Normal phase High Pressure Liquid Chromatography method for combined separation of both polar and neutral lipid classes with application to lipid metabolic flux

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ABSTRACT

Three normal phase HPLC methods were produced to separate lipid classes on a PVA-Sil stationary phase including: 9 polar lipids (method 1); 13 combined polar and neutral lipids (method 2); and a combined method that further separates the neutral lipids into 2-4 subclasses based on the presence of fatty acids containing a polar functional group (e.g. hydroxyl) for a total of 20 lipid classes and subclasses separated in a single run (method 3). Polar lipids separated include: the phosphoglycerolipids PG, PE, PI, PS, PC and LPC; the galactoglycerolipids MGDG and DGDG; and a sulfoglycerolipid SQDG. Neutral lipids include TAG, DAG, and MAG classes and sub-class containing 0-3, 0-2, and 0-1 hydroxy fatty acids, respectively. The hexane/isopropanol/methanol/aqueous system separates polar lipids without the use of chloroform such that it is suitable for radioactivity analysis by in-line flow scintillation counting. Each method was optimized

using the natural lipid standards comprised of diverse molecular species that were detected by ELSD. All molecular species of each lipid class eluted together as single peak detected by ELSD. The methods were demonstrated to be suitable for resolving lipid extracts from animal, microbial, and plant sources as well as application to ^{14}C based metabolic tracing of lipid metabolism in leaves and seeds.

Keywords: Phospholipids, Galactolipids, Triglycerides, Hydroxy fatty acids, Radioactivity, Metabolic flux.

1. INTRODUCTION

Neutral lipids and polar lipids make up the two major categories of glycerolipids. Each category is further subdivided into many different lipid classes based on common chemical structure, and finally each lipid class is composed of many individual molecular species based on the fatty acids esterified to the glycerol backbone. Neutral lipids are mostly hydrophobic and contain few polar functional groups. The major classes of neutral glycerolipids are triacylglycerol (TAG), which is utilized as a carbon and energy storage molecule, and diacylglycerol (DAG), monoacylglycerol (MAG), and free fatty acids (FFA) which are intermediates of glycerolipid metabolism [1-5]. Polar glycerolipid classes are dependent on the polar head group and number of fatty acids attached to the glycerol backbone which include phospholipids, lyso-phospholipids, and glycolipids. Polar glycerolipids are the major components of cell membranes, intermediates of TAG metabolism, and play a key role in molecular signaling, disease, and response to environmental stress in microorganisms, plants, and animals [1, 2, 6-12].

To understand lipid metabolism and its effect on the growth and health of an organism requires methods to identify changes in lipid class abundance and composition, and to track carbon flux through the lipid metabolic reactions. Mass spectrometry based lipidomics has emerged as a sensitive and high throughput method to identify changes to lipid class molecular species abundance in response to disease or

environmental changes [13-16]. Lipidomic analyses commonly involve reverse-phase (RP) high performance liquid chromatography (HPLC) separation of lipid molecular species to simplify the complex mass spectrum. RP-HPLC separates lipids based on hydrophobicity which leads to lipid class molecular species separation and overlapping elution of different lipid classes from the HPLC column which are resolved by either MS/MS or multi-dimensional HPLC-MS approaches within the mass spectrometer [17, 18]. In the recent years, hydrophilic interaction liquid chromatography (HILIC) has emerged as a tool for separation of polar lipid classes, however due to the high water content neutral lipid classes are not resolved in HILIC mode [19-22]. In 2018, Rampler et al. reported a novel online two-dimensional HPLC method where they combined both HILIC and reverse phase modes with MS detection. In this report the polar lipid classes were separated via HILIC in the first dimension and neutral lipids were separated via reverse phase in a second dimension, but the neutral lipid classes were not completely resolved relying on MS to distinguish each lipid class [23]. Due to the high cost and limited availability of mass spectrometry resources for lipidomics, many other lipid class analytical approaches are commonly employed by academic researchers and various industries (i.e. food and biofuel industries) such as: thin layer chromatography (TLC) separation of lipid classes combined with staining or gas chromatography (GC) quantification of fatty acid methyl esters (FAME) derived from eluted lipid classes; or HPLC combined with an ultra-violet (UV), evaporative light scattering detector (ELSD), or charged aerosol detector (CAD) detection of analytes [24-26]. Each of these non-mass selective HPLC detection methods does not distinguish between different lipid classes eluted at the same time, therefore normal phase HPLC methods are used to elute lipid class molecular species as a single peak. In addition, normal phase HPLC is key to the purification of individual lipid classes for further utilization.

Tracking the flux of carbon through lipid metabolism requires the use of isotopically labeled tracers, either stable (e.g. ^{13}C) or radioactive (e.g. ^{14}C) [27, 28]. The incorporation of stable isotopic labeling into lipidomic approaches leads to very complex mass spectra. For instance, a TAG molecule such as triolein can have a labeled mass range from M+1 to M+57. In addition, the increases in mass lead to

overlapping nominal masses of different lipid molecular species, or lipid classes. For example, there is a 0.009 AMU difference between a saturated fatty acid, and a monounsaturated fatty acid containing two ^{13}C. Therefore, targeted ultra-high resolution MS is required to resolve of stable isotopic labeling of lipids [27]. To simplify the isotopomer distribution of ^{13}C lipid flux analysis most studies focus on utilizing tracers that: will only label a few lipid classes (e.g. ^{15}N-serine or ^{15}N-choline [29]); or limit the isotopomer distribution by incorporating a labeled full length fatty acid [30, 31]; or when utilizing a general precursor such as glucose only target specific lipid classes [31, 32], or specific molecular species isotopomers such as just the M+3 isotopomer for labeled glycerol within glycerolipids [33]. However, each of these approaches limit the branches of the lipid metabolic network that can be traced. Radioisotopic metabolic tracers have several benefits over stable isotopes including: enhanced sensitivity, less expensive analytical equipment, ability to quantitatively measure total uptake and release in a non-targeted manner, and the ability for exploratory studies that follow the label into unknown or unanticipated metabolites [28, 34]. The use of radioisotopes to trace lipid metabolism is a common approach to elucidate endogenous metabolic pathways and to understand metabolic perturbations due to disease or genetic engineering in microorganisms, animals, and plants [35-42]. To quantify the radioactivity associated with different lipid classes requires complete resolution of each lipid class, therefore normal phase TLC and HPLC methods are preferred [2, 43].

Our goal was to develop a normal phase HPLC method to simultaneously separate major neutral and polar lipid classes from plant origin suitable for non-mass selective detection (UV or ELSD), and for ^{14}C based lipid metabolic flux analysis. In addition, to resolve neutral lipid sub-classes composed of molecular species containing 0-3 polar hydroxylated fatty acids which accumulate in seed oils of many different plants [44] including the industrially important castor oil crop [45-47], and the emerging industrial crop *Physaria fendleri* [48, 49]. Previously, we demonstrated a polyvinyl alcohol modified silica (PVA-Sil) column to be suitable for separation of hydroxy fatty acid containing neutral lipids with a hexane/isopropanol/methanol/aqueous based mobile phase [43]. PVA-Sil was also utilized to separate most major plant phosphoglycerolipids, and glycoglycerolipids with a tertiary gradient system containing

chloroform [50]. Because chloroform is a potent liquid scintillation quencher we chose to develop a combined neutral and polar lipid method based on a hexane/isopropanol/methanol/aqueous mobile phase. Methods for separating only the non-hydroxy-fatty-acid-containing neutral and polar lipids have been previously reported on silica columns with isooctane/THF/acetone/dichloromethane [51] and isooctane/ethyl acetate/acetone/isopropanol [52] based mobile phases and ELSD detection, yet acetone is not suitable for UV detection of lipids at 210 nm. Here we report three method variations to separate major plant polar lipids (method 1), neutral and polar lipids (method 2), and hydroxy fatty acid containing neutral and polar lipids (method 3). We further demonstrate the suitability of these methods to separate major neutral and polar lipids from microbial, animal, and plant origin, and for quantitative in-line liquid scintillation counting within ^{14}C based lipid flux analysis. The solvents in each method allow the use of non-radioactive standards to establish retention times by UV or ELSD detection. These methods will also be useful for fraction collection of microgram - milligram amounts of individual lipid classes, or quantification by ELSD after establishment of suitable calibration curves for each lipid class on the ELSD [26, 52].

2. MATERIALS AND METHODS:

Materials: Stationary phase, YMC-Pack PVA-Sil (250 × 4.6 mm, 5 μm particle size). All solvents and chemicals are from Fisher Scientific, unless indicated otherwise. Solvents and water were of HPLC or Optima grade. Formic acid (ACS reagent, ≥98%), triethylamine (≥99.5% (GC)) (Millipore Sigma). Polar lipid standards (PG, PE, PI, PS, 95% Soy PC, LPC, SQDG), animal and microbial lipid extracts were purchased from Avanti Polar Lipids. Total extracts used for the analysis were; Bovine Liver (Avanti 181104P), Bovine Heart (Avanti 171201P), Bovine Brain (Avanti 131101C) and *Escherichia coli* (100500P). Non-hydroxy FFA and MAG were prepared from the hydrolysis of canola oil by *Rhizomucor miehei* lipase (Millipore Sigma), and the hydroxy neutral lipid standards and hydroxy free fatty acids (HFFA) were prepared from castor oil and lipase hydrolysis of hydroxy TAGs isolated from castor oil [53].

HPLC instrumentation: Agilent 1260 Infinity II LC quaternary pump, vial sampler, multi column thermostat, diode array detector (DAD), analytical fraction collector, evaporative light scattering detector (ELSD), controlled by OpenLAB CDS Version C.01.09.144 . In line radioactivity detection on a LabLogic β-Ram 6 with a 500µL adjustable volume liquid flow cell set at 300µL, and data acquisition by Laura version 6.0.1.40. Depending on the type of application, the eluate from the DAD was directed to either ELSD, fraction collector, or radio detector. For ELSD nitrogen flow was 1.6 SL/min, nebulizer and evaporation temperatures were 30°C.

3. RESULTS & DISCUSSION:

Method development for the combined separation of neutral and polar lipids was performed on the PVA-Sil stationary phase with ELSD detection. The mobile phase for the analysis is comprised of hexanes, isopropanol, methanol, aqueous formic acid (25 mM) and triethylamine (25 mM) at pH 4.2, and is suitable for both ELSD and UV detection.

3.1 Method 1: Polar Lipid Class Separation

Polar lipids separation on PVA-Sil appeared to have decent peak shapes when a buffer comprising of formic acid and triethylamine was used as the aqueous component rather than just water. The positive affect of triethylamine-formic acid salts on polar lipid peak shapes have also been reported with other solvent systems [43, 54]. The method for polar lipids separation has nine steps with linear gradient and the column was equilibrated for ten minutes at the end of each run (Table 1). The gradient program in the method includes isocratic hold point where the column was flushed with 2-Propanol for 3 minutes to facilitate the mobile phase transition from aqueous phase to organic phase during column equilibration. All samples and standards are dissolved in toluene containing 0.05% BHT for injection. The injection volumes for the individual polar lipid standards were between 5 and 15 µL, and the lipid loaded per injection was

about 30-50µg. Approximately 100µg of lipid standards mixture containing ~ equal mass amounts Monogalactosyldiacylglycerol (MGDG), Digalactosyldiacylglycerol (DGDG), Sulfoquinovosyldiacylglycerol (SQDG), Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylserine (PS), Phosphatidylcholine (PC), lyso phosphatidylcholine (LPC) was injected in 8 µL (Figure 1). All the lipid classes eluted under 23 min with LPC being the last lipid class to elute around 22 minutes.

3.2 Method 2: Separation of neutral and polar lipid classes

A previously optimized method for the separation of just neutral lipids utilizing a hexane/isopropanol/methanol/aqueous system [43] was modified and combined with Method 1 to separate both neutral and polar lipids in a single run. From the previous method, the mobile phase flow was reduced to 1 mL/min, and triethylamine/formic acid system was used instead of water/2-propanol to facilitate separation of polar lipid classes. Polar lipid standards from Figure 1 were combined with the neutral lipids TAG, DAG (both sn-1/3 and sn-1/2 isomers), FFA and MAG and each was resolved under 40 minutes (Figure 2). Total run time is 58 min including equilibration for the next analysis (Table 2). The two isomers of DAG; 1,3-DAG and 1,2-DAG showed complete separation and the former co-eluted with free fatty acids. Because 1,3-DAG is not biologically relevant form of the diacylglycerols, this co-elution is not relevant to most biological extracts. Neutral lipid classes eluted under 18 minutes and MGDG was the first polar lipid class to elute at 19.4 minutes. The baseline on the chromatogram had very minimum noise except for around the 40-minute mark (Figure 2). Running a blank injection confirmed the baseline noise was caused by the method gradient (Supplementary Figure S1).

3.3 Method 3: Separation of HFA-containing neutral and polar lipid classes

A method for the separation of mixture of polar lipids and hydroxy fatty acid (HFA) containing neutral lipids was also developed based off method 2 (Table 3). Here the TAG, DAG and MAG neutral lipid classes separate into sub-classes based on the number of HFA in each molecule. Figure 3 demonstrates the separation of a lipid mixture containing 19 lipid classes under 50 min, with a total run time of 72 min

171 including equilibration. Besides the 19 lipid classes shown in figure 3, we had also analyzed a mixture of
172 PC and LPC using this method and determined that LPC elutes after PC at 50.6 minutes during the analysis
173 for a total of 20 lipid classes separated by method 3 (Supplementary Information Figure S2).
174
175
176 **4. METHODS APPLICATIONS**
177 **4.1 Application of combined neutral and polar lipid methods to biological lipid extracts**
178 Method 2 for combined neutral and polar lipids was used to analyze various lipid extracts from
179 microorganisms, plant, and animal tissues. Partially characterized total lipid extracts of *Escherichia coli*,
180 bovine heart, bovine liver, and bovine brain were purchased from Avanti Polar Lipids. We were able to
181 identify the major polar lipid classes listed on the lipid profile of the corresponding extract (Figure 4). The
182 components listed only as neutral lipids in each purchased extract were also separated and identified. For
183 example, the bovine liver extract only characterizes neutral lipids as 20% of total, we identified that these
184 were mostly TAG, FFA and 1,2-DAG (Figure 4B).
185 Total lipid extracts from *Camelina sativa* and tobacco leaves (Figure 5) were also analyzed by
186 method 2. In addition to the identification of the glycerolipids by ELSD, we monitored the leaf lipid extracts
187 for photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids [55, 56]) by DAD multiple
188 wavelength detection (Supplementary Figure S3). The DAD analysis demonstrated that the mass peaks
189 recorded by ELSD that eluted before the polar lipid classes in leaves are predominantly pigments. We also
190 analyzed transgenic tobacco plants engineered to accumulate the neutral lipid TAG in leaves [41, 57]. The
191 major neutral lipid and polar lipid classes were completely separated from the tobacco leaves
192 (Supplementary Figure S4). Additionally, method 3 that separates neutral lipids (both hydroxy and non-
193 hydroxy) and polar lipids, was used to analyze the total lipid extract from transgenic *Arabidopsis thaliana*
194 seeds engineered to accumulate HFA [58] (Supplementary Figure S5). The HFA-containing and non-HFA-
195 containing lipid classes of the total seed lipid extract were identified based on the retention time (t_R) of the

196 lipid standards from Figure 3. These latter two examples demonstrate the usefulness of these methods for
197 analysis of plant lipid bioengineering.
198
199 **4.2 Application to Radio HPLC analysis**
200 Methods 1, 2 and 3 were used to analyze lipid extracts from *in vivo* ^{14}C based lipid metabolic flux
201 experiments. Lipid extracts from tobacco leaves labeled with [^{14}C]acetate for 30 and 120 min [41] were
202 analyzed for polar lipid resolution by Method 1 (Figure 6). Radioactivity was detected with an in-line liquid
203 scintillation detector with a flow cell volume of 300 µL and an eluant to scintillation cocktail ratio of 1:2,
204 yielding a residence time of 6 seconds for scintillation counting. The peak assignments were made based
205 on the retention times of lipid standards on the ELSD chromatogram (Figure 1). After 30 min of [^{14}C]acetate
206 labeling, the major labeled lipids were PC (36%) followed by MGDG (25%), with lesser amounts in PG
207 (4%), DGDG (0.7%), and others (Figure 6A). The relative proportion of labeled lipids at 30 min of labeling
208 do not match the steady-state mass proportion in wild-type tobacco leaves of 42% MGDG, 22% DGDG,
209 13% PC, and 8% PG [41]. However, the tracing of newly synthesized ([^{14}C]acetate labeled) fatty acids flux
210 into mostly PC here is consistent with the function of the Eukaryotic and Prokaryotic pathways of
211 glycerolipid assembly in plant leaves [2]. In tobacco leaves PG, and some MGDG are produced within the
212 chloroplast through the Prokaryotic pathway [2]. However, PC, most MGDG, and almost all DGDG are
213 produced through the Eukaryotic pathway localized to the endoplasmic reticulum. Within the plant leaf
214 Eukaryotic pathway nascent fatty acids are first incorporated predominantly into the *sn*-2 position of PC as
215 they exit the chloroplast through acyl editing [39, 41, 59]. Subsequently, fatty acids are removed from PC
216 by acyl editing for Kennedy pathway *de novo* PC synthesis. Eukaryotic MGDG and DGDG molecular
217 species are then produced in the chloroplast from DAG derived from *de novo* synthesized PC [2, 39].
218 Therefore, the rapid flux of fatty acids into PC here is consistent with PC as an intermediate to production
219 of the most abundant leaf lipids MGDG and DGDG. In addition, the change in the PC:MGDG labeling
220 ratio from 1.41 at 30 min of [^{14}C]acetate labeling (Figure 6A) to 0.91 at 120 min of labeling (Figure 6B) is

221 consistent with the transfer of labeled fatty acids from PC to MGDG within the Eukaryotic pathway of leaf
222 glycerolipid biosynthesis.

223 To test methods 2 and 3 with radio-HPLC for lipid metabolic flux experiments, we have applied
224 method 2 to analyze the lipid extracts from wild type Arabidopsis seed tissue that was continuously labeled
225 for 30 minutes with ^{14}C acetate (Supplementary Figure S6). Method 3 was used to analyze the seed extracts
226 from HFA producing Arabidopsis that were labeled continuously with ^{14}C glycerol for 45 minutes
227 (Supplementary Figure S7). For both labeling experiments, the substantial labeling of PC at these short time
228 points to levels that are much higher than the steady state mass levels of PC (<5% of seed lipids) are
229 consistent with PC as a key intermediate for acyl and glycerol backbone flux prior to TAG synthesis in
230 plant seeds [1, 28, 60]. The results of the tobacco leaf, and both wild-type and transgenic Arabidopsis seed
231 metabolic labeling experiments are consistent with previous analyses that utilized TLC separation and
232 phosphor imaging to quantify radioactive lipids produced by these tissues [41, 61, 62], indicating the radio-
233 HPLC analysis of lipid metabolic flux experiments presented here represents a more high throughput
234 analytical method than the more traditional analytical procedures for radiolabeled lipid analysis (2, 22).

235

236 **5. Conclusion:**

237 We developed three normal phase HPLC methods for lipid class separation on PVA-Sil stationary
238 phase that utilize hexane/isopropanol/methanol/aqueous based mobile phase and is suitable for ELSD, UV,
239 and in-line liquid scintillation counting detection, or for fraction collection to purify lipids. Method 1
240 resolves polar glycerolipids while neutral lipids elute together at the beginning of the method. Method 2
241 allows separation of neutral and polar lipids containing common saturated and unsaturated fatty acids.
242 Method 3 provides additional separation of the neutral lipids into sub-classes depending on the presence of
243 1-3 fatty acids containing a polar functional group such as a hydroxyl that are found in neutral lipids of
244 various plant species. The lipid classes in each of the extracts were completely separated and identified

based on the retention times of the individual standards. The methods were applied to various total lipid extracts indicating wide applicability for lipid analysis from animal, plant, and microbial systems, as well as for use within radiotracer based metabolic flux experiments.

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FIGURE CAPTIONS

Figure 1: ELSD trace of polar lipid mixture on YMC PVA-Sil column. Identities of peaks are as follows: 1. Monogalactosyldiacylglycerol (MGDG), t_R=6.1 min; 2. Digalactosyldiacylglycerol (DGDG), t_R=8.9 min; 3. Sulfoquinovosyldiacylglycerol (SQDG), t_R=10.8 min; 4. Phosphatidylglycerol (PG), t_R=12.4 min; 5. Phosphatidylethanolamine (PE), t_R=13.8 min; 6. Phosphatidylinositol (PI), t_R=15.9 min; 7. Phosphatidylserine (PS), t_R=18.0 min; 8. Phosphatidylcholine (PC), t_R=19.9 min; 9. Lysophosphatidylcholine (LPC), t_R=22.4 min.

Figure 2: ELSD trace of mixture of polar and non-polar lipid classes on PVA-Sil column. The identities and retentions of the labeled peaks are as follows: 1. Triacylglycerol (TAG), t_R =3.6 min; 2. Free Fatty Acids +1,3-diacylglycerol (FFA+1,3-DAG), t_R = 12.7 min; 3. 1,2- Diacylglycerol (1,2-DAG), t_R = 14.8 min; 4. Monoacylglycerol (MAG), t_R = 17.8 min; 5. Monogalactosyldiacylglycerol (MGDG), t_R = 19.4 min; 6.

444 Digalactosyldiacylglycerol (DGDG), t_R = 22.5 min; 7. Sulfoquinovosyldiacylglycerol (SQDG), t_R = 25.1
445 min; 8. Phosphatidylglycerol (PG), t_R = 27.1 min; 9. Phosphatidylethanolamine (PE), t_R = 28.2 min; 10.
446 Phosphatidylinositol (PI), t_R = 31.2 min; 11. Phosphatidylserine (PS), t_R = 35.5 min; 12. Phosphatidylcholine
447 (PC), t_R = 37.4 min; 13. Lysophosphatidylcholine (LPC), t_R =39.9 min.

448

449 **Figure 3:** ELSD trace of mixture of polar and HFA-containing non-polar lipid standards on PVA-Sil
450 column. The identities and retentions of the labeled peaks are as follows: 1. Triacylglycerol (TAG), t_R
451 =3.6 min; 2. 1-Hydroxy Fatty Acid Triacylglycerol (1-HFA TAG), t_R =10.2 min; 3. Free Fatty Acids
452 +1,3-diacylglycerol (FFA+1,3-DAG), t_R = 12.3 min; 4. 1,2- Diacylglycerol (1,2-DAG), t_R = 14.6 min; 5.
453 2-Hydroxy Fatty Acid Tricylglycerol (2-HFA TAG), t_R =16.5 min; 6. Hydroxy Free Fatty Acids (HFFA),
454 t_R =21.2 min; 7. 1-Hydroxy Fatty Acid Diacylglycerol (1-HFA DAG), t_R = 23.2 min; 8. 3-Hydroxy Fatty
455 Acid Tricylglycerol (3-HFA TAG) t_R = 24.9 min; 9. Monoacylglycerol (MAG), t_R = 27 min; 10. 2-
456 Hydroxy Fatty Acid Diacylglycerol (2-HFA DAG), t_R = 27.3 min; 11. 1-Hydroxy Fatty Acid
457 Monoacylglycerol (1-HFA MAG), t_R =28.6 min; 12. Monogalactosyldiacylglycerol (MGDG), t_R = 29.9
458 min; 13. Digalactosyldiacylglycerol (DGDG), t_R = 33.6 min; 14. Sulfoquinovosyldiacylglycerol (SQDG),
459 t_R = 36.1 min; 15. Phosphatidylglycerol (PG), t_R = 38.2 min; 16. Phosphatidylethanolamine (PE), t_R = 39.3
460 min; 17. Phosphatidylinositol (PI), t_R = 42.3 min; 18. Phosphatidylserine (PS), t_R = 45.9 min; 19.
461 Phosphatidylcholine (PC), t_R = 49.2 min.

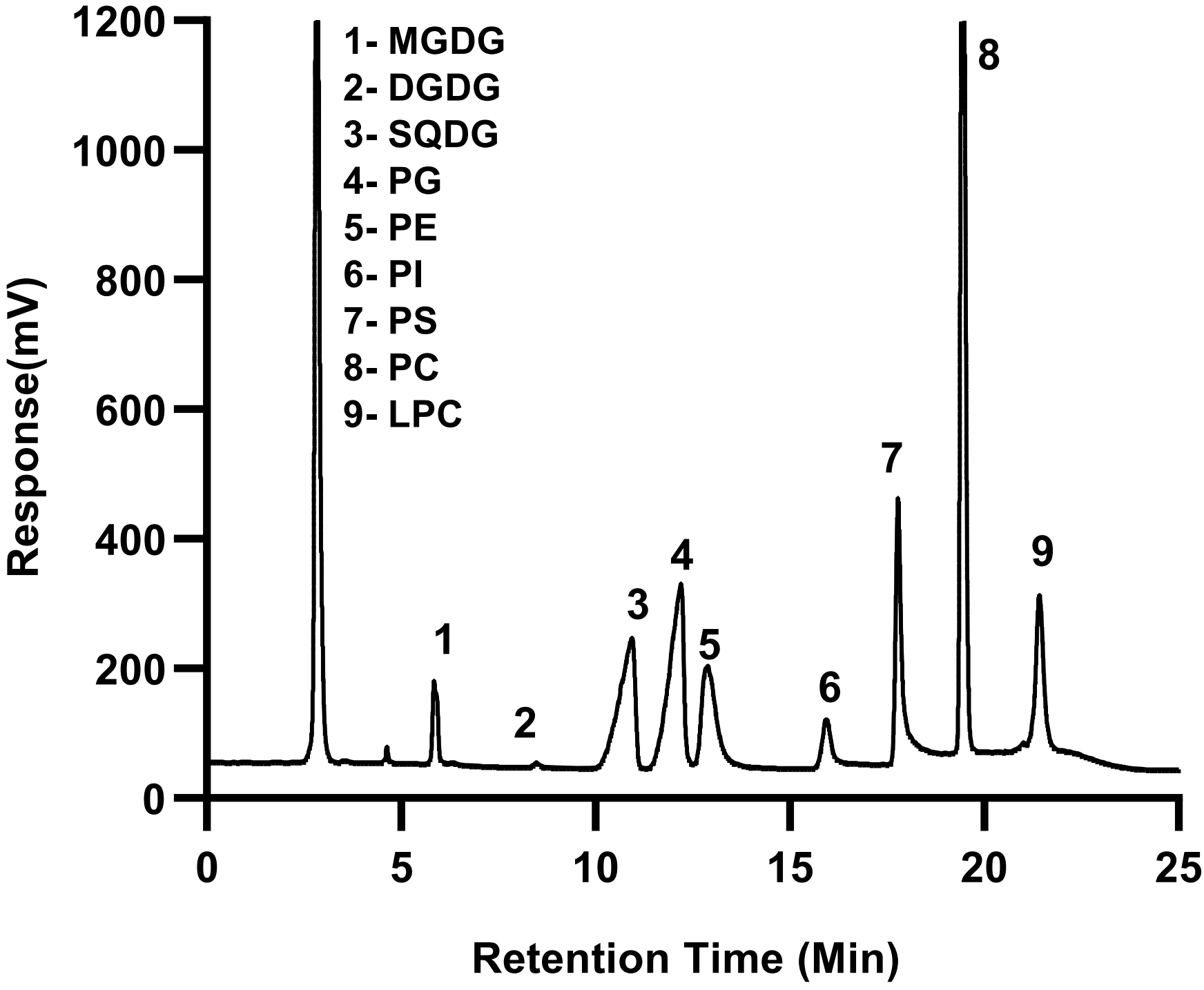
462

463 **Figure 4:** ELSD traces of total lipid extracts from, **A.** *E.coli* (Sample injection: 30µg in 2µl toluene); **B.**
464 Bovine Liver (Sample injection: 60µg in 3µl toluene); **C.** Bovine Heart (Sample injection: 100µg in 4µl
465 toluene); **D.** Bovine Brain (Sample injection: 100µg in 4µl toluene)

466

Figure 5: ELSD traces of leaf lipid extracts of A. *Camelina sativa*; B. Wild type tobacco. Approximately 50µg lipid was injected in 6µl toluene for both analyses

Figure 6: HPLC-Radio traces of Wild type tobacco leaf extracts continuously labeled with ^{14}C acetate for **A.** 30 min (A total of ~42000 CPM was injected in 9µL toluene); **B.**120 min (A total ~54000 CPM was injected in 6µL toluene).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2- DGDG
3- SQDG
4- PG
5- PE
6- PI
7- PS
8- PC
9- LPC
Response(mV)
Retention Time (Min)
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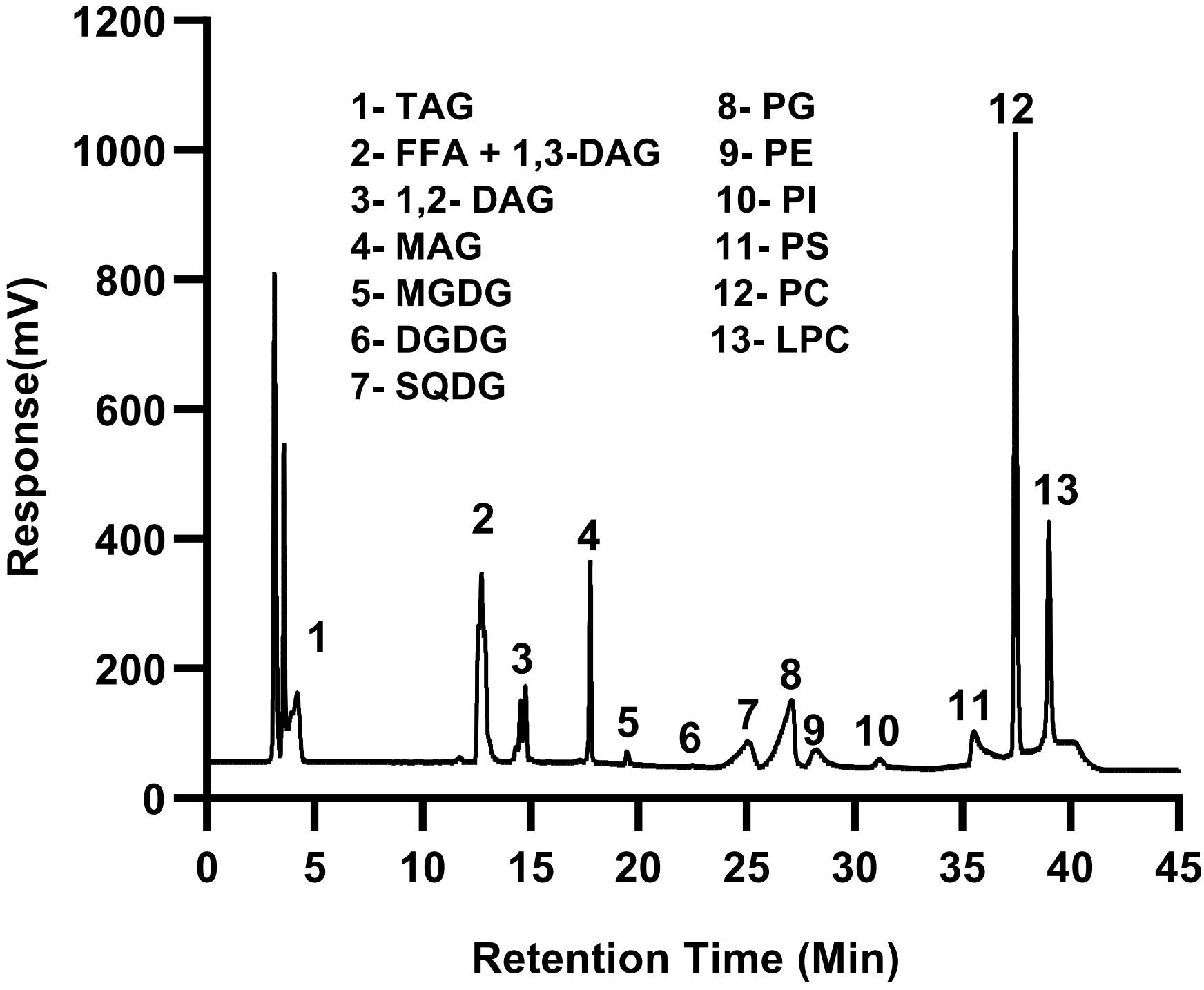
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Response(mV)
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10
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20
25
30
35
40
45
Retention Time (Min)
1- TAG
2- FFA + 1,3-DAG
3- 1,2- DAG
4- MAG
5- MGDG
6- DGDG
7- SQDG
8- PG
9- PE
10- PI
11- PS
12- PC
13- LPC
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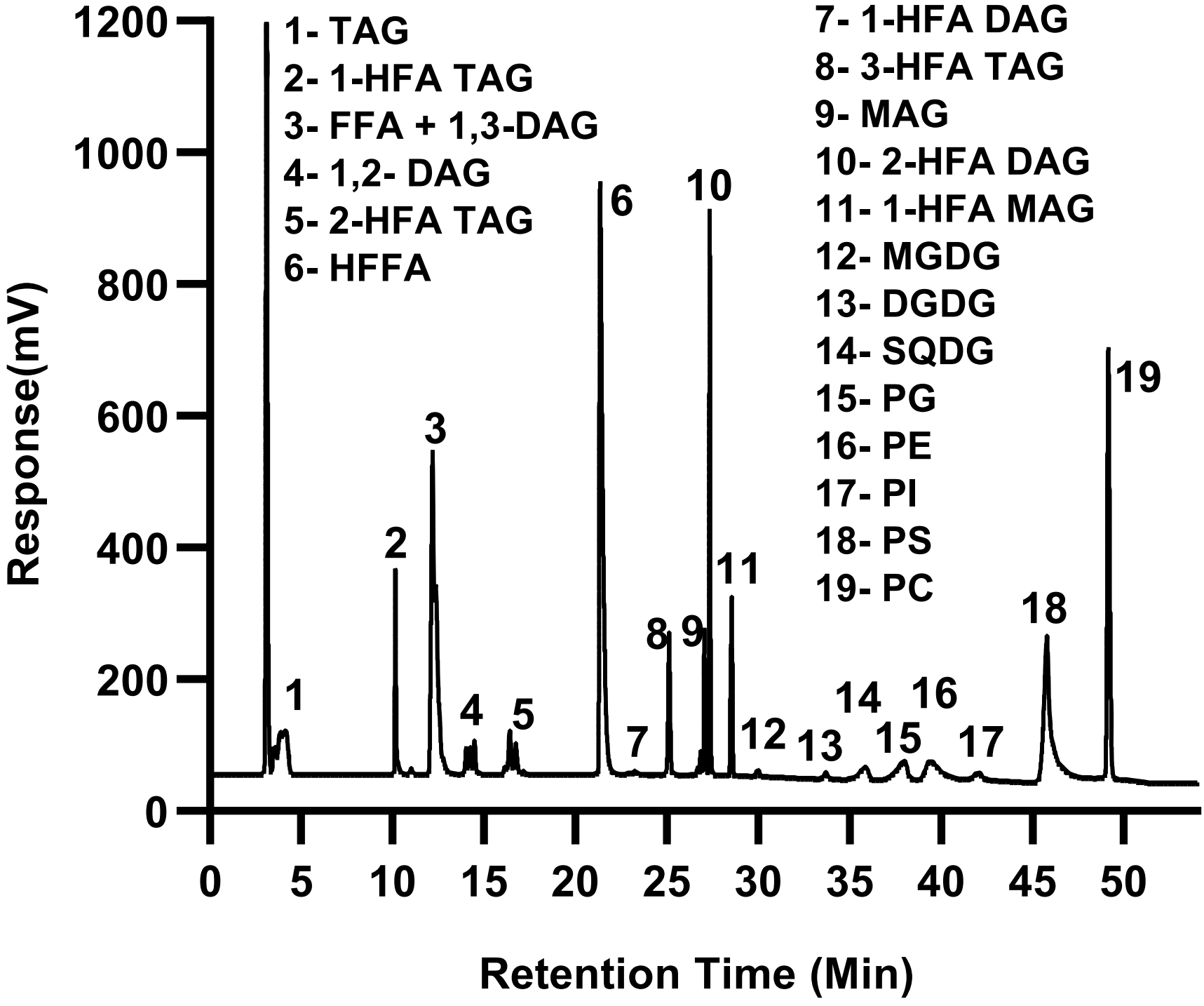
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3- FFA + 1,3-DAG
4- 1,2- DAG
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6- HFFA
7- 1-HFA DAG
8- 3-HFA TAG
9- MAG
10- 2-HFA DAG
11- 1-HFA MAG
12- MGDG
13- DGDG
14- SQDG
15- PG
16- PE
17- PI
18- PS
19- PC
Response(mV)
Retention Time (Min)
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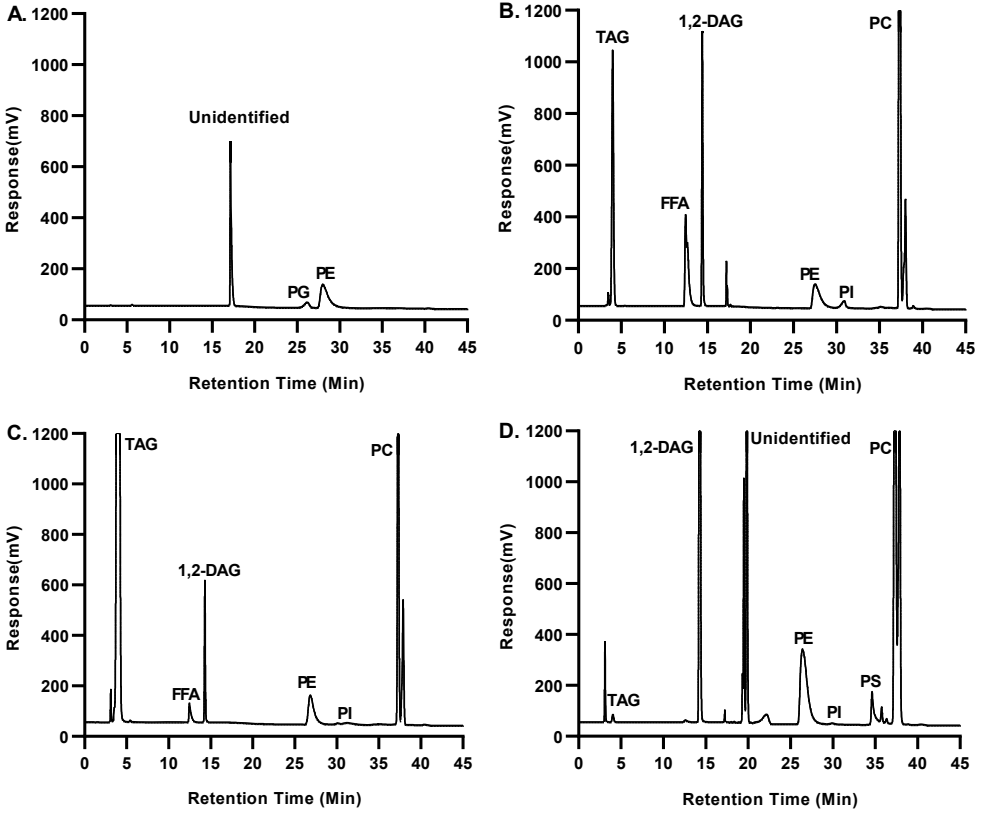
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C.
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PC
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FFA
PE
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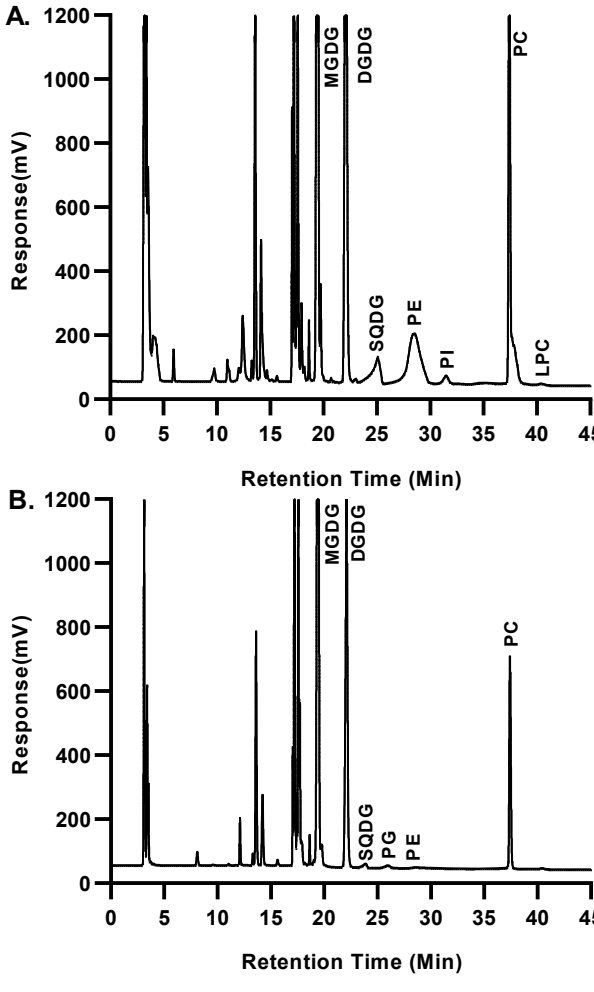
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Retention Time (Min)
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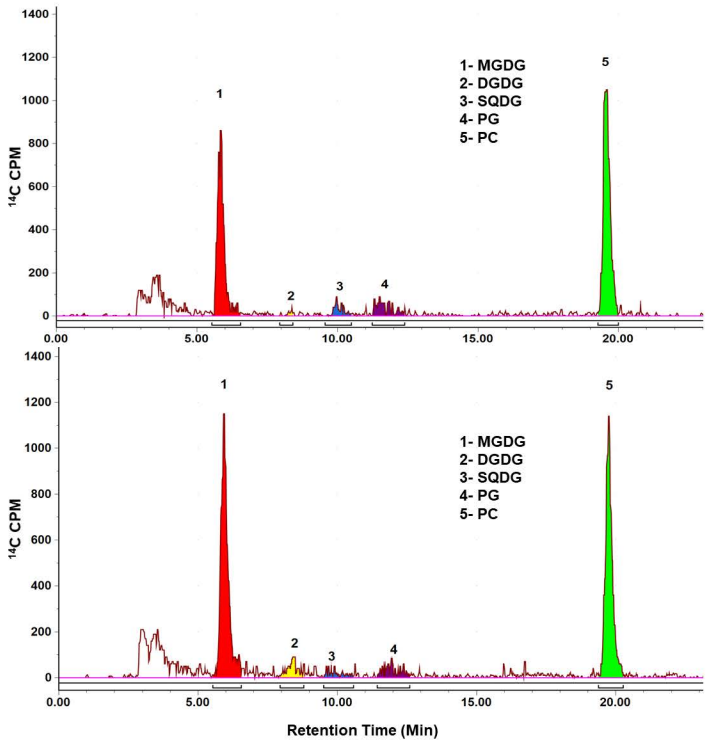
Retention Time (Min)


1- MGDG
2- DGDG
3- SQDG
4- PG
5- PC
^{14}C CPM
Retention Time (Min)