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Determination of Starch Lysophospholipids in Rice Using Liquid Chromatography Mass Spectrometry (LCMS)

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ABSTRACT

Acquisition of a complete understanding of rice starch lysophospholipids (LPLs), their biosynthetic pathways, genetic diversity, and the influence of genotype by environment interactions has been hampered by the lack of efficient high throughput extraction and analysis methods. We hypothesized a single-step aqueous n-propanol extraction combined with liquid chromatography mass spectrometry (LCMS) could be employed to analyze starch LPLs in white rice. Our investigation found different grinding methods showed little effect on the final LPL detected and a simple single-step extraction with 75% n-propanol (8mL/0.15g) heated at 100 ^{o}C for two hours was as effective as an onerous multi-step extraction method. A LCMS method was optimized to simultaneously quantify ten major LPLs in rice starch within 15 minutes. This method enables total and individual starch LPLs analysis of a large number of rice samples at little cost. This approach could be applied to starch LPLs in other cereals.

KEYWORDS:

Rice, lysophospholipid, LCMS, starch lipid, amylose-lipid complex, grain quality.

INTRODUCTION

Cereal starch granules contain endogenous lipids that occur both within and on the surface of starch granules[1]. The starch lipids in this paper refer to those that naturally form inclusion complexes with starch, and amylose in particular, inside the granules[2]. Starch lipids are important because they interact with starch and modify product texture, rheological properties, digestibility and storage stability[3-5].

Cereal starch lipids are mainly monoacyl lipids, such as free fatty acids and lysophopolipids (LPLs, see Supporting Information for Publication for the structures)[1]. The starch LPLs accounts for about 50% starch lipids in rice and are of particular interest to us because they contain bioavailable nutrients such as phosphate, choline and ethanolamine[6]. We recently systematically reviewed the rice phospholipid literature, and evaluated their significance in grain quality and contribution to human health benefits[6]. Although rice starch LPLs are not major sources of dietary phospholipids such as oil seeds, eggs or seafood, they may play an important role for some modern concepts of rice qualities such as glycemic index[6]. In addition, LPLs may have important role in starch biosynthesis and hence impact rice quality. However, there are few reports concerning these LPLs in cereals, which is partially due to the lack of an efficient analytical method.

Rice starch LPLs have previously been analyzed using a combination of classical thin layer chromatography (TLC) and gas chromatography (GC) methods[7]. The major rice starch LPLs were found to be lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE). The fatty acid compositions of these LPLs are mainly palmitic (16:0, 48–63%) and linoleic (18:2, 25–42%) acids, with minor contributions from oleic (18:1, ~5%) and myristic (14:0, ~5%) acids[7]. The TLC-GC

method usually requires extraction from large sample sizes (~100g). Moreover, the sample preparation process is time-consuming with many steps which can lead to errors in the final results. In order to investigate the biosynthetic pathways of rice LPLs, their natural and induced genetic diversity, and the extent to which they are subject to genotype by environment interactions, a large number (500-1000) of small quantity (<5g) samples often need to be analyzed and compared, and this has proven impractical using existing TLC-GC methods[8-10].

Compared to TLC-GC methods, high performance liquid chromatography (HPLC) typically requires simplified sample preparation with no requirement for derivatization. This provides an inherently more direct and tractable approach to LPL analysis. However, since rice starch LPLs only have small chromophores at around 200nm, it is almost impossible to quantify the LPLs with HPLC routinely coupled with an ultraviolet (UV) detector[11]. Recently, detection methods have been developed that are very effective for analyzing phospholipids in animal plasma and liver. These involve HPLC coupled with mass spectrometry (LCMS), and especially MS/MS[12, 13]. More recently, a non-targeted metabolomics investigation of transgenic rice using LCMS/MS identified significant variation in LPE levels[14], although the extraction method used was not suitable for the analysis starch LPLs[6].

LCMS/MS has not been widely applied to research in the agrifood sector, perhaps due to the initial relatively high entry costs for purchase and maintenance of LCMS/MS equipment. Over the past decade, HPLC coupled with single quadrupole mass detector (LCMS) has become significantly cheaper and more accessible to many researchers. We have accumulated considerable experience in successfully analyzing metabolites in cereal

grains using this approach[15, 16]. The aim of this research was to develop a fast, reliable and low-cost LCMS method to analyze starch LPLs in rice grain. Since starch LPLs form inclusion complexes with amylose and require a different procedure for extraction[6], we also investigated the effects of different grinding and extraction methods on total rice starch LPL content. This extraction and analytical strategy is most likely suitable for analyzing starch LPLs in cereals other than rice.

MATERIALS AND METHODS

Plant materials, reagents and standards

Rice accessions (Table 1) were obtained from the Key Laboratory of Zhejiang Province and Chinese Ministry of Agriculture for Nuclear-Agricultural Sciences, Zhejiang University, China. The rice grains were harvested in October 2012. The rice grains were air-dried, stored at room temperature for two months until the physiochemical properties of the rice grain stablised[17, 18], and then stored at 4^{o}C until analyzed in March 2013.

All organic solvents used were HPLC or LCMS grade. The LPL standards, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LPE 18:1), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LPE 16:0), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC 18:1), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC 16:0), were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

Sample grinding and amylose content determination

Rice samples were milled to white rice using a Satake Rice Machine (Satake Corporation, Japan). A laboratory ball mill (Mixer Mill MM301, Retsch) and a cyclone

sample mill (UDY Corporation, Fort Collins, Colorado, USA) were used separately to produce rice flour samples (Figure 1). For the cyclone grinding, the milled rice was ground into flour and passed through a 0.5 mm mesh sieve. For the ball mill, the grinding jar was filled with about 8 g of white rice each time and shaken at 30 r/s for 15 seconds to produce the rice flour samples. The moisture content of the flour was measured by loss on drying, where the flour samples were dried under vacuum (6kPa) and at 60^{o}C overnight until constant weight.

Apparent amylose content was determined using the iodine staining method[19]. The absorbance of the solution was measured at 620 nm against the blank solution using a spectrophotometer. The apparent amylose content was determined in duplicate for each sample.

Multi-step extractions

The multi-step extraction was carried out in triplicate for each sample. In the first step of extraction, rice flour (0.5 g) from each sample was weighed accurately and placed in a 16x125 mm, PYREX culture tube. Chloroform/methanol (2:1, v/v; 8 mL/) was added to each tube which were capped with rubber liners, placed on ice, and the supernatant removed after 15 min[20] (Figure 1). This extraction was repeated two times for each sample (a total of three extractions). The supernatant from each of the three sequential extractions were combined and dried under $N_{2.}$ This concentrated extract (R01BN-R13BN and R01CN-R13CN, Figure 1) was dissolved in isopropanol/acetonitrile (50/50, v/v) for LCMS analysis. The chloroform/methanol extracted rice flour was carefully dried under N_2 in the culture tube before the next extraction step.

In the second step of extraction, the chloroform/methanol extracted rice flour above was extracted with 75% n-propanol (n-propanol/water, 75/25, v/v; 8 mL) for two hours under nitrogen at 100^{o}C in the same culture tube following the method of Morrison and Coventry[21] (Figure 1). The supernatant was removed and the extraction repeated, once for two hours and once for one hour. The supernatant from each sequential extractions were combined in a 25 mL volumetric flask and a 1mL aliquot transferred to a vial for LCMS analysis. The remaining 24 mL of extract (Figure 1; R01BS-R13BS and R01CS-R13CS) was dried using a rotational vacuum concentrator (CHRiST, Osterode am Harz, Germany) and re-dissolved in 1mL 75% n-propanol for LCMS analysis.

Single-step extraction

Rice flour (~0.15 g) from each sample was weighed accurately, placed in a culture tube and extracted for two hours under nitrogen at 100^{o}C with 75% n-propanol (8 mL). The culture tubes were weighed before and after heat extraction to ensure there was no loss of solvent which would have affected the solvent/sample ratio (w/w). A 1mL aliquot of the extract (Figure 1; R01B1-R13B1 and R01C1-R13C1) was transferred to a vial for the LCMS analyses. Triplicate extractions of each sample were undertaken.

LCMS analysis

The analysis of extracts was undertaken using an Agilent HPLC (Series 1290) equipped with a vacuum degasser, binary pump, auto-injector, diode array detector(DAD, 1260), coupled to an Agilent quadrupole mass detector (MSD, 6120). A Phenomenex Luna C18 column (5 μm; 150×4.6 mm internal diameter) was used for analysis of rice grain lipids. Column temperature was set at 40^{o}C. Absorbance was monitored between

190 nm and 600 nm. The injection volume was set at 5 μl per injection. The LCMS system was controlled using ChemStation software.

Two linear gradient elution programs based on our previous research of the lipophilic components in wheat grain[15, 16, 22] were used for the qualitative analysis of concentrated rice lipid extracts (Figure 1; R01BN-R13BN, R01CN-R13CN, R01BS-R13BS and R01CS-R13CS). The first linear gradient contained methanol with 0.005% trifluoroacetic acid (TFA) and 2-propanol with 0.005%TFA. The solvent gradient was programmed from 10% to 95% 2-propanol with 0.005% TFA in 15 min with a flow rate of 1.0 mL/min. The second linear gradient contained acetonitrile with 0.005% TFA and water with 0.005% TFA. The solvent gradient was programmed from 10% to 99% acetonitrile in 30 minutes with a flow rate of 1.0 mL/min and held at 99% acetonitrile for 5 min.

The MSD was carried out in two ionization modes: atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). The APCI mode used parameters of: scan mass range 100–1200; fragmentor 150; capillary voltage, 3000 V (positive); corona current, 4.0 μA; drying gas flow, 5.0 L/min; drying gas temperature, 350^{o}C; vaporizer temperature, 350^{o}C; nebulizer pressure, 35 psig. The ESI mode used parameters of: scan mass rang 100–1200; fragmentor 150; capillary voltage, 3000 V (positive); drying gas flow, 12.0 L/min; drying gas temperature, 350 ^{o}C; vaporizer temperature, 350 ^{o}C; nebulizer pressure, 35 psig. All LCMS settings and parameters above were optimized based on the manufacturer's recommendations and a number of flow injection experiments.

Quantification of starch LPLs

The same Agilent LCMS equipment above was used to quantify LPLs. A much shorter and lower volume Agilent Eclipse Plus C18 RRHD column (1.8 μm; 50×2.1 mm internal diameter) was used for the quantitative analysis of rice starch LPLs. A shorter linear gradient elution program was used for the quantitative analysis, with a mobile phase containing water and 0.005% TFA and acetonitrile with 0.005% TFA programmed from 10% to 99% acetonitrile in 10 min with a flow rate of 0.3 mL/min and held at 99% acetonitrile for 1.5 min. For this quantitative analysis, the injection volume was set at 3 μl per injection.

The rice starch LPLs were quantified using an external standard method. The MSD was operated using the same parameters as for the ESI method above except that Single Ion Monitor (SIM) mode was used rather than Scan mode. Four available MSD signal channels were employed to detect ten ions simultaneously as shown in Table 2. Standards were prepared at concentration of 32pM, 160pM, 800pM, 4000pM, 20,000pM, 100,000pM, 200,000pM. LPLs eluted at similar retention time were allocated to different signal channels (Table 2). The LPLs without commercially available standards were quantified using LPLs of similar structure (Table 2). The method was validated for linearity, precision, limit of detection, limit of quantification and working concentration range prior to quantification.

Statistical analysis

All the LPLs data were measured at least in triplicate. Data analyses were performed with The SAS program version 9.1 (SAS Institute Inc., Cary, Nc). The analysis of variance (ANOVA) were performed to determine the effects of grinding methods,

extraction methods on the starch lipids and non-starch lipids with the general linear model procedure (Proc GLM). The multiple comparison of different mean values for test of significance were determined with Proc means and Proc tukey at $P<0.05$. The correlation coefficients between the parameters were conducted with Proc corr.

RESULTS AND DISCUSSION

Sample grinding and multi-step extraction

Ball and cyclone sample mills were chosen for this experiment as they are the two most commonly used sample grinding methods for plant material prior to solvent extraction and instrumental analysis. Ball mills are widely available in most analytical laboratories where they are used for general purpose plant sample preparation, whilst cyclone sample mills are often used for grain quality assessment, such as Rapid Visco-Analyzer (RVA) test of pasting viscosity. In this experiment, we used a very gentle ball mill method (30 r/s for 15 seconds) as severe ball-milling may destroy starch granule structure and affect quantitative extraction of lipids[21]. The rice flour produced by this gentle ball mill method has visibly larger particle size than flour generated by the cyclone sample mill. The moisture content of the flour was between 10.39- 11.47% (Table 1).

The multi-step extraction conditions were chosen carefully based on our previous review[6]. The first step of multi-step extraction with chloroform/methanol was designed to extract non-starch lipids and it is a classic method for lipid extraction. The second step of multi-step extraction with n-propanol aqueous solution was designed to extract starch lipids. As rice starch lipids (including the LPLs) are protected by the impermeable starch granules and form inclusion complexes with amylose, they are not readily extracted by organic solvent at room temperature. Efficient extraction of internal starch LPLs requires

both water and heat to swell or gelatinize the native starch granule to permit the alcohol to penetrate and extract the lipids[21]. The starch lipids were initially suspected to be an artefact formed during starch isolation. Later, ^{13}C-cross-polarisation/magic angle spinning nuclear magnetic resonance (^{13}C CP/MAS-NMR) analysis has confirmed that the LPLs exist *in vivo* in native rice starch granules[23].

The multi-step extraction procedure included three repeated extraction for each step in order to exhaustively extract and separate non-starch and starch lipids in rice endosperm. The extraction of non-starch lipid was carried out at low temperature (0 ^{o}C) as a precautionary measure to slow enzymatic reactions during extraction, and to reduce the co-extraction of non-lipid components[6]. These preventative measures were proven to be effective to reduce the action of lipase and phosphatase as no degradation products (such as phosphatidic acids) were found by subsequent LCMS analysis. Through the multi-step extraction, less than 1% of starch LPLs was found in the non-starch lipid fraction by subsequent LCMS analysis, suggesting the multi-step sequential extraction of rice flour was capable of separating the non-starch lipids from starch lipids.

The yields (%) of non-starch lipids extracted by chloroform/ methanol (2/1, v/v) varied from 0.10% (R04CN) to 1.34% (R02CN) (Table 1, Figure 1&2). For some samples such as R02, R03, R07, R08 and R09, the yields (%) of non-starch lipid extracts of cyclone-milled samples were significant higher than those of ball-milled samples ($P < 0.001$), which may be due to the effects of different grinding methods. The yields (%) of starch lipids extracted by 75% n-propanol were higher than the non-starch lipid extracted by chloroform/methanol ($P<0.05$) and varied from 0.70% (R01BS) to 2.24% (R12CS) (Figure 2). In general, the yields of extracted lipids are higher from the cyclone milled

flour than from the ball milled flour which may be attributed to the finer flour generated by the cyclone mill which have higher contact surface with the extraction solutions. However, the two grinding methods were proved to have no significant effects on the quantification of total LPLs by subsequent LCMS analysis ($P = 0.363$). As yield (%) calculations were based on extraction from a very small quantity of rice flour (0.5g for each extraction), these results should only be considered as a preliminary indication of the relative non-starch lipid and starch lipid content in rice grain. Although different rice samples were used, the yields obtained are higher than those reported by Juliano[24] for non-starch lipid (0.41%-0.81%) and starch lipid (0.12%-0.57%) ($P<0.05$), suggesting a possible co-extraction of non-lipid components in our experiment.

LCMS analysis of lipids extracted from multi-step extraction

The concentrated extracts from multi-step extraction (Figure 1; R01BN-R13BN, R01CN-R13CN, R01BS-R13BS and R01CS-R13CS) were analyzed using the 2-propanol/methanol LCMS method. The total ion chromatograph (TIC) clearly indicated that the major components in the first step extract of multi-step extraction (non-starch lipids) were the relatively non-polar triglycerides (eluted at 8-11 min) as discussed in our previous reports (Figure 3a, TICs of R10BN was used as an example)[15, 16]. The extract (starch lipids) from the second step of multi-step extraction contained relatively more polar lipids (Figure 3a, TICs of R10BS was used as an example) which are further investigated using the acetonitrile/water gradient (Figure 3b).

The extracts (starch lipids) from the second step of multi-step extraction were better separated in the acetonitrile/water gradient than in the 2-propanol/methanol gradient (Figure 3b, TICs of R10BS was used as an example). There was little response observed

for the starch lipid samples with the mass detector (MSD) working in the atmospheric pressure chemical ionization (APCI) mode (Figure 3b), with only two LPEs (LPE 18:2, *m/z* 478 [$M+H^+$] and LPE 16:0, *m/z* 454 [$M+H^+$]) identified in the TIC of starch lipids (Figure 3b). We explored many parameters in the APCI mode, but were unable to detect any LPC. A method using electrospray ionization (ESI) mode was developed and optimized, based on Xia and Jemal[25]. It is apparent that LPLs, including LPEs and LPCs, have a much stronger response in the ESI than in the APCI mode (Figure 3b). Seven peaks including both LPCs and LPEs were identified between 19 and 26 min in the ESI modes (Figure 3b), and so this was used for all subsequent experiments (see Supporting Information for Publication for the mass spectra).

The LPL composition of rice grains was analyzed based on the starch LPLs reported by Maningat and Juliano[7] using an extract ion method in the TIC of starch lipids (Figure 3c). Ten major LPLs, including five LPCs and five LPEs, were clearly identified and co-elution of some LPLs was observed (Figure 3c). Different mobile phases and columns were used in an attempt to separate the co-eluted components. However, we were unable to obtain an improved separation, indicating that single ion monitor (SIM) may be the only option to quantify this class of LPLs.

Interestingly, Figure 3c shows two peaks for each of LPCs or LPEs, whereas to date there has been no mention of this phenomenon in the literature on the quantification of LPLs using LCMS. The standards purchased from Avanti also displayed two peaks for the same compounds, suggesting the two peaks for one compound could result from the amphoteric or zwitterionic nature of the LPLs, containing both–O–$P(O_2^-)$–O– (anionic phosphate) and $–N^+(CH_3)_3$ (cationic quaternary ammonium for LPC) or $–NH_3^+$

(ammonium ion for LPE). Based on the report of Xia and Jemal[25], we attempted to run the samples and standards at different pH (2.7, 3.2, 4.4 and 9.3) and at different column temperatures (30°C, 40°C, 50°C and 60°C), but the two peaks for each LPL remained in all conditions. Subsequent ^{1}H NMR analysis (see the Supporting Information for Publication) demonstrated that the two peaks were actually positional isomers of 1-acyl-2-hydroxy and 2-acyl-1-hydroxy LPLs, which were formed via acyl migration[26]. We tried to isolate pure 1-acyl-2-hydroxy LPC/LPE, the thermodynamically favored isomer[26], using preparative HPLC. However, it underwent migration during separation attempts, forming a mixture of 1-acyl-2-hydroxy and 2-acyl-1-hydroxy isomers and so we were unable to obtain pure 1-acyl-2-hydroxy or 2-acyl-1-hydroxy LPC/LPE within the scope of this study. The ^{1}H NMR analysis indicated that the isomer mixtures generally contained up to 10% 2-acyl-1-hydroxy isomer.

LCMS quantification of LPLs

The LCMS method used for the qualitative analysis could also be used to quantify LPLs with modification of mass detection from Scan mode to SIM mode. However, this method was not suited for testing a large number of samples as it took 45 min and consumed approximately 25 mL acetonitrile and 540 liters of high purity N_2 for each analysis. Therefore, an ultra performance liquid chromatography (UPLC) approach was introduced, with the optimized method involving about 30% of the time (45 → 15 min) and 10% cost (e.g. 25 → 2.5 mL acetonitrile) for each analysis. In this UPLC method, the effect of column length reduction (150→50 mm) on chromatography resolution was compensated by the finer column packing particles (5 → 1.8 μm).

As some LPL standards are not commercially available and every LPL standard has two positional isomers, quantification has to be based on the assumption that LPLs with similar structures have similar response factors (area/concentration) in our LCMS analysis. The area under each peak was integrated by the ChemStation software and the area of two positional isomers were summed and considered as one LPL. Plots of the LPL concentrations and the areas were used to obtain second order polynomial regression equations (Figure 4a). The response factors of LPCs are larger than LPEs. Compared to the LPE, the two LPCs have very similar response factors (Figure 4a).

In this report, the limit of detection (LOD) was defined as the concentration of LPL that was required to produce a signal greater than three (3) times the standard deviation of the noise level (S/N>3). The limit of quantification (LOQ) was defined as the concentration of LPL that was required to produce a signal greater than ten (10) times the standard deviation of the noise level (S/N>10). The Signal-to-Noise ratio (S/N) was calculated using an Agilent Chemstation Software (Rev. B04.03). Under the LCMS conditions used in this study, the limit of detection (LOD) was about 6 pM (about 3 pg/mL) and the limit of quantification (LOQ) was about 30 pM (about 15 pg/mL). Based on the current extraction and sample preparation methods, we can detect and quantify each LPL when its concentration in the rice grain is above 0.75 ng/g (about 1 ppb).

The R^2 of the LPL standard curves were all greater than 0.9996 over the working concentration range (between 32 pM and 200,000 pM). The precision of the instrument was obtained by injecting a standard mix solution six times (n=6). The average of the relative standard deviation (RSD) values for each LPLs was about 1% of the six repeated analyzes.

Total starch LPLs by multi-step extraction

The ten major starch LPLs extracted by the second step extraction of multi-step extraction were quantified using the LCMS method above and added together as the total starch LPLs. The total starch LPLs (from R01CS - R02CS and R01BS - R02BS, Figure 1) varied between 516-675μg/g for the waxy rice (or glutinous rice, amylose content less than 2%) and 5733-8139μg/g for the non-waxy rice (R03CS-R12BS) (Figure 4b). Our starch LPLs results were greater (P<0.05) than those (~3mg/g for non-waxy rice) reported by Choudhury and Juliano[27] but similar to those (4070-8110 μg/g for non-waxy rice) reported by Morrison et al.[9]. Our method could quantify low levels of starch LPLs in waxy rice with less than 2% amylose content, which is more sensitive than the TLC-GC method used by Morrison et al.[9]. The lower starch LPL values for the non-waxy rice reported by Choudhury and Juliano[27] may be due to the limitation of column chromatography (CC) and gravimetric method used at that time. However, the difference may be also due to the nature of samples themselves, as different rice samples have been used in this report.

Total starch LPLs had good correlation with apparent amylose content (P < 0.001) when waxy and non-waxy rice were considered together (Figure 4c), similar to the finding reported by Morrison et al.[9]. However, this correlation did not exist when the non-waxy rice were considered alone. The correlation between total LPLs and amylose content has been found in other waxy and non-waxy cereal grains, such as barley[28]. This correlation is possibly affected by both varietal and environmental factors in rice[9]. Therefore, such a correlation, if it exists, can only be revealed by investigating a sufficient number of rice samples grown in controlled environments.

Individual starch LPLs by multi-step extraction

We found that LPL16:0 is the single most abundant (45.4–63.2%) starch LPL in both waxy and non-waxy rice, followed by LPC 18:2 (10.5 – 23.4%) (Table 3), in agreement with Maniñgat and Juliano[7]. Palmitic acid (16:0) (up to ~76%) and linoleic acid (18:2) (up to ~29%) are the two major fatty acids within both starch LPC and LPE. The fatty acids of LPLs in waxy rice were more saturated than those found in non-waxy rice. Although the minor LPLs in waxy rice, such as LPC 18:3 and LPE 18:1, are below the limit of quantification, their presence can still be clearly identified in the chromatogram.

The percentage of individual LPLs can sometimes vary by almost four fold between different rice genotypes. For example, LPC 14:0 varied from 3.1% to 11.6% amongst the rice accessions analyzed (Table 3). As reported by Chang et al.[14], the individual LPL differences observed in this study may be caused by the combination of varietal and environmental effects. To unravel how environmental variation contributes to the rice starch LPLs, growing environments should be studied at different stages of rice development.

Increase sample throughput by single-step extraction method

The ten major starch LPLs extracted by the single-step extraction were quantified using the LCMS method above and added together as the total LPLs in rice. ANOVA analysis indicated that extraction methods had significant effect on total LPLs ($P<0.001$). In most cases, total LPLs were slightly higher in the single-step extraction than total starch LPLs in the multi-step extraction (Figure 4b). This difference is possibly due to the minimal amount of non-starch LPLs in rice[10], which only represented less than 1% of starch LPLs in our study and was included in the total LPLs by the single-step extraction

but not included for the total starch LPLs by the multi-step extraction. However, the difference may be also due to loss through the extended multi-step extraction.

To analyze a large number of samples for genetic and metabolomics studies, reducing the time for a single sample preparation is paramount. The multi-step extraction method (6 samples in triplicate for 2 d) takes about 6-8 times longer compared to the single-step extraction (24 samples in triplicate for 1 d) for each sample prepared. The simplification from multi-step to single-step extraction can significantly reduce the sample preparation time, and the risk of human error in removing and combining supernatants. As the total LPLs are almost same as total starch LPLs, we recommend the single-step extraction method for screening large numbers of samples followed by a multi-step extraction of samples of particular interest identified by the initial screening.

This is the first report of the determination of starch LPLs in rice using LCMS. Compared to previous TLC-GC methods, the LCMS method developed here is more efficient and sensitive, and can simultaneously quantify individual LPLs. The comparison of grinding methods indicates that ball or cyclone mills are equally effective for determination of total starch LPLs. The rapid single-step extraction method significantly simplifies sample preparation, improves consistency and increases throughput for lower cost. The combination of efficient extraction and LCMS method for the first time provides an opportunity to analyze starch LPLs within extensive collections of rice grain samples and should contribute to unravelling genetic and environmental contributions to variation in starch LPLs, and may also help to improve the understanding of the effects of starch LPLs on rice grain quality. Starch LPLs in other cereal grains may be also

379 determined using this strategy and this should be further explored for grain research and
380 development.

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384 **REFERENCES**

385 1. Morrison, W. R., Lipids in cereal starches: A review. *J. Cereal Sci.* **1988,** *8*, 1-15.

386 2. Morrison, W. R., Starch lipids and how they relate to starch granule structure and
387 functionality. *Cereal Foods World* **1995,** *40*, 437–446.

388 3. Blazek, J. Role of amylose in structure-function relationship in starches from
389 Australian wheat varieties. The University of Sydney, Sydney, 2008.

390 4. Putseys, J. A.; Derde, L. J.; Lamberts, L.; Östman, E.; Björck, I. M.; Delcour, J.
391 A., Functionality of short chain amylose−lipid complexes in starch−water systems and
392 their impact on in *vitro* starch degradation. *J. Agric. Food Chem.* **2009,** *58*, 1939-1945.

393 5. Putseys, J. A.; Lamberts, L.; Delcour, J. A., Amylose-inclusion complexes:
394 Formation, identity and physico-chemical properties. *J. Cereal Sci.* **2010,** *51*, 238-247.

395 6. Liu, L.; Waters, D. L. E.; Rose, T. J.; Bao, J.; King, G. J., Phospholipids in rice:
396 Significance in grain quality and health benefits: A review. *Food Chem.* **2013,** *139*, 1133-
397 1145.

398 7. Maniñgat, C. C.; Juliano, B. O., Starch lipids and their effect on rice starch
399 properties. *Starch/Staerke* **1980,** *32*, 76-82.

8. Azudin, M. N.; Morrison, W. R., Non-starch lipids and starch lipids in milled rice. *J. Cereal Sci.* **1986,** *4*, 23-31.

9. Morrison, W. R.; Milligan, T. P.; Azudin, M. N., A relationship between the amylose and lipid contents of starches from diploid cereals. *J. Cereal Sci.* **1984,** *2*, 257-271.

10. Choudhury, N. H.; Juliano, B. O., Effect of amylose content on the lipids of mature rice grain. *Phytochemistry* **1980,** *19*, 1385-1389.

11. Christie, W., Chromatographic analysis of phospholipids. *Chemistry and Materials Science* **1985,** *181*, 171-182.

12. Hu, C.; van Dommelen, J.; van der Heijden, R.; Spijksma, G.; Reijmers, T. H.; Wang, M.; Slee, E.; Lu, X.; Xu, G.; van der Greef, J.; Hankemeier, T., RPLC-Ion-Trap-FTMS method for lipid profiling of plasma: method validation and application to p53 mutant mouse model. *J. Proteome Res.* **2008,** *7*, 4982-4991.

13. Rectra, K. Schistosomal lysophosphatidylserine: an immunomodulatory factor Utrecht University, Netherlands, 2007.

14. Chang, Y.; Zhao, C.; Zhu, Z.; Wu, Z.; Zhou, J.; Zhao, Y.; Lu, X.; Xu, G., Metabolic profiling based on LC/MS to evaluate unintended effects of transgenic rice with cry1Ac and sck genes. *Plant Mol. Biol.* **2012,** *78*, 477-87.

15. Liu, L.; Deseo, M. A.; Morris, C.; Winter, K. M.; Leach, D. N., Investigation of a-glucosidase inhibitory activity of wheat bran and germ. *Food Chem.* **2011,** *126*, 553-561.

16. Liu, L.; Winter, K. M.; Stevenson, L.; Morris, C.; Leach, D. N., Wheat bran lipophilic compounds with in vitro anticancer effects. *Food Chem.* **2012,** *130*, 156-164.

17. Juliano, B. O., Criteria and tests for rice grain qualities. In *Rice Chemistry and Technology*, Juliano, B. O., Ed. American Association of Cereal Chemists, Inc.: 1985; p 403.

18. Juliano, B. O., Rice: overview. In *Encyclopedia of grain science*, Wrigley, C.; Corke, H.; Walker, C., Eds. Academic Press. : 2004; Vol. 3, pp 41-48.

19. Bao, J. S.; Shen, S. Q.; Sun, M.; Corke, H., Analysis of genotypic diversity in the starch physicochemical properties of nonwaxy rice: apparent amylose content, pasting viscosity and gel texture. *Starch/Staerke* **2006,** *58*, 259-267.

20. Yoshida, H.; Tanigawa, T.; Yoshida, N.; Kuriyama, I.; Tomiyama, Y.; Mizushina, Y., Lipid components, fatty acid distributions of triacylglycerols and phospholipids in rice brans. *Food Chem.* **2011,** *129*, 479-484.

21. Morrison, W. R.; Coventry, A. M., Extraction of lipids from cereal starches with hot aqueous alcohols. *Starch/Staerke* **1985,** *37*, 83-87.

22. Liu, L. Phytochemical and Pharmacological Perspectives of Wheat Grain and Lupin Seed. Southern Cross University, Lismore, NSW, Australia, 2009.

23. Morrison, W. R.; Law, R. V.; Snape, C. E., Evidence for Inclusion Complexes of Lipids with V-amylose in Maize, Rice and Oat Starches. *Journal of Cereal Science* **1993,** *18*, 107-109.

24. Juliano, B. O., Lipids in rice and rice processing. In *Lipids in Cereal Technology*, Barnes, P. J., Ed. Academic Press: London, UK, 1983; pp 305–330.

25. Xia, Y. Q.; Jemal, M., Phospholipids in liquid chromatography/mass spectrometry bioanalysis: comparison of three tandem mass spectrometric techniques for monitoring plasma phospholipids, the effect of mobile phase composition on phospholipids elution

445 and the association of phospholipids with matrix effects. *Rapid Commun. Mass Spectrom.*
446 **2009,** *23*, 2125-38.
447 26. Plueckthun, A.; Dennis, E. A., Acyl and phosphoryl migration in
448 lysophospholipids: importance in phospholipid synthesis and phospholipase specificity.
449 *Biochemistry* **1982,** *21*, 1743-1750.
450 27. Choudhury, N. H.; Juliano, B. O., Lipids in developing and mature rice grain.
451 *Phytochemistry* **1980,** *19*, 1063-1069.
452 28. Morrison, W. R.; Tester, R. F. S., C. E.; Law, R.; Gidley, M. J., Swelling and
453 gelatinization of cereal starches. IV. Some effects of lipid-complexed amylose and free
454 amylose in waxy and normal barley starches. *Cereal Chem.* **1993,** *70*, 385-391.
455

Figure Captions

Figure 1. Rice samples and their grinding, extraction and LCMS analysis of rice lipids.
[a]Rice sample numbers are the same as in Table 1.

Figure 2. Extraction yields (%) of non-starch and starch lipids of rice by multi-step extraction

Figure 3.LCMS profiles of rice non-starch (R10BN) and starch (R10BS) lipids

Figure 4. Quantification of total LPLs using different grinding and extracting methods.

Table 1. Information about rice accessions

Sample ID	Accession	Moisture content (%)	Apparent Amylose (%)	Comments
R01	Yunanheixiannuo	10.73 ± 0.02	0.9 ± 0.1	Waxy
R02	Youzaonuo	10.82 ± 0.05	1.2± 0.1	Waxy
R03	Zhefu 504	10.74 ± 0.08	13.1± 0.1	Low amylose
R04	Yixiang B	10.94 ± 0.20	14.2 ± 0.1	Low amylose
R05	Ce 482	11.10± 0.39	14.8 ± 0.6	Low amylose
R06	Zheda 104	10.84± 0.21	17.2 ± 0.5	Low amylose
R07	Xiushui 110	11.06± 0.31	18.0 ± 0.5	Low amylose
R08	Lemont	11.07 ± 0.06	22.8 ± 0.4	Intermediate
R09	II32B	11.47± 0.12	24.8 ± 0.4	High amylose
R10	Guangluai 4	10.86 ± 0.09	25.5 ± 0.2	High amylose
R11	Xinnong 170	10.39 ± 0.12	25.7 ± 0.1	High amylose
R12	Zhaiyeqing 8	10.99± 0.03	25.9 ± 0.1	High amylose
R13	Longtefu B	11.13± 0.15	26.4 ± 0.2	High amylose

Table 2.LCMS single-ion-monitor (SIM) mode setting for quantification of starch lysophospholipids in rice.

Time (min)	Group	SIM Ion	Fragmentor	Gain	Dwell (msec)	%Rel Dwell
Signal 1 (%cycle time: 25); Mode: SIM; Polarity: Positive.						
5.00	LPC 18:3[a]	518	150	1.00	20	100.0
6.15	LPC 18:2[b]	520	150	1.00	20	100.0
6.75	LPC 18:1[c]	522	150	1.00	110	100.0
Signal 2 (**%cycle time: 25)**; Mode: SIM; Polarity: Positive.						
5.00	LPC 14:0[d]	468	150	1.00	20	100.0
6.25	LPC 16:0[e]	496	150	1.00	20	100.0
Signal 3 (%cycle time: 25); Mode: SIM; Polarity: Positive.						
5.00	LPE 18:3[f]	476	150	1.00	20	100.0
6.15	LPE 18:2[g]	478	150	1.00	20	100.0
6.70	LPE 18:1[h]	480	150	1.00	50	100.0
Signal 4 (**%cycle time: 25)**; Mode: SIM; Polarity: Positive.						
5.00	LPE 14:0[i]	426	150	1.00	20	100.0
6.25	LPE 16:0[j]	454	150	1.00	20	100.0

[a]LPC 18:3 for 1-linolenoyl-2-hydroxy-*sn*-glycero-3-phosphocholine;
[b]LPC 18:2 for 1-linoleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine;
[c]LPC 18:1 for 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine;
[d]LPC 14:0 for 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine;
[e]LPC 16:0 for 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine;
[f]LPE 18:3 for 1-linolenoyl -2-hydroxy-*sn*-glycero-3-phosphoethanolamine;
[g]LPE 18:2 for 1-linoleoyl -2-hydroxy-*sn*-glycero-3-phosphoethanolamine;
[h]LPE 18:1 for 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine;
[i]LPE 14:0 for 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine;
[j]LPE 16:0 for 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine.

Table 3. Individual starch LPL/total starch LPLs (%) in waxy and non-waxy rice[a].

Individual LPL[b]	% of total LPLs in waxy Rice	% of total LPLs in non-waxy Rice
LPC 18:3	n/a[(c)]	0.2-0.7
LPC 18:2	10.5-15.0	14.7–23.4
LPC 18:1	5.9-7.1	4.9-12.7
LPC 14:0	5.6-11.6	3.1-7.6
LPC 16:0	56.5-63.2	45.4-53.4
LPE 18:3	n/a[c]	0-0.1
LPE 18:2	0.6 - 0.9	3.6–5.9
LPE 18:1	0 - 0.3	0.7-1.9
LPE 14:0	0.1-0.6	0.4-1.0
LPE 16:0	9.1-12.3	8.8-13.3

[a]The data are obtained from ball milled samples with multi-step extraction for the starch LPLs;
[b]LPL codes are same as in Table 2;
[c]Lower than quantification limit.

Figure 1.

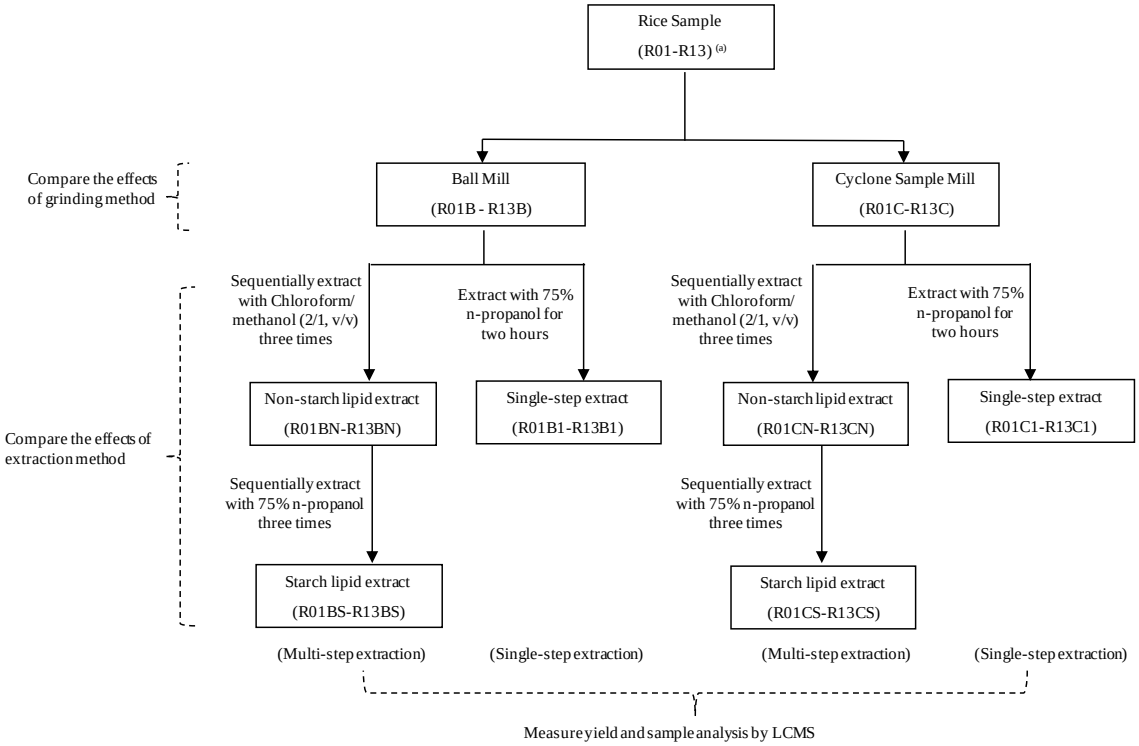

Rice Sample
(R01-R13) [a]
Compare the effects of grinding method
Ball Mill
(R01B - R13B)
Cyclone Sample Mill
(R01C-R13C)
Sequentially extract with Chloroform/ methanol (2/1, v/v) three times
Extract with 75% n-propanol for two hours
Sequentially extract with Chloroform/ methanol (2/1, v/v) three times
Extract with 75% n-propanol for two hours
Compare the effects of extraction method
Non-starch lipid extract
(R01BN-R13BN)
Single-step extract
(R01B1-R13B1)
Non-starch lipid extract
(R01CN-R13CN)
Single-step extract
(R01C1-R13C1)
Sequentially extract with 75% n-propanol three times
Sequentially extract with 75% n-propanol three times
Starch lipid extract
(R01BS-R13BS)
Starch lipid extract
(R01CS-R13CS)
(Multi-step extraction)
(Single-step extraction)
(Multi-step extraction)
(Single-step extraction)
Measure yield and sample analysis by LCMS

Figure 2.

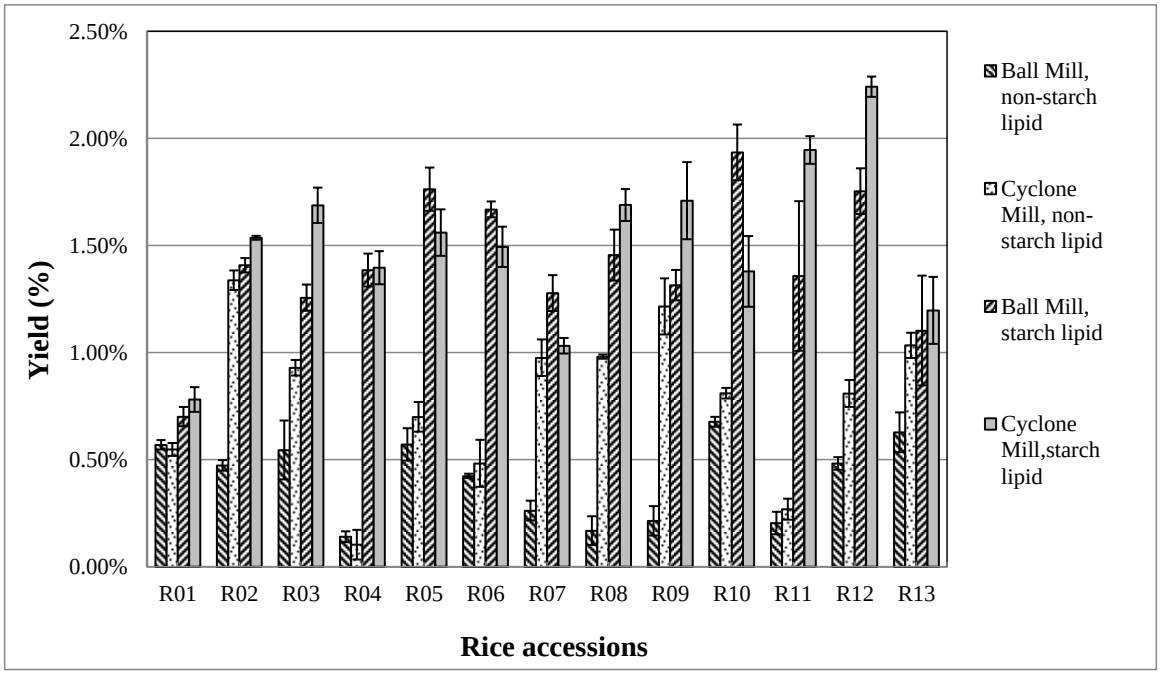
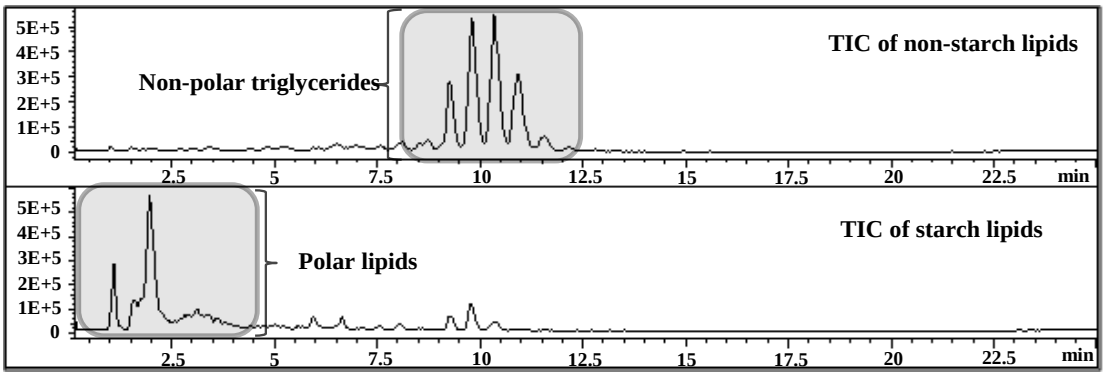
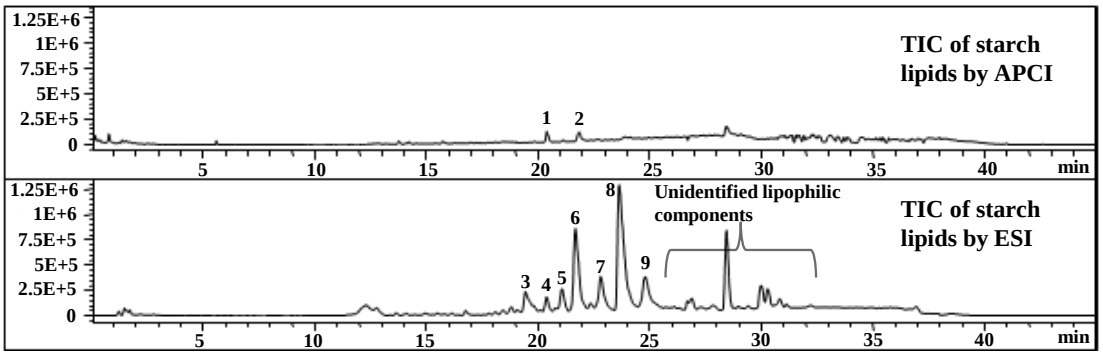

2.50%
2.00%
1.50%
1.00%
0.50%
0.00%
Yield (%)
R01
R02
R03
R04
R05
R06
R07
R08
R09
R10
R11
R12
R13
Rice accessions
Ball Mill, non-starch lipid
Cyclone Mill, non-starch lipid
Ball Mill, starch lipid
Cyclone Mill,starch lipid

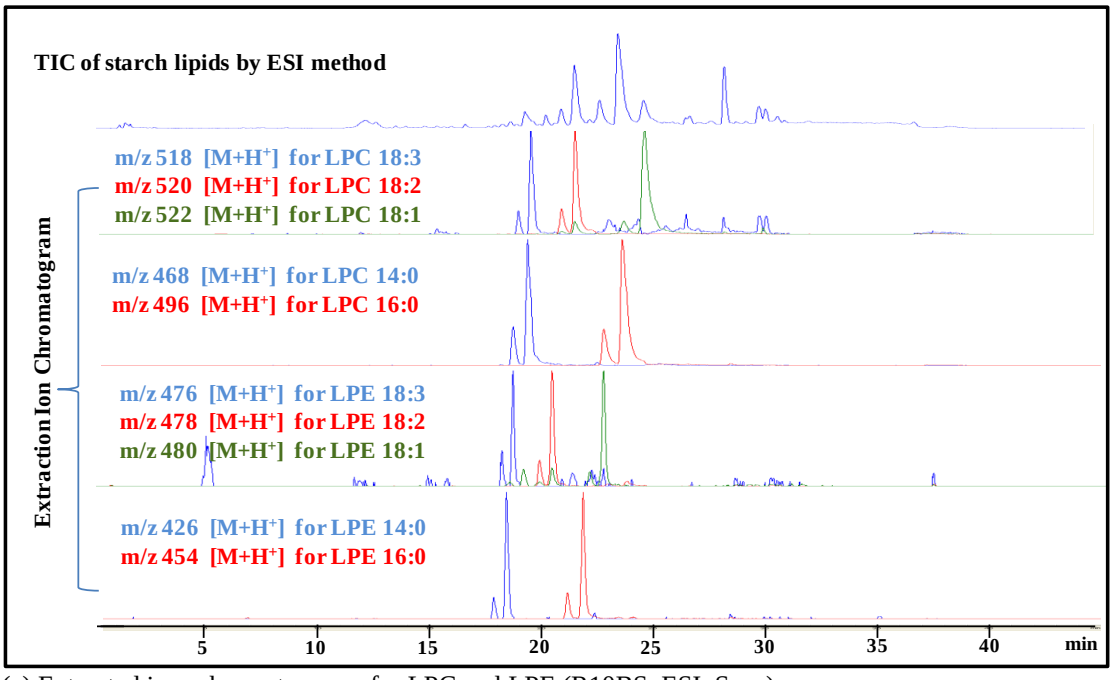
Figure 3.



(a) TIC of non-starch (R10BN) and starch (R10BS) lipids using 2-propanol/methanol gradient (APCI, Scan).

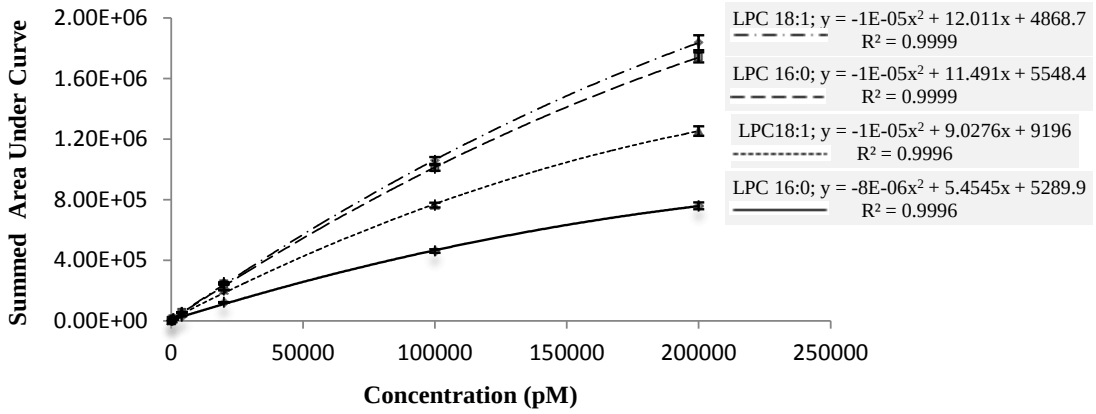


(b) TIC of starch lipids (R10BS) using acetonitrile/water gradient in APCI and ESI modes (Scan).
Peak-1: LPE18:2; Peak-2: LPE16:0; Peak-3: LPC 14:0;Peak-4: LPE18:2; Peak-5 and 6: LPC 18:2; Peak-7 and 8: LPC16:0; Peak-9: LPC 18:1.

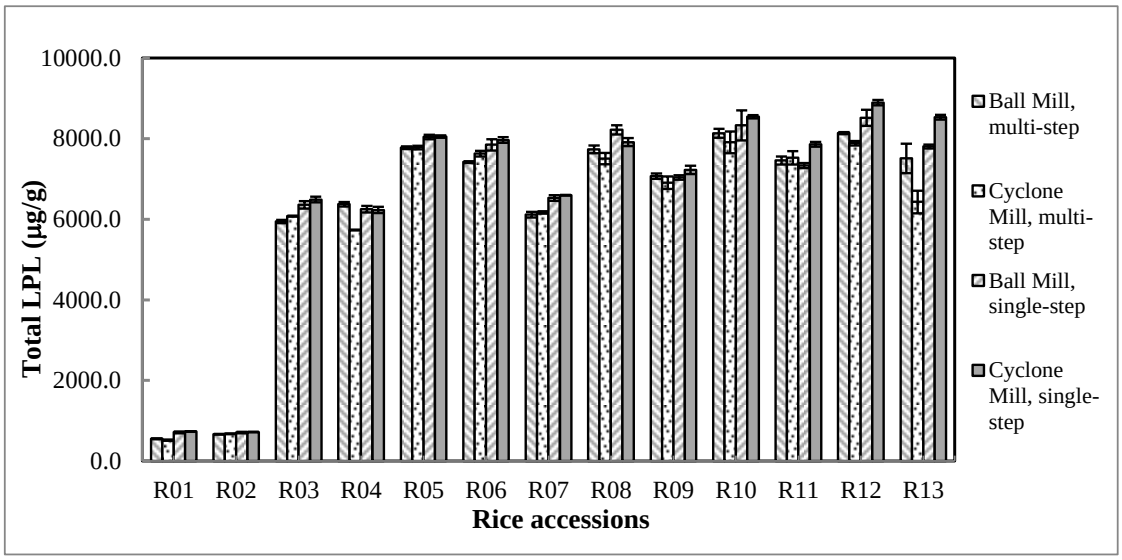


(c) Extracted ions chromatograms for LPC and LPE (R10BS, ESI, Scan).

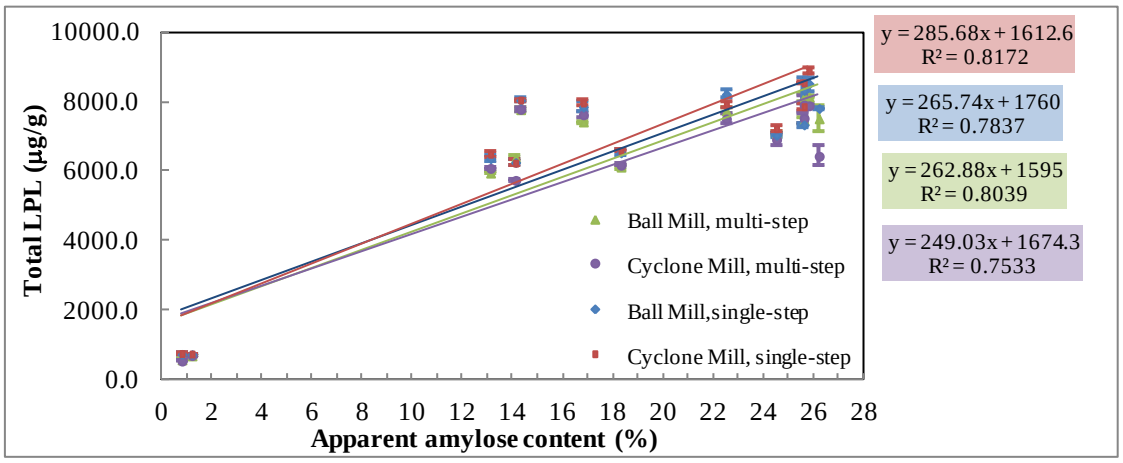
Figure 4.



(a) Standard curves for LPCs and LPEs.

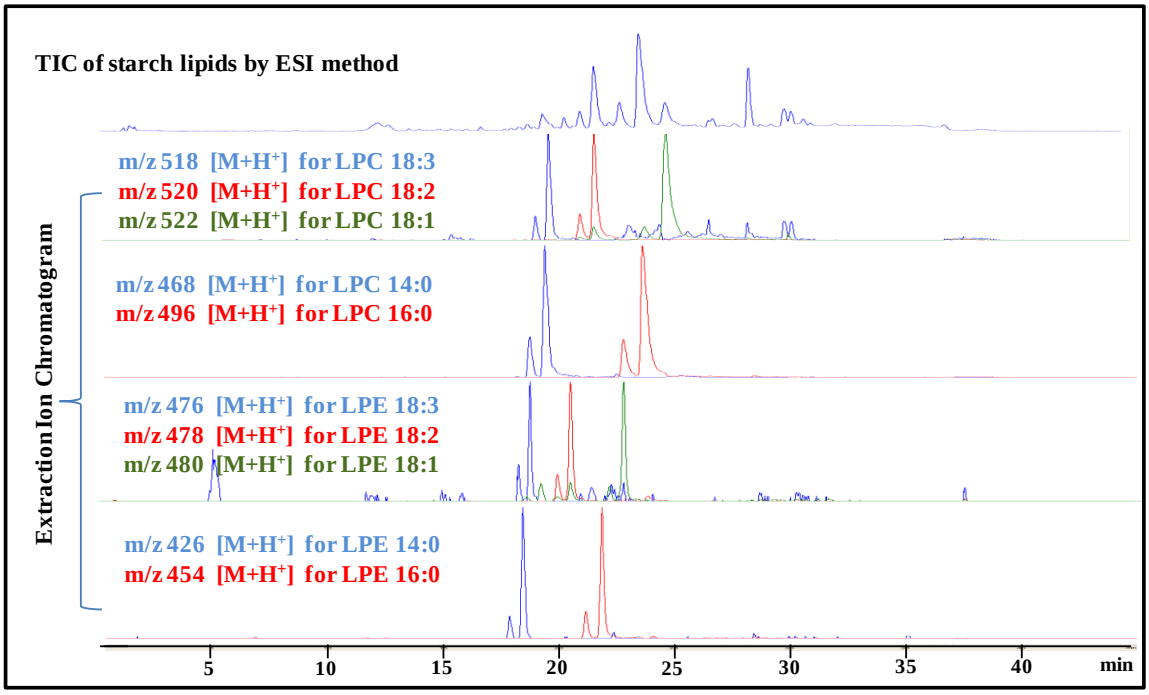


(b) Total LPLs quantified using different grinding and extracting methods.

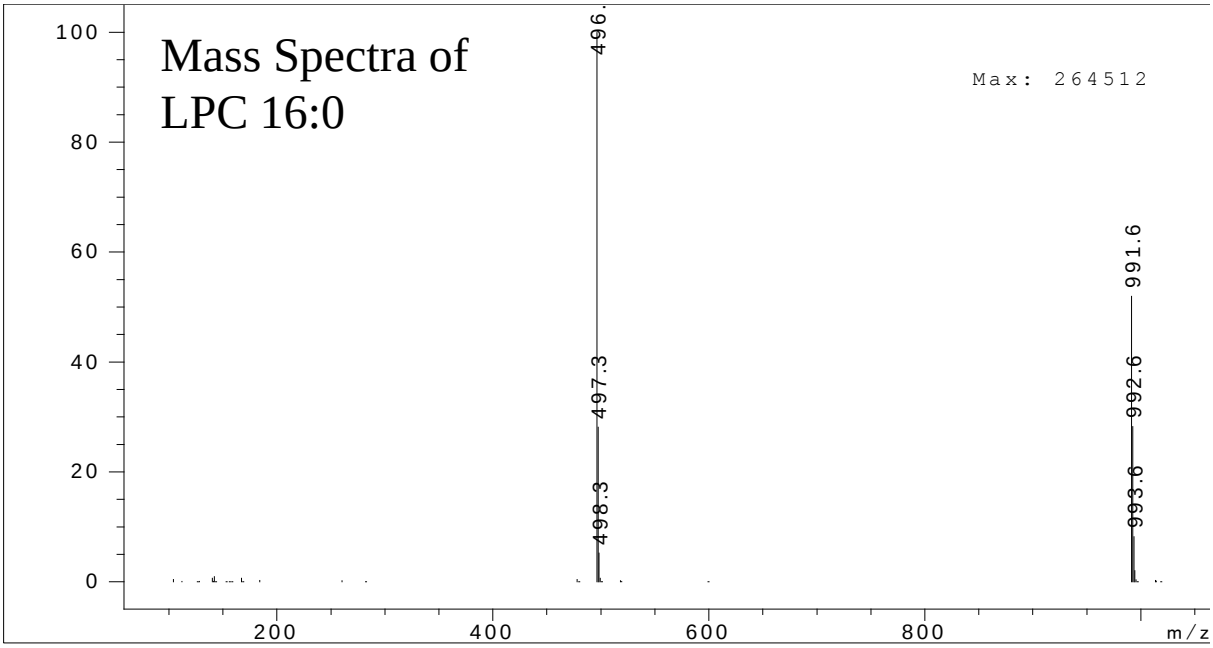


(c) Correlation between total LPLs and apparent amylose contents.

Graphic for table of contents



Supporting Information for Publication (Mass spectra and compound structure)

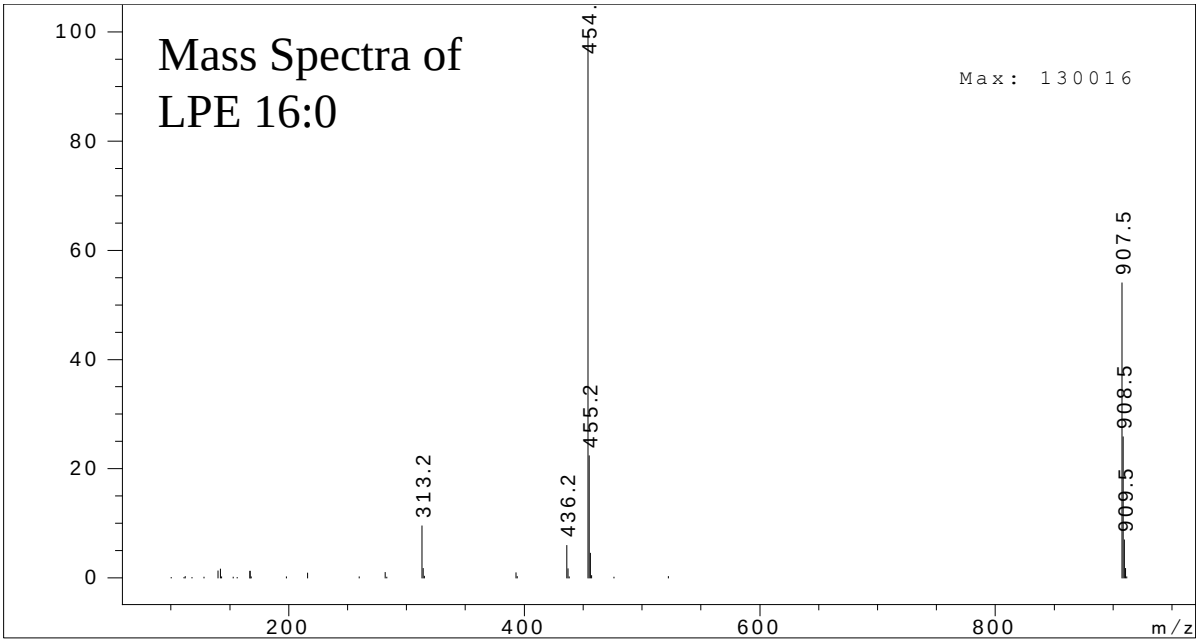


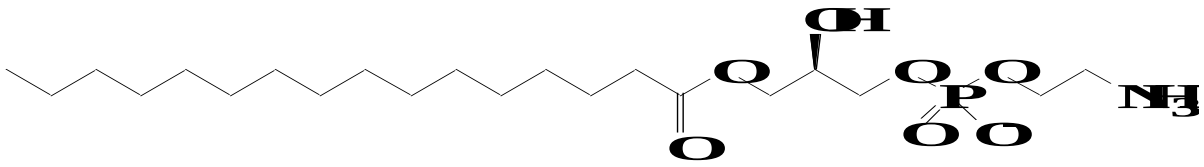
Structure of LPC 16:0

Abbreviation: LPC16:0

Compound Name: 1-palmitoyl-*sn*-glycero-3-phosphocholine

Supporting Information for Publication (Mass spectra and compound structure)



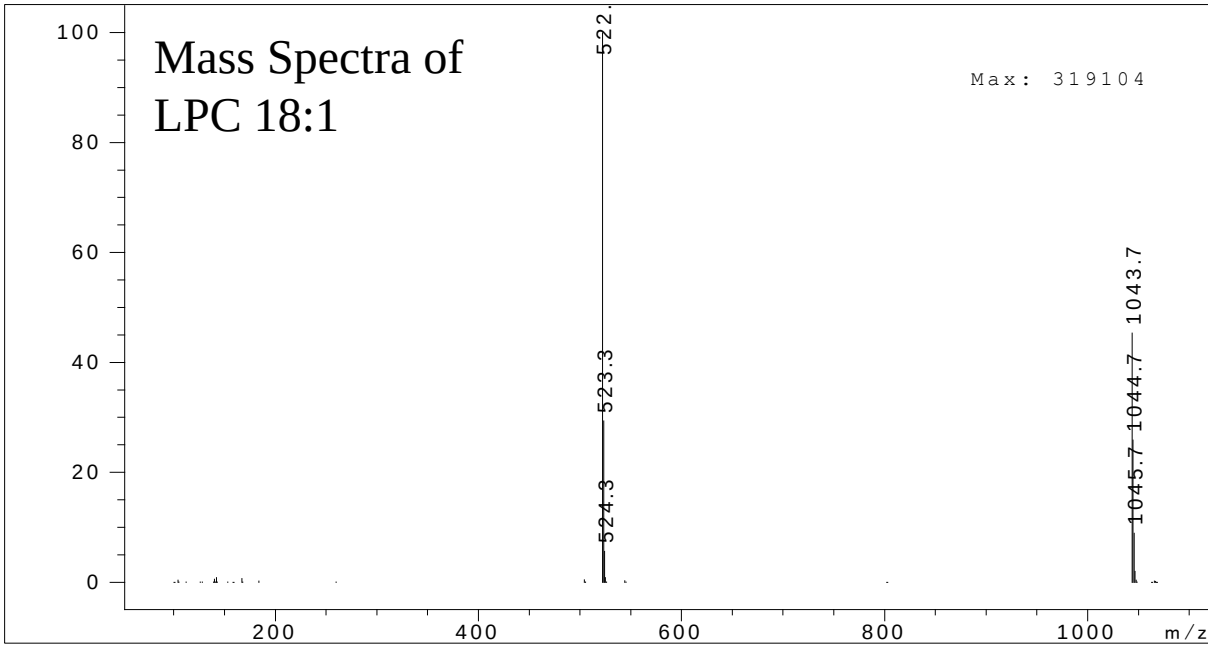


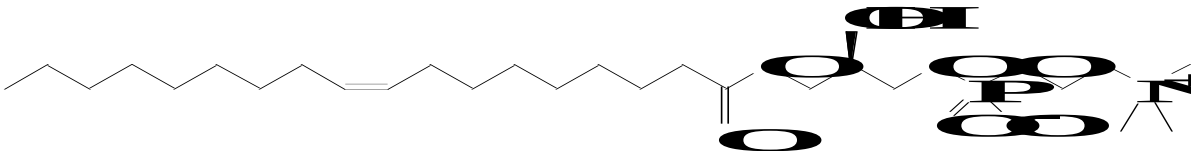
Structure of LPE 16:0

Abbreviation: LPE16:0

Compound Name: 1-palmitoyl-*sn*-glycero-3-phosphoethanolamine

Supporting Information for Publication (Mass spectra and compound structure)



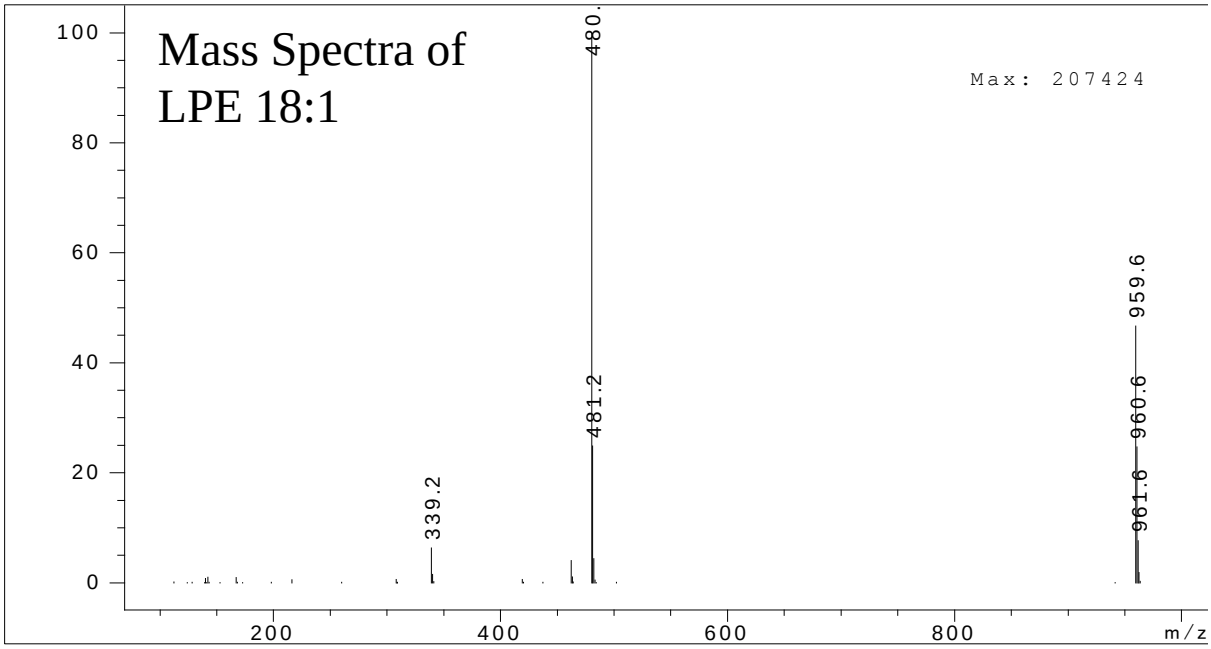


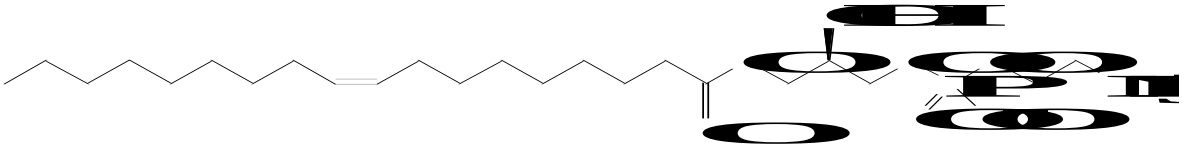
Structure of LPC 18:1

Abbreviation: LPC18:1

Compound Name: 1-oleoyl-*sn*-glycero-3-phosphocholine

Supporting Information for Publication (Mass spectra and compound structure)





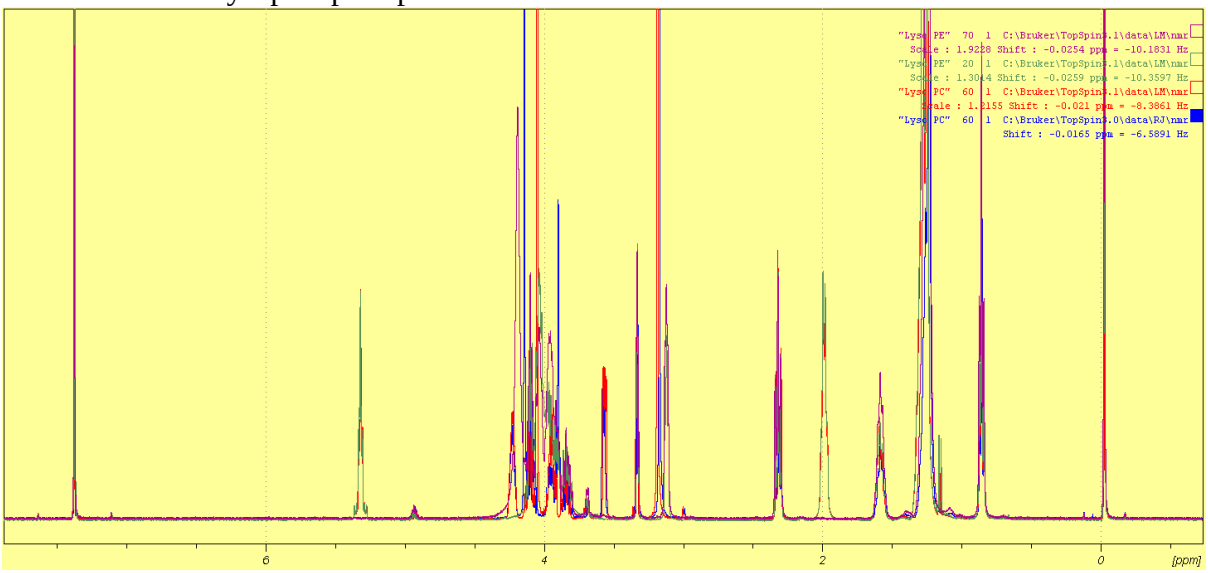
Structure of LPE 18:1

Abbreviation: LPE18:1

Compound Name: 1-oleoyl-*sn*-glycero-3-phosphoethanolamine

Supporting Information for Publication (^{1}H NMR analysis)

^{1}H NMR for the lysophospholipids standards.



Expansion of the section of spectra indicating the presence of 2-acyl-1-hydroxy isomers (around 4.92ppm)