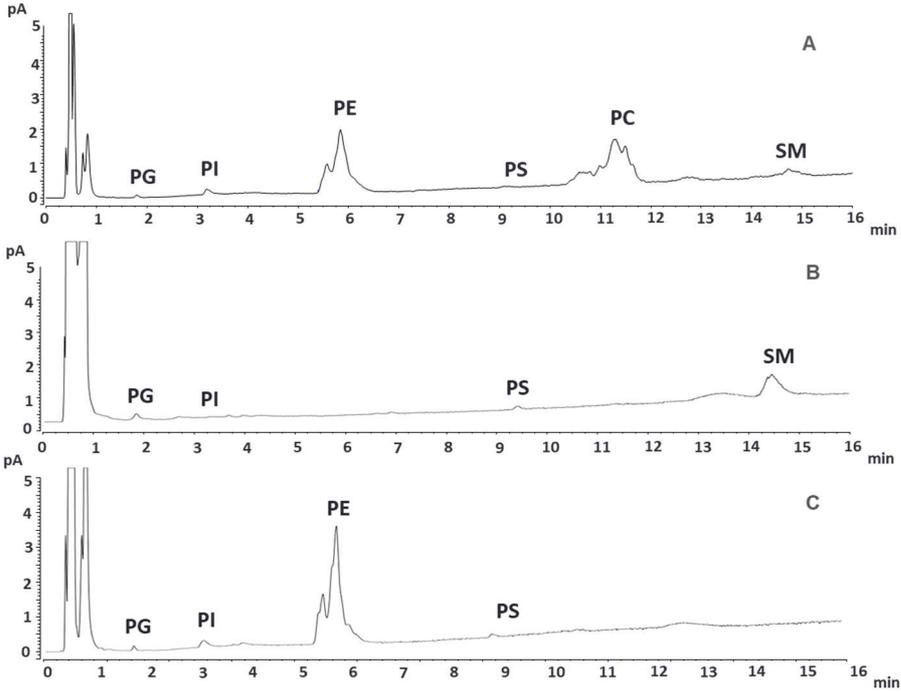


Fig. 3.

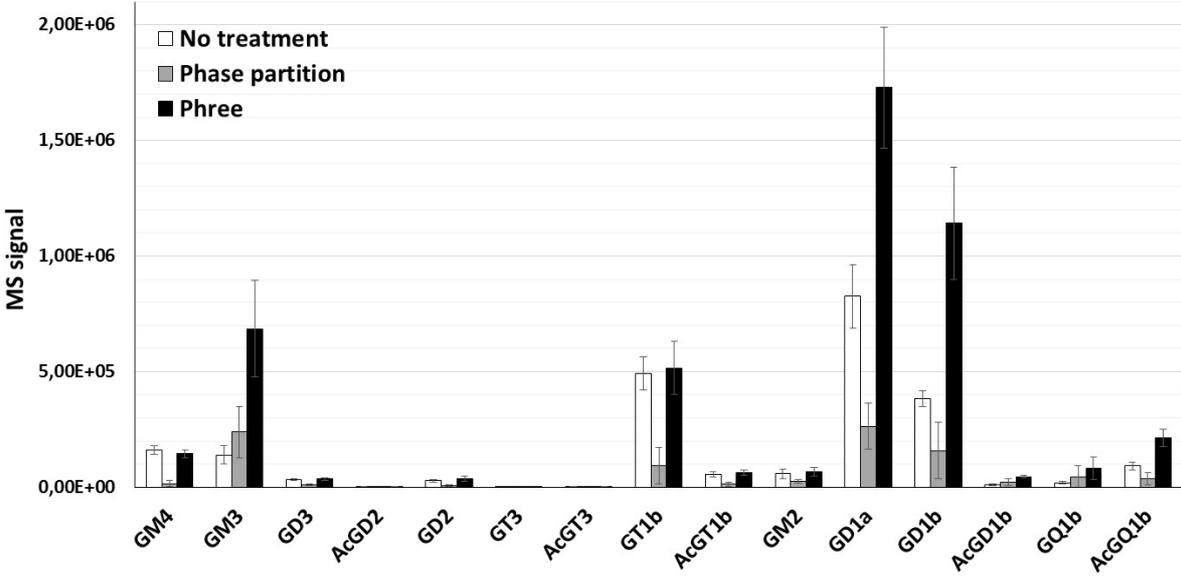


Fig 4.

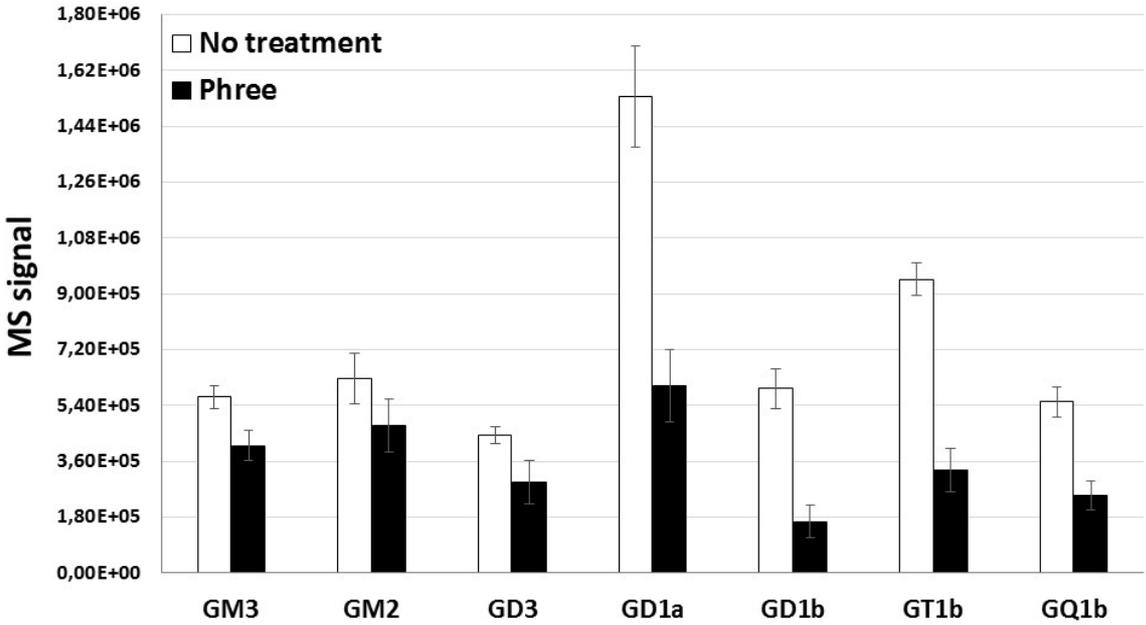


Fig 5.

