Quantitative $^{31}$P NMR Method for Individual and Concomitant Determination of Phospholipid Classes in Polar Lipid Samples

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Abstract: Herein, to achieve individual and concomitant quantifications of phospholipid classes, an absolute quantification $^{31}$P NMR method using an internal standard was examined. Phospholipid standards and dietary foods were dispersed to prepare test solutions in an anionic surfactant (sodium cholate) solution containing EDTA, as a modification based on a reported method. Each phospholipid class showed a reproducible chemical shift at a near-neutral test solution pH of 6.90±0.04 and temperature of 30.0±0.1°C. The quantity of synthetic phosphatidylcholine measured using $^{31}$P NMR was consistent with that measured by $^1$H NMR using an internal standard. As the principal phospholipid class of soybean and egg yolk lecithin is phosphatidylcholine, the measurement conditions of $^{31}$P NMR (pulse interval time and number of scans) were optimized such that minor phospholipids, including lysophospholipids, also present in lecithin could be quantified simultaneously. Phospholipid classes in commercial polar lipid samples derived from porcine brain, yeast, and soybean were individually quantified using the above conditions. Using phosphoserine as the internal standard material allowed the absolute molar quantity of the phospholipid class to be precisely determined with traceability to the SI. The determined molar amounts of phospholipid classes were then translated to the weight amount by assuming that each phospholipid class contained two stearic acid molecules as the constituent fatty acid. The calculated total contents of each phospholipid class by $^{31}$P NMR were in good agreement with those obtained by molybdenum blue colorimetry. Furthermore, the quantitative values of the principal phospholipid classes in the polar lipid samples obtained by $^{31}$P NMR corresponded in a broad view, however, was more informative for the separation of individual phospholipid species rather than the quantitative 2D thin-layer chromatography.

Key words: phospholipid, quantitative nuclear magnetic resonance, phosphorus nuclear magnetic resonance, accurate quantitative NMR with internal reference substance (AQARI)

Abbreviations: PA, Phosphatidic acid; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PI, Phosphatidylinositol; PS, Phosphatidylycerine; SM, Sphingomyelin; DHSM, Dihydrosphingomyelin; DOPC, Dioleoylphosphatidylcholine; DOPS, Dioleoylphosphatidylserine; DSPA, Distearoylphosphatidic acid; DSPE, Distearoylphosphatidyl ethanolamine; POPE, Palmitoyloleoylphosphatidylethanolamine; DSPG, Distearoylphosphatidylglycerol; POPG, Palmitoyloleoylphosphatidylglycerol; SOPC, Stearoyloleoylphosphatidylcholine; 2-LOPA, 2-Lyso-oleoylphosphatidic acid; 2-LPA, 2-Lysophosphatidic acid; 2-LPC, 2-Lysophosphatidylcholine; 1-LPC, 1-Lysophosphatidylcholine; 2-LPE, 2-Lysophosphatidylethanolamine; 1-LPE, 1-Lysophosphatidylethanolamine; 2-LPI, 2-Lysophosphatidylinositol; 1-LPI, 1-Lysophosphatidylinositol; 2-OPS, 2-Lysophosphatidylethanolamine; PSER, Phosphoserine; 1,4-BTMSB-d$_4$, 1,4-bis(trimethylsilyl)benzene-d$_4$; $T_1$, relaxation time; TRM, traceable reference material; CRM, certified reference material; EDTA, ethylenediaminetetraacetic acid disodium salt; 2D-TLC, two-dimensional thin-layer chromatography; SI, International System of Units; ELSD, evaporative light scattering detectors.

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Accepted June 25, 2018 (received for review April 6, 2018)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jjocs
1 INTRODUCTION

Phospholipids are polar lipids with amphiphilic and bilayer-forming characteristics, and are widely used as additives in foods, cosmetics, or dietary supplements. Furthermore, phospholipids form liposomes in aqueous solution, which has received much attention as a medium for transporting pharmaceutical ingredients.

The properties of the resultant emulsion and bilayer are strongly dependent on the phospholipid species and concentration. Consequently, an accurate determination of phospholipid quantity is accordingly required, and particularly required for individual quantification of phospholipid classes. Conventionally, a laborious 2D-TLC method has been used for phospholipid quantification, in which phospholipid classes are individually separated by 2D-TLC, extracted from the TLC plate, and then determined colorimetrically using molybdic acid\textsuperscript{1-2}. This method is reliable, but requires careful operation. Recently, an HPLC method using an ELSD\textsuperscript{4} that achieves excellent separation of the phospholipid classes has been widely adopted. Unfortunately, the dynamic range of this method is relatively narrow owing to the nonlinear response of the detector\textsuperscript{5}.

In contrast, phosphorus nuclear magnetic resonance ($^{31}$P NMR)$^\text{7}$ can realize the mutual separation of phospholipid classes in a relatively easy operation. NMR provides well separated signals of individual phospholipid classes with chemical shifts based on structural differences in hydrophilic groups. Furthermore, as the $^{31}$P NMR signal area ratio represents the molar ratio of phosphorus nuclei, this method can directly determine the molar ratio of phospholipid classes in samples without using a reference standard. In previously reported $^{31}$P NMR quantification methods, phospholipids have been dissolved in a mixed solvent (chloroform–methanol) containing a limited amount of aqueous EDTA solution to improve the spectrum line width\textsuperscript{8-9}. In other methods, a homogeneous test solution was obtained by using cholic acid as a surfactant. Cholic acid forms tiny micelles in aqueous solution that incorporate phospholipids and suppress liposome formation by phospholipids. As a result, $^{31}$P NMR of an aqueous solution of phospholipids containing cholic acid and EDTA gives highly resolved signals\textsuperscript{10-12}.

Recently, $^1$H NMR that enables accurate quantification, known as qNMR, has become a widely used method\textsuperscript{13,14}. In particular, the AQARI\textsuperscript{15} or equivalence method\textsuperscript{16}, using CRM as an internal standard, has been successfully used to precisely determine the quantity and purity of low-molecular-weight organic compounds\textsuperscript{17,18}. AQARI is an absolute quantitative method\textsuperscript{19} that can obtain quantitative values with SI traceability and is highly reliable, with sufficiently advanced verification\textsuperscript{20-22}.

In the present report, the phospholipid quantification method by $^{31}$P NMR was carefully optimized using an aqueous sodium cholate–EDTA solution system with the presence of phosphoserine (PSER) as a certified reference material, and with controlling the pH of the sample in order to separate and quantify at most phospholipid classes effectively and concomitantly. The improved method was successfully applied to the absolute quantification of phospholipids in commercially distributed soybean lecithin and dietary foods. Furthermore, the results of the present $^{31}$P NMR method were verified by comparison with the AQARI-based $^1$H NMR method using 1,4-bis(trimethylsilyl)benzene-$d_4$ of a traceable reference material. The quantified results of phospholipid classes from $^{31}$P NMR were also compared with those of conventional molybdenum blue colorimetry and 2D-TLC methods. The present method was recognized in comparison with 2D-TLC method that the total phospholipid contents of polar lipids were in good agreement with those obtained by molybdenum blue colorimetry, however, the quantitative values for phospholipid classes were varied depending their content for more than 10% or less.

2 EXPERIMENTAL

2.1 Materials and chemical reagents

Phospholipid samples soy lecithin 1 (95 g of phospholipid per 100 g, including 28 g of PC) and soy lecithin 2 (1.2 g of phospholipid per soft capsule, including 0.18 g of PC) were purchased from an internet store (https://www.amazon.co.jp/). Dietary supplement food 1 (containing 50 mg of PS and 30 mg of PC per hard capsule) and dietary supplement food 2 (containing 100 mg of PS per soft capsule) were also purchased from the same internet store. Synthetic phospholipids DOPC, DOPS, DSPA, DSPE, POPE, DSPG, and POPG, and natural phospholipids soybean PC and egg yolk SM, were purchased from NOF Corporation (Tokyo, Japan). Synthetic 2-LOPA, soybean PE, PI, LPI, egg yolk PE, PA, LPC, and LPE were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA) and polar lipids extracted from soybean, porcine brain, and yeast were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The PSER TRM (purity 99.5%, expanded uncertainty was 0.9%, $k = 2$) and the 1,4-BTMSB-$d_4$ CRM (purity 99.8%, expanded uncertainty was 0.5%, $k = 2$) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sodium cholate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). EDTA was obtained from Dojindo Laboratories (Kumamoto, Japan), deuterium oxide (99.9% deuterium content) was obtained from Merck KGaA (Darmstadt, Germany), deuterated dichloromethane (99.8% deuterium content) was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan), Silica gel 60 HPTLC plates (size 10 × 10 cm) were obtained from Merck KGaA (Darmstadt, Germany) and NMR test tubes with 5-mm outer diameter were purchased from Wako Pure Chemical Industries, Ltd.
2.2 Apparatus

Phospholipids and samples were weighed using a semi-micro balance (MS205DU, Mettler-Toledo, Greifensee, Switzerland). PSER and 1,4-BTMSB-d4 were precisely weighed using an ultra-micro balance (MSE2.7S, Sartorius AG, Göttingen, Germany). ¹H NMR spectra were measured using an NMR spectrometer equipped with a 5-mm indirect probe (Varian NMR System 500, Varian Technologies, Palo Alto, CA, USA). ³¹P NMR measurements were performed using a JNM-ECA500II spectrometer (JEOL Ltd., Tokyo, Japan) equipped with a 5-mm FG/RO Digital Auto Tune Probe.

2.3 ³¹P NMR measurements

A surfactant solution (20%) was prepared by dissolving sodium cholate (20 g) in deuterium oxide (100 mL). EDTA 2Na (2.5 g) was dissolved in Tris–HCl buffer (25 mL, 1 M, pH 7.0) to prepare an EDTA solution (100 mg/mL). A mixture of accurately weighed PSER (20 mg) and EDTA solution (2 mL) was made up to 20 mL by adding Tris–HCl (1 M, pH 7.0) and used as an internal standard solution (1 mg/mL). The synthetic phospholipid was dissolved in the surfactant solution at concentrations ranging from 0.5% to 2%. Soybean lecithin powder was dispersed in the surfactant solution at concentrations ranging from 0.4 to 0.8 mg/mL and used as an internal standard solution (1 mg/mL). The dissection of dietary supplements 1 and 2 containing PS were prepared using the same procedure as for the soy lecithin powder, but without their capsules. The obtained mixture of phospholipid extract and internal standard was a cloudy liquid that was then centrifuged at 15,000 rpm. The pH of the obtained supernatant was adjusted to 6.90 ± 0.04 using 1 M NaOH or 1 M HCl solutions and then used for ³¹P NMR measurements. ³¹P NMR spectra were measured using the following conditions: irradiation frequency, 202 MHz; acquisition time, 5.453 s; probe temperature, 30°C; spectral width, 80 ppm; FID data points, 65,536; spinning, 15 Hz; dummy scans, 4; ¹H decoupling, Waltz16; pulse angle, 90°; pulse width, 14.8 μs.

2.4 Absolute quantification of DOPC by ³¹P NMR and ¹H NMR

DOPC (20.21–20.89 mg) and PSER (1.12–1.67 mg) as internal standards were accurately weighed and dissolved in the surfactant solution (2 mL) to prepare a test solution. This test solution was used for ³¹P NMR measurement under the conditions mentioned above (section 2.3). Another test solution was prepared by dissolving accurately weighed DOPC (20.17–20.26 mg) and 1,4-BTMSB-d4 (1.00–1.01 mg) as internal standard in deuterated dichloromethane (0.9 mL). This test solution was used for ¹H NMR measurement. The ¹H NMR relaxation delay expected to recover over 99% of z magnetization was set to six times the longest T2 of ¹H signals. T1 was determined using an inversion-recovery experiment. ¹H NMR spectra were measured under the following optimized conditions: irradiation frequency, 500 MHz; acquisition time, 4 s; relaxation delay, 40 s; probe temperature, 25°C; spectral width, 40 ppm; FID data points, 161,290; number of scans, 8; spinning, off; dummy scans, 2; ¹³C decoupling, MFP8; pulse angle, 90°; pulse width, 10.4 μs.

2.5 Calculation of phospholipid quantity

The purity of DOPC was determined from ¹H and ³¹P NMR spectra. The purity value of DOPC was determined by ¹H NMR with 1,4-BTMSB-d4 as internal standard using Equation (1). The purity (Ps) of 1,4-BTMSB-d4 was traceable to the SI, therefore traceability was inherited in the purity (Ps) of DOPC obtained by calculation. Quantitative values were calculated from ³¹P NMR using PSER as the internal standard traceable to the SI.

\[
P_A = \frac{I_A}{I_{IS}} \times \frac{N_{IS}}{N_{IS}} \times \frac{M_{IS}}{M_{IS}} \times \frac{W_{IS}}{W_A} \times P_{IS}
\]  

(1)

where \( P_{IS} \) is the purity of DOPC (%), \( I_A \) is the area ratio of the DOPC signal, \( I_{IS} \) is the area ratio of the internal standard, \( N_{IS} \) is the number of protons or phosphorus atoms in the internal standard, \( N_A \) is the number of protons or phosphorus atoms in DOPC, \( M_A \) is the molecular weight of DOPC, \( M_{IS} \) is the molecular weight of the internal standard, \( W_{IS} \) is the weight of internal standard used (g), \( W_A \) is the weight of DOPC used (g), and \( P_{IS} \) is the purity of the internal standard (%).

The molar amount of phospholipid in the analyte was determined from the ³¹P NMR spectrum of the test solution. The molar amounts of individual phospholipid classes were determined from the ³¹P NMR spectrum of the test solution with PSER as internal standard using Equation (2). Furthermore, the contents (%) of individual phospholipid classes were calculated using Equation (3), assuming that fatty acids bind to the sn-1 and sn-2 positions of the phospholipids as stearic acid.

\[
Mol_A = \frac{I_A}{I_{IS}} \times Mol_{IS}
\]  

(2)

\[
Value_A = \frac{I_A}{I_{IS}} \times \frac{N_{IS}}{N_A} \times \frac{M_{IS}}{M_A} \times \frac{W_{IS}}{W_A} \times P_{IS}
\]  

(3)

where \( Mol_A \) is the molar amount of the phospholipid, \( Mol_{IS} \) is the molar amount of the internal standard, \( Value_A \) is the quantity (%) of phospholipid, \( I_A \) is the area ratio of the phospholipid, \( I_{IS} \) the area ratio of PSER, \( N_{IS} \) the number of phosphorus atoms in PSER, \( N_A \) the number of phosphorus atoms in the phospholipid, \( M_A \) the molecular weight of the phospholipid, \( M_{IS} \) the molecular weight of PSER, \( W_{IS} \) the weight of the internal standard, \( W_A \) the weight of the polar lipid sample, and \( P_{IS} \) is the purity (%) of PSER.
2.6 Colorimetric quantification of total phospholipids using molybdenum blue

The total phospholipids contained in polar lipid samples of soybean, porcine brain, and yeast were quantified by a colorimetric method using molybdenum blue. Specific procedures are listed in standard method 2.4.11-2013 (JOCS 4.3.3.1-1996) \(^2\) from Standard Methods for the Analysis of Fats, Oils and Related Materials, which is the official oil analysis method in Japan established by Japan Oil Chemists Society. The sample amounts collected were 112.1 mg of soybean polar lipid, 99.0 mg of porcine brain polar lipid, and 99.0 mg of yeast polar lipid. Each sample was taken up in chloroform (50 mL), fractionated into 2-mL crucibles, and solvent was then removed by blowing with nitrogen gas before testing.

2.7 Quantification of individual phospholipids using 2D-TLC

The separation and classification of phospholipids by 2D-TLC was conducted according to standard method 4.3.3.3-1996 (JOCS 4.3.3.1-1996) \(^2\) from Standard Methods for the Analysis of Fats, Oils and Related Materials. However, the polar lipids extracted from samples were prepared by the alternative procedure described in Section 2.6 (18.87–19.88 mg of soybean polar lipid, 15.95–16.34 mg of porcine brain polar lipid, and 16.76–17.07 mg of yeast polar lipid were used) and then dissolved in chloroform to give 2 mg/mL polar lipids solutions. The polar lipids solutions (20 μL) were separated by 2D-TLC for collecting of individual phospholipid using solvent 1 and 2, with the amount of phosphorus measured and the composition ratio calculated for each phospholipid class. Solvent 1 contained tetrahydrofuran, acetone, methanol, and water (50:20:40:8, v/v), while solvent 2 contained chloroform, acetone, methanol, acetic acid, and water (50:20:10:15:5, v/v). Each phospholipid spot was clearly visualized by the Dittmer reagent and individually scraped from Silica gel 60 HPTLC plate and transferred into the digestion tube. These recovered phospholipids were quantified by the molybdenum blue method according to Section 2.6. Quantitative values of individual phospholipid classes were calculated by multiplying the total phospholipid amount obtained (see Section 2.6) by the composition ratio for each class.

### Table 1 Calculated purity of DOPC using \(^1\)H NMR \((N=3)\).

| Signals | S/N ratio | Number of nuclei | Purity (%) | SD (%) |
|---------|-----------|------------------|------------|-------|
| B       | 1100      | 4                | 99.1       | 0.3   |
| C       | 1400      | 4                | 99.1       | 0.3   |
| D       | 22000     | 9                | 99.1       | 0.3   |
| E       | 2500      | 4                | 99.4       | 0.4   |
| F       | 4600      | 8                | 99.2       | 0.3   |
| G       | 1600      | 4                | 100.4      | 0.7   |
| H       | 15000     | 40               | 99.6       | 0.2   |
| I       | 11000     | 6                | 99.7       | 0.1   |
| 1,4-BTMSB-d\(_4\) | 19000 | 18               | –          | –     |

Average: – – 99.4 0.3

3 RESULTS AND DISCUSSION

3.1 Determination of DOPC quantity by \(^1\)H NMR

A typical \(^1\)H NMR spectrum of DOPC is shown in Fig. 1. This experiment was conducted as three parallel tests, and the quantitative values obtained are shown in Table 1. Signals A–I, derived from the partial structure of DOPC (oleic acid, choline, and glycerol), were observed in the spectrum. Among these signals, signal A was not used for quantitative calculation because it overlapped with the solvent signal (dichloromethane). The purity was calculated using signals B–I, which were separated by sufficient intervals, and tended to be higher when higher-field signals were used in calculations instead of low-field signals. The values were particularly high when using signals G–I, assigned to methyl and methylene groups of fatty acid chains. However, signal D of the N-methyl group of choline was a strong single peak with a narrow line width and no interference from any impurities. Signal D was accordingly considered the most reliable signal for determining the true value. Consequently, the purity of DOPC was determined as 99.1 ± 0.3% by \(^1\)H NMR.

3.2 Optimization of phospholipid chemical shifts

In the \(^{31}\)P NMR spectra of phospholipids, the phosphate
For the precise quantification of phospholipids, it is important to obtain an accurate area ratio from signals resulting from the phospholipids and internal standard PSER. Pulse interval time is among the most important parameters for determining the area of an NMR signal accurately. The $T_1$ of the focusing signal was first measured and then used to calculate an appropriate pulse interval time to be employed. Validation was conducted using three sample categories: (i) DOPC, DOPS, and SM as synthetic high-purity phospholipids; (ii) soy lecithins 1 and 2 as polar lipids extracted from soybean, which are mixtures of phospholipids; and (iii) dietary supplement foods 1 and 2, which contain various components other than phospholipids. Samples from each category were dissolved in the surfactant solution and $T_1$ parameters were measured using the reverse recovery method (Table 3). The $T_1$ of PC and PS was approximately 0.8–1.1 s, regardless of the molar ratio of cholic acid, the total phospholipid content, and the type of sample. Although the $T_1$ of PI, PE, and LPC tended to increase at a lower molar ratio of total phospholipids to surfactant, it remained below 2 s. SM from egg yolk, which was highly pure, seemed to have $T_1$ values similar to those of phospholipids such as PC. However, PA showed a longer $T_1$ of 1.6–1.7 s, which was different to those of other phospholipids, even when the test solutions contained rather higher phospholipid molar ratio to the surfactant. As the content of PA in the sample was lower than that of other phospholipids, its $T_1$ could not be measured. However, it was estimated that corresponding $T_1$ was in the range of approximately 1.5–2.5 s. In contrast, the $T_1$ of PSER was significantly different to that of other phospholipids, at 1.3–4.3 s depending on the molar ratio to phospholipid. At higher phospholipid concentrations, the $T_1$ values of phospholipid and PSER became shorter. As the aggregation number of sodium cholate is reportedly as low as 4$^{20}$ at 20–25°C, the ratio of soy lecithin 1 to surfactant in the experiment of 4.3:1 implied that one lecithin molecule was incorporated into a small micelle formed by four sodium cholate species (Table 3). Therefore, a further increase of

### Table 2

| Phospholipid          | Chemical shift (ppm) | Phospholipid          | Chemical shift (ppm) |
|-----------------------|----------------------|-----------------------|----------------------|
| DOPC                  | −0.091               | DSPE                  | 0.508 ±0.001         |
| PC from soy           | −0.070, −0.090       | SM from egg yolk      | 0.541 ±0.001         |
| PI from soy           | 0.059                | DSHM from egg yolk    | 0.638                |
| 1-LPC from egg yolk   | 0.208                | 1-LPE from egg yolk   | 0.791                |
| DOPS                  | 0.250 ±0.001         | 2-LPE from egg yolk   | 0.930                |
| 1-LPI from soy        | 0.385                | POPG                  | 0.987                |
| 2-LPC from egg yolk   | 0.386                | DSPG                  | 1.000                |
| POPE                  | 0.486                | PA from egg yolk      | 3.844                |
| PE from egg yolk      | 0.471, 0.487         | DSPA                  | 3.903 ±0.016         |
| PE from soy           | 0.473, 0.486         | 2-LOPA                | 4.464                |
| 2-LPI from soy        | 0.498                | PSER                  | 4.543 ±0.017         |

Chemical shift was affected by the pH of the test solution. Particularly significant chemical shift changes were observed for PE, PS, and their lyso forms$^{12}$. Further improvements in the mutual separation of each phospholipid class would be possible through pH control. However, under alkaline conditions, acyl group hydrolysis and lysophospholipid formation would be accelerated. Furthermore, sodium cholate precipitation was anticipated under acidic conditions. Consequently, the conditions were optimized to pH 6.90 ±0.04 at 30.0 ±0.1°C (Table 2). No appreciable change ($\pm0.001$ ppm) was observed in the chemical shifts of DOPC, DOPS, DSPG, SM, and DSPE when varying the concentration from 0.1 to 1%, or in that of DSPE at concentrations of 0.025–0.5%, respectively. LPI content in the polar lipid samples was very small, the quantitative values of PE and 2-LPC should not come into significant problem. Except for SM, the phospholipids from natural products, including egg yolk and soybean, showed signals with multiple apexes owing to heterogeneity in fatty acid composition. Furthermore, the PC signal from soybean comprised two peaks$^{31}$, the resolution of which decreased at concentrations of 1% or higher.

### 3.3 Measurement of relaxation time to determine pulse interval time

SI-traceable PSER was used in the present study as a reference standard material for quantification by $^{31}$P-NMR. For the precise quantification of phospholipids, it is important to obtain an accurate area ratio from signals resulting from the phospholipids and internal standard PSER. Pulse
the ratio of phospholipid to surfactant should be avoided.

Using the same solution of DOPC used to measure $T_1$ in Table 3, $^{31}$P NMR spectra were obtained by changing the pulse interval time from 0.2 to 20 s in seven steps to compare the area ratio between DOPC and PSER signals. The area of DOPC increased slightly until the pulse interval time reached 1 s, which was close to $T_1$, and decreased thereafter (Fig. 2). Furthermore, for PSER, the signal area ratio increased until 4 s, which was again close to $T_1$, and then decreased after 4 s.

The area intensity of an NMR spectrum generally increases with the recovery of magnetization when the pulse interval time is extended, and eventually stabilizes with constant intensity. For quantitative NMR experiments using AQARI, to ensure that the signal area intensity for an observed nucleus correlated with the nuclear number, a pulse interval time more than five times longer than $T_1$ is required for stabilization. However, in the present $^{31}$P NMR of phospholipids in the surfactant solution, the area intensity decreased unexpectedly as the pulse interval time increased. Table 4 shows the quantitative values of DOPC at each pulse interval time. As mentioned in section 3.1, the purity of DOPC was determined to be 99.1% from the quantitative $^1$H NMR result referencing the N-methyl group. Quantitative values of 98.8 and 99.0% were also obtained by $^{31}$P NMR when the pulse interval time was 20 and 40 s, which were more than five times longer than the $T_1$ of DOPC (0.9 s) and PSER (4.2 s), respectively. An equivalent purity (98.7%) was also obtained at a pulse interval time of 2 s, which was half the $T_1$ of DOPC and PSER.

Test solutions of $^{31}$P NMR were prepared for soy lecithin 1 and dietary supplement food 2. The phospholipid quantities were determined by changing the pulse interval time from 1 to 40 s over six steps. The obtained signal area of each phospholipid was compared with that of PSER, with the relationship between signal area and pulse interval time shown in Fig. 3. The phospholipid classes of soy lecithin 1 and PSER had $T_1$ values as low as 1 s, with the area intensity of these signals decreasing with increasing pulse interval time. The plots of all phospholipids and PSER showed a similar tendency of downward-sloping curves, and reasonable quantitative values with less than 1% relative standard deviation (RSD) were obtained for PC, PI, and PE at all pulse interval times. Owing to the relatively low total phospholipid concentrations in dietary supplement food 2, the curves indicated that the area intensities of phospholipids and PSER in various sample matrices by $^{31}$P NMR.

| Sample            | Mole ratio of cholic acid and total phospholipid including PSER | $T_1$ (s) |
|-------------------|---------------------------------------------------------------|----------|
| DOPC (High purity)| 14:1                                                          | 0.9      |
| DOPS (High purity)| 14:1                                                          | 0.9      |
| SM (High purity)  | 14:1                                                          | 0.9      |
| Soy lecithin 1    | 4.3:1                                                         | 0.9      |
| Soy lecithin 2    | 6.9:1                                                         | 1.0      |
| Dietary supplement food 1 | 24:1                                                   | 0.9      |
| Dietary supplement food 2 | 9.8:1                                                   | 0.8      |

All test solution concentrations determined using the $^{31}$P NMR method.

![Fig. 2](image_url)

Relative change in signal area ratio (DOPC and PSER) by $^{31}$P NMR using various pulse interval times.

Area of initial point (0.2 s) set to 1, area at each pulse interval time expressed as a relative value. DOPC (9.96 mg/mL) in surfactant solution with PSER (0.45 mg) as internal standard. Measurement conditions: probe temp, 30°C; 2048 transients; spinning, on; $^1$H decoupling, WALTZ.
P NMR Quantification of Phospholipids

Table 4 Phospholipid quantities using various relaxation delays.

| Sample                  | Phospholipid | Relaxation delay (s) | Mean (%) | RSD (%) |
|-------------------------|--------------|----------------------|----------|---------|
| DOPC                    | DOPC         | 1  2  4  8  10  20  40 |          |         |
| Soy lecithin 1          | PC           | 24.2 24.1 24.3 24.4 24.5 − 24.1 24.3 0.7 |
|                         | PI           | 13.8 13.7 13.8 14 13.7 − 13.8 13.8 0.8 |
|                        | PE           | 24.9 24.6 24.8 25 24.8 − 24.8 24.8 0.5 |
|                        | PA           | 5.3 5.4 5.3 5.2 5.1 − 5.1 5.2 2.3 |
| Dietary supplement 2    | PS           | 20.5 19.5 18.7 18.7 18.9 − 19.4 19.3 3.6 |
|                         | PA           | 2.6 2.5 2.4 2.3 2.3 − 2.4 2.4 4.8 |
|                        | 2-LPS        | 0.6 0.6 0.6 0.6 0.6 − 0.5 0.6 7.0 |

Fig. 3 Relative change in signal area ratio (soy lecithin 1 and dietary supplement food 2) by 31P NMR using various pulse interval times.

(a) Soy lecithin 1 (0.1 g) in surfactant solution (1.8 mL) with PSER (1.2 mg) as internal standard; probe temp, 30°C; 2048 transients; spinning, on; 1H decoupling, WALTZ. (b) Dietary supplement food 2 (0.1 g) in surfactant solution (1.8 mL) with PSER (0.98 mg) as internal standard; probe temp, 30°C; 2048 transients; spinning, on; 1H decoupling, WALTZ.

Each compound had different tendencies at each pulse interval time, with the dispersion of quantitative values (4–7% RSD) becoming larger than that of soy lecithin 1 (>1% RSD). Quantitative values fluctuated up and down, even when the pulse interval time was extended to be close to T1. In addition, as the similar case of the present study, the quantification of phospholipid classes by 31P NMR using sodium cholate did not show a significant difference in signal intensities between the case where the pulse interval time is 1 s and that of 10 s. Moreover, acceptable signal intensity was also obtained from LPE of minor component at 3 s. A relatively short interval time of 2 s was accordingly employed in the present study, not only for obtaining adequate quantification results, but also for reducing the time needed for data acquisition.

For accurate quantification of the samples containing unknown classes and concentrations of phospholipids, a suitably long pulse interval time compared with T1 is desirable. However, such a strategy was not effective in the present case. In the surfactant solution, it was difficult to investigate the reason why the signal area of phospholipids and PSER decreased with the increasing the pulse interval time. As shown in the above results, for 31P NMR quantification of 1–4 wt% phospholipid samples with a large molar ratio of surfactant, a short interval time of 2 s was effective for reducing the quantitative error to less than 5%. Therefore, the present method was confirmed as a reliable quantitative method.

3.4 Effect of signal-to-noise ratio on quantitative area ratio by 31P NMR

The test solution of soy lecithin 1 and dietary supplement food 2 was measured by 31P NMR using a pulse interval time of 2 s while changing the number of integrations from 2 to 2048. As a result, the signal intensities of PS spectra were increased and the signal-to-noise ratios (S/N)
were improved from 10 to 270. The signal area ratio of PS to PSER was calculated to be 103% from the spectrum with a S/N of 20, based on that from the spectrum with a S/N of 270 as 100%. However, the area ratio increased to 114% due to the influence of noise at S/N = 10. As soybean lecithin contained minor components, such as PG and 2-LPA, more than 1024 scans were required to obtain an S/N of at least 20 using a 500-MHz NMR spectrometer.

3.5 Determination of phospholipids in polar lipids

$^{31}$P NMR spectra of polar lipid samples prepared from soy, brain, and yeast were measured and are shown in Fig. 4. The total phospholipid contents in the samples were 76.0 ± 0.3, 68.0 ± 0.3, and 82.6 ± 0.3, respectively, determined by molybdenum blue colorimetry. As NMR enables determination of the molar amount in principal, the individual quantities of different phospholipid classes were directly calculated as molar amounts per 100 g of sample (Tables 5–7). The major phospholipid classes with contents of 10 mmol or more showed good repeatability, with RSDs of 0.5–2.1%. For phospholipid classes with contents of > 1 mmol and < 1 mmol, the repeatability varied significantly, with RSDs of 0.7–11% and 4.2–40%, respectively. Phospholipids and lysophospholipids are generally attached to one and two fatty acids, respectively, with carbon numbers of 16–20. Therefore, their molecular weights were calculated as species containing one and two stearic acid (C18) moieties as a typical fatty acid. Furthermore, the molybdenum blue colorimetric and 2D-TLC methods conventionally attributed the phospholipid content as SOPC. The results of $^{31}$P NMR were also translated into SOPC quantities for comparison. The total phospholipid contents of polar lipids prepared from soy, brain, and yeast obtained by $^{31}$P NMR were in good agreement with those obtained by molybdenum blue colorimetry, in the range 97–102%. However, in comparison with 2D-TLC method, the quantitative values provided by $^{31}$P NMR were almost 10% higher (99–107%) because additional separation and extraction steps were not required for $^{31}$P NMR. The main phospholipid class, with contents of 10% or more for all polar lipids, gave quantities by $^{31}$P NMR up to 20% higher than those provided by the 2D-TLC method (97–117%). Conversely, in phospholipid classes with contents of less than 10%, excluding PA, the $^{31}$P NMR quantities remained low, at 13–79%. For the brain polar lipid, a low PA content was detected (0.2%) by $^{31}$P NMR, but a quantitative value 10 times higher was obtained by 2D-TLC. Therefore, although differences between the results of $^{31}$P NMR and 2D-TLC were relatively large for the individual quantification of phospholipid classes, $^{31}$P NMR had the fundamental advantage of being less susceptible to handling errors during operation. Furthermore, $^{31}$P NMR was able to quantify 1-lyso and 2-lyso forms, which are difficult to differentiate using the conventional method. Additionally, by converting results to an amount of SOPC, the $^{31}$P NMR results were easily compared with quantities obtained by the conventional method. These two methods would be cooperative in case for the situation aimed for accuracy and contemporaneousness, such as availability of an NMR instrument and/or number of samples to be treated.

4 CONCLUSIONS

To accurately determine phospholipid classes in commercial foods and dietary supplements, we optimized the $^{31}$P NMR quantification method using a surfactant solution of cholic acid and verified its performance. Chemical shifts of phospholipids were highly reproducible when the test solution pH was adjusted to 6.90 ± 0.04. The pulse interval time and number of scans were optimized to obtain NMR signals suitable for quantitative calculation. The NMR signal of the phospholipids in surfactant micelles showed a decreased signal area ratio at longer pulse interval times. However, data could be collected without losing precision, even at an interval time of 2 s, which was relatively short compared with $T_1$. The optimized pulse interval time was...
successfully applied to quantify samples rich or moderately rich in phospholipids, such as lecithin, prepared from soybean, egg yolk, and dietary supplements. Using PSER as an internal standard TRM, the present method achieved SI traceability and confirmed its robustness in comparison with AQARI-based $^1$H NMR using 1,4-BTMSB-$d_4$ CRM. Furthermore, $^{31}$P NMR also accomplished concomitant analysis of many phospholipid classes, which is difficult to achieve using $^1$H NMR. The optimized $^{31}$P NMR method was successfully applied to the quantification of phospholipid classes in three types of polar lipid samples. The phospholipid amount determined by $^{31}$P NMR was in good agreement with total values obtained by conventional molybdenum blue colorimetry. In particular, the present method provided superior individual quantification of phospholipid classes compared with 2D-TLC, which required careful operation.

### Table 5  Phospholipid class quantities in soy polar extract (100 g).

| Method | 31P NMR | 2D-TLC |
|--------|---------|--------|
| Result | PL content (mmol) | DS form PL (wt%) | SOPC (wt%) | SOPC (wt%) |
| PC     | 32.16 ± 0.27 | 25.4 ± 0.2 | 25.4 ± 0.2 | 25.3 ± 1.0 |
| PE     | 32.17 ± 0.21 | 24.1 ± 0.2 | 25.4 ± 0.2 | 21.8 ± 0.7 |
| PI     | 17.18 ± 0.09 | 14.9 ± 0.1 | 13.6 ± 0.07 | 14.0 ± 1.0 |
| PA     | 11.06 ± 0.14 | 7.8 ± 0.1 | 8.7 ± 0.1 | 4.3 ± 0.7 |
| Class  |          |         |         |         |
| PG     | 1.79 ± 0.05 | 1.4 ± 0.0 | 1.4 ± 0.04 | 3.8 ± 0.7 |
| PS     | 0.75 ± 0.07 | 0.6 ± 0.1 | 0.6 ± 0.05 | – |
| 2-LPC  | 1.05 ± 0.09 | 0.6 ± 0.0 | 0.8 ± 0.07 | 3.1 ± 0.3 |
| 2-LPE  | 0.83 ± 0.04 | 0.4 ± 0.0 | 0.7 ± 0.03 | – |
| 2-LPA  | 0.48 ± 0.07 | 0.2 ± 0.0 | 0.4 ± 0.05 | – |
| Total  | 97.47 ± 0.37 | 75.3 ± 0.3 | 76.9 ± 0.3 | 72.2 ± 1.7 (76.0 ± 0.3*) |

DS from PL ($^{31}$P NMR): Absolute quantification calculated by multiplying the molar amount (mmol) by the molecular weight of distearoyl or lysostearoylphospholipid.

SOPC ($^{31}$P NMR): Absolute quantification calculated by multiplying the molar amount (mmol) by the molecular weight of stearoyloleoylphosphatidylcholine (SOPC).

*Quantitative value before separation by 2D TLC.

### Table 6  Phospholipid class quantities in brain (porcine) polar extract (100 g).

| Method | 31P NMR | 2D-TLC |
|--------|---------|--------|
| Result | PL content (mmol) | DS form PL (wt%) | SOPC (wt%) | SOPC (wt%) |
| PC     | 17.84 ± 0.35 | 14.1 ± 0.3 | 14.1 ± 0.3 | 13.9 ± 0.1 |
| PE     | 15.48 ± 0.15 | 32.4 ± 0.7 | 34.2 ± 0.7 | 29.5 ± 1.2 |
| PI     | 3.32 ± 0.03 | 2.9 ± 0.1 | 2.6 ± 0.02 | 3.6 ± 0.3 |
| PA     | 0.25 ± 0.05 | 0.2 ± 0.1 | 0.2 ± 0.04 | 2.9 ± 0.4 |
| Class  |          |         |         |         |
| PS     | 16.17 ± 0.16 | 12.8 ± 0.1 | 12.8 ± 0.1 | 11.2 ± 0.5 |
| 2-LPE  | 0.33 ± 0.12 | 0.2 ± 0.1 | 0.3 ± 0.09 | 2.3 ± 0.5 |
| SM     | 1.99 ± 0.14 | 1.4 ± 0.1 | 1.6 ± 0.1 | 2.7 ± 0.3 |
| 2-LPA  | 0.12 ± 0.05 | 0.1 ± 0.1 | 0.1 ± 0.04 | – |
| Total  | 83.38 ± 1.04 | 64.0 ± 0.8 | 65.8 ± 0.8 | 66.2 ± 0.5 (68.0 ± 0.3*) |

DS from PL ($^{31}$P NMR): Absolute quantification calculated by multiplying the molar amount (mmol) by the molecular weight of distearoyl or lysostearoylphospholipid.

SOPC ($^{31}$P NMR): Absolute quantification calculated by multiplying the molar amount (mmol) by the molecular weight of stearoyloleoylphosphatidylcholine (SOPC).

*Quantitative value before separation by 2D TLC.
Table 7  Phospholipid class quantities in yeast polar extract (100 g).

| Method   | Result | 31P NMR | 2D-TLC |
|----------|--------|---------|--------|
|          | PL content (mmol) | DS form PL (wt%) | SOPC (wt%) | SOPC (wt%) |
| PC       | 32.81 ± 0.49       | 26.0 ± 0.4     | 25.9 ± 0.4 | 23.4 ± 1.0 |
| PE       | 9.76 ± 0.12        | 7.3 ± 0.1     | 7.7 ± 0.1 | 8.2 ± 0.4 |
| PI       | 43.09 ± 0.22       | 37.4 ± 0.2   | 34.0 ± 0.2 | 30.0 ± 1.2 |
| PA       | 7.10 ± 0.10        | 5.0 ± 0.1    | 5.6 ± 0.1 | 5.0 ± 0.6 |
| PG       | 0.35 ± 0.15        | 0.3 ± 0.1    | 0.3 ± 0.1 | –          |
| PS       | 3.03 ± 0.06        | 2.4 ± 0.1    | 2.4 ± 0.1 | –          |
| Class    | LPC               | 7.51 ± 0.07  | 3.9 ± 0.1 | 5.9 ± 0.1 | 7.5 ± 0.9 |
|          | 2-LPC             | 6.37 ± 0.19  | 3.3 ± 0.1 | 5.0 ± 0.1 | –          |
|          | 1-LPC             | 1.14 ± 0.12  | 0.6 ± 0.1 | 0.9 ± 0.1 | –          |
|          | LPE               | 2.84 ± 0.04  | 1.4 ± 0.1 | 2.2 ± 0.1 | 4.7 ± 0.2 |
|          | 2-LPE             | 2.52 ± 0.06  | 1.2 ± 0.1 | 2.0 ± 0.1 | –          |
|          | 1-LPE             | 0.32 ± 0.03  | 0.2 ± 0.1 | 0.3 ± 0.1 | –          |
|          | 2-LPA             | 0.52 ± 0.05  | 0.2 ± 0.1 | 0.4 ± 0.1 | –          |
| Total    | 107.00 ± 0.55      | 83.8 ± 0.4   | 84.4 ± 0.4 | 78.8 ± 0.7 |

DS from PL (31P NMR): Absolute quantification calculated by multiplying the molar amount (mmol) by the molecular weight of distearoyl or lysostearoyl-phospholipid.

SOPC (31P NMR): Absolute quantification calculated by multiplying the molar amount (mmol) by the molecular weight of stearyl-oleoyl-phosphatidylcholine (SOPC).

*Quantitative value before separation by 2D TLC.

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