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WHITE PAPER

Quantitative ^{31}P NMR Analysis of Lecithins and Phospholipid Products: Chemistry, Sample Preparation, Process Checkpoints and the PNA Method

Soybean, sunflower, rapeseed and niche-oilseed lecithins; egg yolk; dairy milk-fat-globule-membrane materials; krill and marine phospholipids; high-PC and phosphatidylserine products

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Abstract

Phospholipid ingredients are specified, traded and regulated on the basis of their class composition, yet the classes that define their value—phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), sphingomyelin (SM) and the lyso-, N-acyl and ether-linked analogues—are chemically similar and are not quantified reliably by chromatography without class-specific calibration. Phosphorus-31 NMR spectroscopy resolves these classes through the chemical shift of the single phosphorus atom in each head group, and because every phosphorus nucleus contributes equally to the integrated signal, molar amounts are obtained against one internal standard without response factors. In this white paper the chemistry that governs the ^{31}P chemical shift of phospholipids is reviewed, the principal sample-preparation systems (biphasic $CDCl_3$/methanol/Cs-EDTA reagents, monophasic organic solvents, and aqueous cholate or deoxycholate micelles) are compared, and the method used routinely at Process NMR Associates (PNA) is described in full, including the acquisition parameters recorded in the spectrometer files, the integration windows applied in practice and the calculation conventions. The phospholipid chemistry of soybean, sunflower, rapeseed and niche-oilseed lecithins, egg yolk, dairy milk-fat-globule-membrane ingredients, krill oil, fish roe and shellfish is summarized, and the manufacturing routes for fluid, deoiled, fractionated, enzyme-modified, high-PC and PS products are mapped against twenty-seven ^{31}P NMR checkpoints. Genericized spectra from four recent PNA analyses (a sunflower lecithin, a PS-enriched sunflower phospholipid powder, a high-purity PS softgel fill and a PS capsule blend) are presented with recomputed results, a PI-anchored balance for phospholipase D conversion, and an integration-strategy comparison obtained by re-processing a raw free-induction decay. Synthesized spectra are used to illustrate products and process stages for which PNA data are not shown, the effect of magnetic field strength (including 60 and 80 MHz benchtop instruments) is modeled, and an exploratory principal component analysis of class profiles drawn from PNA and published data is reported. The critical assessment identifies the integration model (which changed the PS result of a capsule blend by about 7 %), the completeness of partition into the measured phase, assignment in the alkaline reagent (including the possible presence of lyso-PS among minor signals), chemical-shift referencing, molecular-weight and reporting conventions, and decision rules close to specification limits as the dominant sources of error, and method refinements are proposed for implementation under ISO/IEC 17025.

Keywords: ^{31}P NMR; quantitative NMR; lecithin; phospholipids; phosphatidylcholine; phosphatidylserine; phospholipase D; krill oil; milk fat globule membrane; egg yolk; degumming; process monitoring; ISO/IEC 17025

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Abbreviations and Symbols

AAPC	Alkylacyl-phosphatidylcholine	**PA**	Phosphatidic acid
AI	Acetone insolubles	**PC**	Phosphatidylcholine
APE (NAPE)	N-Acyl-phosphatidylethanolamine	**PCA**	Principal component analysis
BPC	Buttermilk protein concentrate	**PCP, PEP**	PC and PE plasmalogens
CDTA	trans-1,2-Diaminocyclohexanetetraacetic acid	**PE**	Phosphatidylethanolamine
CL (DPG)	Cardiolipin (diphosphatidylglycerol)	**PG**	Phosphatidylglycerol
CP, EP, IP	Choline, ethanolamine and inositol phosphates	**PI**	Phosphatidylinositol
CSA	Chemical-shift anisotropy	**PL**	Phospholipid
DAG	Diacylglycerol	**PLA_1, PLA_2**	Phospholipases A_1 and A_2
DAG-AEP	Diacylglyceryl-(2-aminoethyl)phosphonate	**PLC, PLD**	Phospholipases C and D
DHSM	Dihydrosphingomyelin	**PLS**	Partial least squares
DOPC, DOPS	Dioleoyl-PC, dioleoyl-PS	**PMG**	Phosphonomethylglycine (glyphosate)
DPP	Diphenyl phosphate	**P-mol %**	Mole percent of total phospholipid phosphorus
EDTA	Ethylenediaminetetraacetic acid	**PS**	Phosphatidylserine
EMEY	Enzyme-modified egg yolk	**PSER**	O-Phospho-L-serine
FFA	Free fatty acids	**qNMR**	Quantitative NMR
FID	Free-induction decay	**RSD, RSDr, RSDR**	Relative standard deviation; repeatability; reproducibility
FWHM	Full width at half-height	**S/N**	Signal-to-noise ratio
ICP-OES	Inductively coupled plasma optical emission spectroscopy	**SM**	Sphingomyelin
IS	Internal standard	**T_1**	Spin–lattice (longitudinal) relaxation time
LAAPC	Lyso-alkylacyl-phosphatidylcholine	**TBP**	Tributyl phosphate
LoD, LoQ	Limits of detection and quantification	**TIP**	Triisopropyl phosphate
LPA	Lysophosphatidic acid	**TMP**	Trisodium trimetaphosphate
LPC	Lysophosphatidylcholine (1-acyl and 2-acyl isomers)	**TPP**	Triphenyl phosphate (PNA internal standard)
LPE	Lysophosphatidylethanolamine	**TR**	Repetition time (acquisition time + relaxation delay)
LPI	Lysophosphatidylinositol	**U, u**	Expanded and standard uncertainty
LPS	Lysophosphatidylserine	**WPPC**	Whey protein phospholipid concentrate
MFGM	Milk-fat-globule membrane	**wt %**	Percent by weight of sample
NOE	Nuclear Overhauser effect		

1. Introduction

Phospholipids occupy an unusual position among food and supplement ingredients. In the form of lecithin they are among the most widely used emulsifiers in chocolate, bakery, margarine, instant powders and release agents; in concentrated or purified form they are sold as dietary ingredients in their own right, most visibly as phosphatidylcholine (PC) concentrates, phosphatidylserine (PS) powders and softgels, and krill oil, whose value proposition rests on the fraction of its omega-3 fatty acids that is carried in phospholipid rather than triacylglycerol form. Dairy phospholipid concentrates prepared from buttermilk and butter serum, egg-yolk phospholipids and their enzyme-modified derivatives, and the lecithins recovered from sunflower, rapeseed and a growing list of niche oilseeds complete the commercial landscape[1,2]. In every one of these materials the functional and nutritional properties, and very often the label claim, are defined by the distribution of phospholipid classes rather than by the total phosphorus content alone.

The analytical difficulty is that the classes are chemically similar. They share a glycerophosphate or sphingosine phosphate backbone, carry the same families of fatty acyl chains, and differ only in the small polar group esterified to the phosphate. Thin-layer chromatography with phosphorus determination was for many years the reference approach, and normal-phase HPLC with evaporative light-scattering or charged-aerosol detection is now widely used, but chromatographic detectors respond to mass in a nonlinear, composition-dependent manner and must be calibrated class by class with standards whose fatty-acid composition matches the sample; recoveries from TLC plates are incomplete, and lysophospholipids and acidic classes are handled poorly by several of the published solvent systems[3–5]. Phosphorus-31 NMR avoids most of these difficulties. The ^{31}P nucleus has a spin of ½ and 100 % natural abundance, each glycerophospholipid molecule contains exactly one phosphorus atom (cardiolipin contains two), and the ^{31}P chemical shift is governed primarily by the head group, so that each class gives one resolved resonance, or a small cluster of resonances, within a window of roughly 6 ppm. Because the integrated signal is proportional to the number of phosphorus nuclei, the molar ratios of all classes are obtained directly from the spectrum, and absolute amounts follow from a single weighed internal standard[6–9].

The technique has a long history. Henderson, Glonek and Myers described the ^{31}P spectra of the principal phospholipids in 1974[6], London and Feigenson showed in 1979 that detergent micelles collapse the broad bilayer signals into narrow isotropic lines suitable for class analysis[10], and quantitative ^{31}P analysis of phospholipid mixtures was demonstrated by Sotirhos and co-workers in 1986[7]. The practical breakthrough for extracted lipids came with the chloroform/methanol/aqueous cesium-EDTA reagent of Meneses and Glonek[11], in which divalent and paramagnetic cations are sequestered, cesium becomes the common counter-ion, and line widths of a few hertz are obtained. That reagent, refined by Diehl and colleagues for commercial lecithins and krill oil and subsequently tested in interlaboratory proficiency schemes, is the direct ancestor of the procedure used at Process NMR Associates (PNA)[12–14]. In parallel, aqueous cholate and deoxycholate micellar systems have been developed and validated for dairy phospholipids,

edible oils and egg-yolk emulsions[3,15,16], and benchtop ^{31}P spectrometers operating at 1–2 T have been evaluated for routine lipid analysis[17].

PNA has applied ^{31}P NMR on a routine basis for more than a decade to vegetable lecithins, PC and PS concentrates, finished PS and PC supplements and krill oils. The purpose of this white paper is to consolidate the chemistry, the methodology and the process knowledge that underpin that work, with four objectives. The first is to explain why each class resonates where it does and why the positions move with the sample-preparation system, since assignment errors, not signal-to-noise, are the principal failure mode of the method. The second is to describe the current PNA procedure exactly as it is executed, including the acquisition parameters recorded in the spectrometer files and the integration windows used in practice, so that the method can be audited and improved. The third is to map the manufacturing routes of the major phospholipid sources and products and to identify the points at which a ^{31}P measurement answers a specific process question. The fourth is to examine, critically, the sources of error that an assessor, a client or an opposing expert would be expected to probe, and to propose refinements appropriate to an ISO/IEC 17025 quality system[18]. Spectra from four recent PNA analyses are reproduced in genericized form, and synthesized spectra are used where a product or process stage is discussed for which PNA data are not shown; every synthesized spectrum is labeled as such.

2. Phospholipid Chemistry Relevant to ^{31}P NMR

2.1 Glycerophospholipid architecture and the observed phosphorus

The glycerophospholipids that dominate lecithins and most phospholipid ingredients are built on *sn*-glycero-3-phosphate. Fatty acids are esterified at the *sn*-1 and *sn*-2 positions, and the phosphate at *sn*-3 is esterified in turn to a polar alcohol—choline, ethanolamine, L-serine, *myo*-inositol or glycerol—or left unsubstituted, as in phosphatidic acid (Figure 1). The observed phosphorus is therefore, in all classes except PA and lyso-PA, a phosphodiester carrying one negative charge on its non-bridging oxygens at the pH values encountered in analysis, bonded through oxygen to two carbon atoms: the glycerol C-3 and the first carbon of the head group. The ^{31}P chemical shift of such a phosphodiester is determined chiefly by the O–P–O bond angles and the torsional arrangement about the P–O ester bonds, which are in turn set by the size, charge and hydrogen-bonding capacity of the head group and by any intramolecular contacts between the head group and the phosphate[6,19–21]. The head-group dependence is the reason ^{31}P NMR resolves classes rather than molecular species; the fatty acyl chains are remote from the phosphorus, and their influence appears only as small shift dispersions and line-shape asymmetries that become visible at high field or with highly unsaturated marine lipids[22,23].

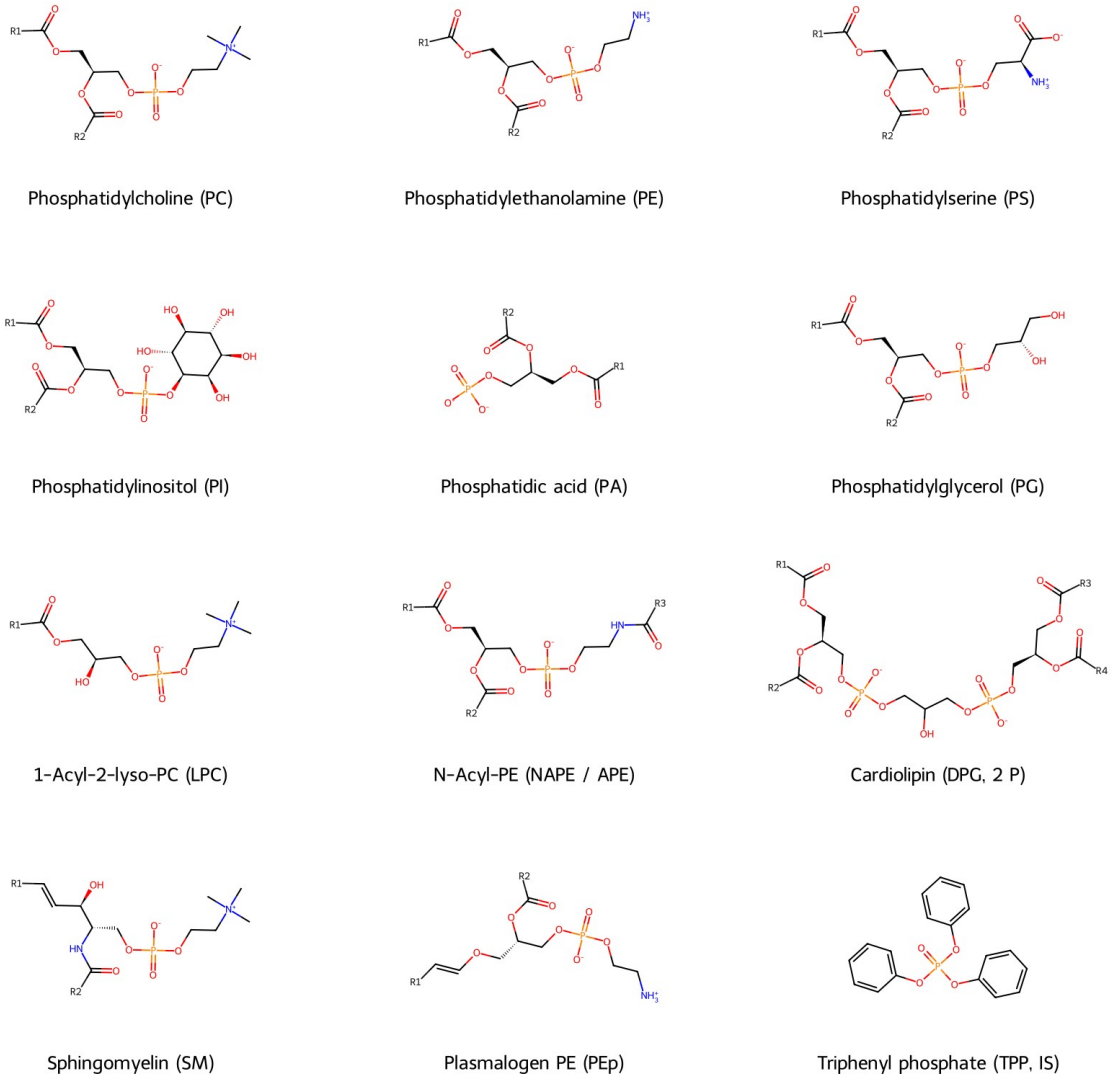


Figure 1. Structures of the principal phospholipid classes discussed in this paper, drawn as the ionic forms expected under alkaline analysis conditions, together with the internal standard triphenyl phosphate (TPP). R denotes fatty acyl (or, for sphingomyelin and plasmalogens, the sphingoid or alk-1′-enyl) chains. Cardiolipin (diphosphatidylglycerol, DPG) is the only common class carrying two phosphorus atoms per molecule.

Two consequences follow directly for quantitation. First, a ^{31}P integral counts phosphorus atoms, not molecules and not mass. The mole fraction of phosphorus in each class is therefore the primary result of the measurement, and any conversion to weight percent requires an assumed molecular weight that depends on the fatty-acid composition and, for anionic classes, on the counter-ion (Section 2.7). Second, because only the phosphorus is observed, the acyl chains, the neutral lipids and the non-phosphorus matrix of a finished product are invisible; triacylglycerol, sterols, carbohydrate carriers, capsule shell components and most excipients contribute nothing to the spectrum, which is the principal reason ^{31}P NMR can be applied directly to softgel fills, capsule blends and lecithin granules without chromatographic clean-up.

2.2 The head-group classes

Phosphatidylcholine carries a quaternary ammonium head group and is zwitterionic at all practical pH values; its phosphodiester resonance is the most shielded (lowest-frequency) of the common classes in every solvent system examined in this paper and is therefore the natural internal chemical-shift reference for relative scales[23,24]. Phosphatidylethanolamine differs from PC only by the replacement of three methyl groups by hydrogen, but the primary ammonium group of PE can hydrogen-bond to its own phosphate and can be deprotonated at high pH, so that the PE resonance is more deshielded than PC and moves with pH and with the cation environment. Phosphatidylserine carries an α-amino acid in its head group and therefore has an ammonium group, a carboxylate and the phosphodiester; it is net anionic at neutral pH and its resonance is sensitive to both pH and divalent cations, which it binds strongly. Phosphatidylinositol carries the bulky, polyhydroxylated *myo*-inositol ring and is anionic; its resonance lies between PC and PE in organic Cs-EDTA reagents but moves close to PC in cholate micelles, which is one of the clearest illustrations of the solvent dependence of the method (Section 4). Phosphatidylglycerol, the dominant anionic phospholipid of plant plastids and bacteria and a minor component of lecithins, resonates downfield of PE[4,23].

Phosphatidic acid is the exception to the phosphodiester pattern. With only one ester bond, the phosphorus in PA is a phosphomonoester that can carry one or two negative charges, and the second ionization falls in the neutral to mildly alkaline range. The chemical shift of the monoanion and dianion differ by several parts per million, so the PA resonance is the most pH-sensitive signal in a phospholipid spectrum: in cholate micelles it has been observed to move from about 2.5 to 3.9 ppm as the pH is raised from 7.4 to 12[25], and in the alkaline biphasic reagent used at PNA it appears near 3.9 ppm, well separated from the phosphodiester envelope, whereas in a near-neutral $CDCl_3$/CD_3OD/Cs-EDTA system it lies within 1.1 ppm of PC[23,24]. The same behavior, amplified, is shown by lyso-PA. Cardiolipin (diphosphatidylglycerol, DPG, often abbreviated CL) consists of two phosphatidyl units bridged by glycerol; it contributes two phosphorus atoms per molecule, which must be accounted for when molar or weight results are calculated.

2.3 Lysophospholipids, acyl migration and a note on nomenclature

Lysophospholipids carry a single acyl chain. They are formed enzymatically by phospholipases A_1 and A_2, chemically by alkaline or acidic hydrolysis, and during storage of wet or poorly stabilized materials. Removal of one chain changes the local environment of the phosphate enough to move the ^{31}P resonance by several tenths of a part per million relative to the diacyl parent, so lyso-PC (LPC), lyso-PE (LPE), lyso-PA (LPA) and, in PS products, lyso-PS (LPS) appear as separate signals. Each lyso class exists as two positional isomers: the 1-acyl-2-lyso form, which is the product of PLA_2, and the 2-acyl-1-lyso form, which is the product of PLA_1. The two interconvert by intramolecular acyl migration, a reaction catalyzed by both acid and base, and the equilibrium mixture contains roughly 90 % of the 1-acyl isomer because the primary ester is thermodynamically favored[26,27]. Van Rijn and co-workers observed that at pH 9 the equilibrium composition is reached essentially immediately after extraction[15]. The ratio of the two isomer

signals in a ^{31}P spectrum therefore reports the enzymatic origin of the lyso fraction only when the sample has been handled under conditions that do not accelerate migration.

The literature is not consistent in naming the isomers. Some authors name them by the position of the remaining acyl chain (1-acyl-LPC), others by the position of the free hydroxyl (2-lyso-PC), and the shorthand "1-LPC" and "2-LPC" has been used in both senses; at least one widely cited paper appears to use one convention in its abbreviation list and the other in its discussion. In PNA reports the major isomer, observed immediately upfield of PS, is labeled "2-LPC" (2-lyso-1-acyl-PC) and the minor isomer, observed between PI and PC, is labeled "1-LPC" (1-lyso-2-acyl-PC). In this white paper the unambiguous forms 1-acyl-LPC and 2-acyl-LPC are used wherever the assignment is known, and the PNA labels are retained only where the routine integration windows are reproduced.

2.4 N-acyl phosphatidylethanolamine and other low-polarity phospholipids

The primary amine of PE can be acylated. N-acyl-PE (NAPE, abbreviated APE in PNA reports) occurs naturally in oilseeds and cereals, is formed during seed germination and stress, and is also produced deliberately when lecithin is acetylated with acetic anhydride to improve its hydrophilicity, in which case the N-acetyl derivative is formed[1]. Because acylation removes the ammonium–phosphate interaction, the ^{31}P resonance of APE lies between PE and the DPG/LPE region in the PNA reagent. Low-polarity phospholipids of this type, including N-acyl-PE, N-acyl-lyso-PE and acyl-PG, have been characterized by TLC and ^{31}P NMR by Vyssotski, MacKenzie and Scott[28], and their presence at the percent level is normal in soybean and sunflower lecithins. An elevated APE fraction is nevertheless diagnostic: in a non-acetylated lecithin it indicates seed or process history, and in an acetylated lecithin it measures the degree of modification directly.

2.5 Ether lipids, plasmalogens and sphingomyelin

Animal and marine phospholipids contain classes that are rare in plant lecithins. In the ether-linked (alkyl-acyl) and plasmalogen (alk-1′-enyl-acyl) forms the *sn*-1 chain is attached through an ether or vinyl-ether bond rather than an ester. The phosphorus environment is only slightly perturbed, and in $CDCl_3$/methanol/Cs-EDTA systems the ether and plasmalogen analogues resonate a few hundredths of a part per million downfield of their diacyl counterparts: plasmalogen PC is observed about 0.05 ppm from PC and lyso-PC plasmalogen about 0.04–0.07 ppm from LPC[23,24]. Alkylacyl-PC (AAPC) and lyso-alkylacyl-PC (LAAPC) are characteristic of krill oil and have been quantified in it by ^{31}P NMR[25,29]. At 7 T these species are resolved only as shoulders on the dominant PC and LPC signals, and their quantitation is correspondingly less certain than that of the head-group classes.

Sphingomyelin is built on sphingosine rather than glycerol, with the fatty acid bound as an amide, and carries the same phosphocholine head group as PC. It is a major phospholipid of milk (roughly one quarter of the phosphorus in milk-fat-globule membrane material), a minor component of egg yolk, and absent from plant lecithins. The saturated analogue dihydrosphingomyelin (DHSM) is

resolved from SM by ³¹P NMR in cholate micelles and in $CDCl_3$/CD_3OD/Cs-EDTA, which is an advantage of the method over chromatographic techniques that co-elute the two[3,23]. Marine invertebrates add a further class: phosphonolipids containing a direct carbon–phosphorus bond (for example ceramide or diacylglyceryl 2-aminoethylphosphonate) resonate near 20–23 ppm, far from all phosphate esters, and were quantified by ³¹P NMR in squid mantle and mussel oils[24].

2.6 Ionization, counter-ions and salt forms

With the exception of PC and SM, whose zwitterionic head groups carry charges that do not change with pH, the phospholipid classes carry groups whose ionization depends on pH, and all of them interact with metal cations. Calcium and magnesium bind to the phosphate and, in PS and PA, to the carboxylate and phosphomonoester groups, broadening the ³¹P lines through exchange and aggregation and shifting them in a concentration-dependent way; iron, copper and manganese broaden the lines paramagnetically[11,30]. The role of EDTA in every reagent discussed in this paper is to remove these cations, and the role of the cesium counter-ion in the biphasic reagents is to replace them with a single, large, weakly binding monovalent cation that renders the anionic classes soluble in the chloroform-rich phase and gives reproducible shifts[11,30]. This chemistry has practical consequences for commercial products. PS is usually supplied as the sodium or calcium salt, PA-rich fractions can contain calcium and magnesium carried over from non-hydratable phosphatides, and krill oils and egg products contain metal ions from the raw material. All of these must be fully exchanged for the phospholipid to partition quantitatively into the phase that is measured, which is why the volume and concentration of the Cs-EDTA solution are critical parameters of the method.

2.7 Molecular-weight conventions and the phosphorus basis of quantitation

Because a ³¹P integral counts phosphorus atoms, the conversion of an integral ratio into a mass requires a molecular weight for each class, and natural phospholipids are mixtures of molecular species whose average molecular weight depends on chain length and unsaturation. The values used by PNA, adopted from the literature methods on which the procedure is based, are listed in Table 1. They correspond approximately to species carrying C_{18} chains with the degree of unsaturation typical of soybean lecithin and to the free-acid or zwitterionic form, and they are appropriate for vegetable lecithins and PS derived from them. For marine phospholipids rich in eicosapentaenoic and docosahexaenoic acids, for dairy phospholipids rich in saturated chains, and for PS supplied as a sodium or calcium salt, the true average molecular weight can differ from the tabulated value by 1–3 %, and the resulting bias is carried directly into every weight-percent result. Kato and co-workers addressed the same problem by reporting results both as moles and as "distearoyl-equivalent" or "stearoyl-oleoyl-PC-equivalent" masses[4]. The most defensible primary result is therefore the molar content (µmol of each class per gram of sample, or mol % of phospholipid phosphorus), with weight percent reported as a derived quantity whose molecular-weight basis is stated. Two further conventions deserve explicit statement. For cardiolipin, which carries two phosphorus atoms, the integral must be halved before multiplication by the molecular

weight of the whole molecule (equivalently, 725 g mol^{-1} per phosphorus). For resonances that cannot be assigned, a nominal molecular weight of 770 g mol^{-1} (that of PC) is applied, and the resulting "other phospholipid" value is an estimate whose uncertainty is dominated by the unknown identity.

Table 1. Phospholipid classes quantified by the PNA method, with the molar masses applied in the calculations, the number of phosphorus atoms per molecule and the range of positions observed on the routine PNA scale in the four analyses of Section 8.

Class	Abbrev.	Head group / character	P per molecule	Molar mass used (g mol^{-1})	Observed position, PNA scale (ppm)
Phosphatidylcholine	PC	Choline; zwitterion	1	770	−0.72 to −0.78
Lyso-PC, major isomer (1-acyl-2-lyso; PNA label "2-LPC")	LPC	Choline; one acyl chain	1	515	≈ −0.20 to −0.30
Lyso-PC, minor isomer (2-acyl-1-lyso; PNA label "1-LPC")	LPC	Choline; one acyl chain	1	515	≈ −0.35 to −0.60
Phosphatidylinositol	PI	myo-Inositol; anionic	1	835	−0.27 to −0.42
Phosphatidylserine	PS	L-Serine; net anionic	1	797	−0.07 to −0.13
Phosphatidylethanolamine	PE	Ethanolamine; zwitterion	1	725	−0.03 to +0.02
N-Acyl-phosphatidylethanolamine	APE (NAPE)	N-Acylethanolamine; anionic	1	990	+0.05 to +0.20
Diphosphatidylglycerol (cardiolipin)	DPG (CL)	Glycerol-bridged; dianionic	2	1450 (725 per P)	+0.25 to +0.40
Lyso-phosphatidylethanolamine	LPE	Ethanolamine; one acyl chain	1	470	+0.26 to +0.50
Phosphatidylglycerol	PG	Glycerol; anionic	1	758	+0.33 to +0.57
Phosphatidic acid	PA	Phosphomonoester; dianion at pH 9.7	1	685	+3.73 to +3.97
Lyso-phosphatidic acid	LPA	Phosphomonoester; one acyl chain	1	430	≈ +4.5 and ≈ +5.0 (see text)
Unassigned signals	Other	—	1[a]	770 (nominal)	Various
Triphenyl phosphate (internal standard)	TPP	Phosphate triester	1	326.28	−17.80 (reference)

[a] Assumed. Molar masses correspond approximately to C_{18} species in the free-acid or zwitterionic form. SM and DHSM, which are absent from plant lecithins, are not included in the routine PNA table. Observed positions are peak positions or window limits on the routine scale and vary with the matrix (Section 5.7).

3. Principles of Quantitative ^{31}P NMR of Phospholipids

3.1 Sensitivity and the phosphorus basis of the measurement

The ^{31}P nucleus is, in several respects, the most convenient heteronucleus for quantitative work. It has a nuclear spin of ½, so no quadrupolar broadening is encountered, it is present at 100 % natural abundance, and its magnetogyric ratio is about 40 % of that of ^{1}H, which gives a receptivity of roughly 6.6 % of the proton at equal numbers of nuclei. In phospholipid work the practical sensitivity is further improved because the concentration of phosphorus in a lecithin or phospholipid concentrate is high (a 70 % phospholipid lecithin contains close to 0.9 mmol of phospholipid phosphorus per gram) and because the entire phospholipid signal is compressed into a few parts per million, so that very few scans are required to reach signal-to-noise ratios of several hundred for the major classes[8,9]. The limitations are the converse of these advantages. The phosphodiester resonances of the different classes are separated by only a few tenths of a part per million, the separations depend on the chemical environment in the tube, and the integrals must be obtained from partially overlapped lines. Most of the analytical effort in this field has therefore been directed not at sensitivity but at the preparation of samples that give narrow, reproducibly positioned lines, and at the assignment of the resulting signals[11,31,32].

The quantity that is actually measured is the number of phosphorus nuclei resonating within a defined frequency interval. Provided that every nucleus is excited equally, that longitudinal magnetization has recovered fully between scans, that no nuclear Overhauser enhancement is present and that the integration captures the whole of each line, the ratio of two integrals is equal to the ratio of the numbers of phosphorus atoms that produce them[9]. The four conditions are satisfied in different ways by the different methods discussed below, and each of them corresponds to a term in the uncertainty budget developed in Section 10. It deserves emphasis that no calibration against the analyte is involved; a single internal standard of known purity is sufficient for every class in the spectrum, including classes for which no commercial reference material exists, and this is the property that distinguishes ^{31}P NMR from all of the chromatographic methods with which it is compared[3–5].

3.2 Origins of the chemical shift and its sensitivity to the sample environment

The ^{31}P shielding of a phosphate ester is governed by the electronic structure of the PO_4 tetrahedron, and the empirical correlations established for phosphate esters show that the isotropic shift is most sensitive to the O–P–O bond angles, to the torsional angles about the P–O ester bonds and to the charge and protonation state of the non-bridging oxygens[6,19–21]. The head group of a phospholipid influences all three. A bulky or hydrogen-bonding head group alters the preferred conformation about the P–O(C) bonds, an ammonium group that can approach the phosphate (as in PE and PS) changes the local electrostatic field, and an ionizable group on the phosphorus itself (PA and LPA) changes the charge on the tetrahedron directly. Because these influences are specific to each head group, the resonances are dispersed by class, which is the foundation of the method.

The same sensitivity makes the shifts dependent on the medium. The pH of the aqueous phase with which the lipid has equilibrated sets the ionization state of PA, LPA, PS and, at high pH, PE; the identity and concentration of the counter-ion determine the extent of ion pairing at the phosphate; the ratio of chloroform, methanol and water in organic reagents determines the degree of hydration of the head groups and the size of the reverse micelles in which they reside; and the concentration of lipid and the presence of other amphiphiles (triacylglycerol, free fatty acids, monoacylglycerols, cholate) modify the aggregation state[30,31,33,34]. Temperature produces smaller but measurable shifts that are not identical for all classes, and Puppato and co-workers showed that in cholate micelles the relative positions of several classes change with both temperature and pH, so that signals can cross[34]. Lutz and Cozzone formalized these observations as a multiparametric optimization problem, in which solvent composition, pH, temperature and cation concentration are adjusted together to maximize the separation of the classes present in a given tissue extract[31,32].

The acyl chains contribute a second-order effect. Kaffarnik and co-workers observed that PC from fish, rich in polyunsaturated chains, resonated 0.026 ppm upfield of PC from cheese when both were referenced to triphenyl phosphate, and that the PC signal of fish lipids carried a broad high-field shoulder that was attributed to polyunsaturated molecular species; similar shoulders were seen on the other classes[23]. Pearce and Komoroski had earlier resolved molecular-species structure within the brain phospholipid classes at high field[22]. For the quantitative analyst the lesson is that a single class can occupy a band of 0.05–0.1 ppm, particularly in marine materials, and that integration windows must be wide enough to accommodate it. The matrix effect is of the same order. In the same study, the PE shift varied by almost 0.2 ppm among four cheese extracts, and PE and its ether-linked analogue exchanged their order in two extracts that differed only in their preparation; the authors concluded that such matrix effects differ from class to class and cannot be removed by referencing the spectrum to PC[23]. The practical consequence, which is revisited in the discussion of the PNA method (Section 5.7), is that fixed shift tables are guides to assignment rather than definitions of it.

3.3 Line width, aggregation and chemical-shift anisotropy

The phosphorus in a phospholipid has a large chemical-shift anisotropy (CSA), of the order of 40–50 ppm in bilayers[35], and in any aggregate whose reorientation is slow the anisotropy is incompletely averaged. In unilamellar or multilamellar dispersions this produces the characteristic broad bilayer powder pattern, which is useless for class analysis. The purpose of all of the sample-preparation systems described in Section 4 is to place the phospholipid in a small, rapidly tumbling aggregate: a mixed micelle with cholate or deoxycholate in water[10,27,35], or a small reverse micelle or solvated ion pair in chloroform–methanol mixtures in which the water content and counter-ion have been controlled[11,33]. In either case the residual line width is determined by the effective correlation time of the aggregate, by chemical exchange between environments, by paramagnetic impurities, and, at high field, by CSA relaxation.

The last point has practical consequences for the choice of spectrometer. Because the CSA contribution to transverse relaxation scales with the square of the magnetic field, the lines of

micellar phospholipids broaden as the field is raised, while the separations between classes, expressed in hertz, increase only linearly. Mayar and co-workers calculated the CSA contribution from measured self-diffusion coefficients for phospholipids in cholate micelles and in $CDCl_3$, and they showed that the gain in resolution with field strength levels off for the micellar system; a 9.4 T (400 MHz) instrument was judged to be a fair compromise between sensitivity and CSA broadening, and measurements at 16.4 T gave only a modest improvement in precision despite a 1.4-fold higher signal-to-noise ratio[16]. The same authors noted that the ^{31}P T_1 values of the micellar phospholipids were shorter at 16.4 T than at 9.4 T, which is the signature of CSA-dominated relaxation, whereas the small internal standards were unaffected[16]. In organic reagents, where the aggregates are smaller, the CSA penalty is less severe, and line widths of about 2–3.5 Hz are obtained at 7 T for the major phosphodiester classes, and about 1.3 Hz for the internal standard (Section 5.7).

3.4 Longitudinal relaxation, repetition time and flip angle

Quantitative accuracy requires that longitudinal magnetization recover completely, or to a known and constant extent, between scans. For a pulse of flip angle θ repeated at an interval TR (the sum of the acquisition time and the relaxation delay), the steady-state signal of a nucleus with relaxation time T_1 is proportional to the expression given in Eq. (1), and the ratio of two integrals is unbiased only when this factor is the same for the analyte and the standard. With θ = 90° the recovery after TR = 5T_1 is 99.3 %, and after 7T_1 it is 99.9 %.

$$S \propto \sin\theta \cdot (1 - E_1) / (1 - E_1 \cos\theta), \quad \text{where } E_1 = \exp(-TR/T_1) \tag{1}$$

The phospholipids themselves relax relatively quickly. In cholate micelles Kato and co-workers measured T_1 values of 0.9–1.1 s for PC, 0.6–1.0 s for PI, 0.9–1.4 s for PE, 0.8–0.9 s for PS, 1.6–1.7 s for PA and 0.8–1.4 s for LPC, and van Rijn and co-workers reported values of 0.9–1.4 s for the phospholipid and lysophospholipid classes in deoxycholate micelles at pH 9[4,15]. At 1 T the phospholipid T_1 fell to about 0.37 s[17]. The difficulty lies with the internal standards and with the small, water-soluble phosphorus compounds, which relax much more slowly. Triisopropyl phosphate had T_1 = 3.6 s in the deoxycholate system, inorganic phosphate 10.6 s and choline phosphate 8.4 s, so that an interpulse delay of 15 s recovered 94–100 % of the phospholipid signals but only 89 % of the phosphate signal[15]. Phosphoserine, used by Kato and co-workers, had a T_1 that varied from 1.3 s to 4.3 s depending on the ratio of cholate to phospholipid in the sample, which illustrates that the relaxation of the standard is itself matrix-dependent[4]. Diphenyl phosphate and tributyl phosphate relaxed with T_1 values of 4.1 s and 14 s at 1 T, which led Gouilleux and co-workers to investigate a gadolinium relaxation agent and a cross-polarization scheme as alternatives to long delays[17]. Representative values are collected in Table 2.

Table 2. Representative ^{31}P spin–lattice relaxation times (T_1) of phospholipids, internal standards and related species reported in the literature.

Medium and conditions	Species and T_1 (s)	Reference
Sodium cholate–EDTA micelles, 30 °C	PC 0.9–1.1; PI 0.6–1.0; PE 0.9–1.4; PS 0.8–0.9; PA 1.6–1.7; LPC 0.8–1.4	[4]
Sodium cholate–EDTA micelles, 30 °C	Phosphoserine (internal standard) 1.3–4.3, depending on the cholate:phospholipid ratio	[4]
Deoxycholate–EDTA–TRIS, pH 9.0	PC 1.1; PI 0.9; LPC 1.4; PE/LPI 1.1; LPE 1.4	[15]
Deoxycholate–EDTA–TRIS, pH 9.0	Triisopropyl phosphate (internal standard) 3.6; inorganic phosphate 10.6; choline phosphate 8.4	[15]
Cholate buffer, pH 7.5; 9.4 and 16.4 T	Trimetaphosphate 1.7; phosphonomethylglycine 0.7; phospholipid T_1 shorter at 16.4 T than at 9.4 T (CSA relaxation)	[16]
$CDCl_3$/MeOH/Cs-CDTA (ternary), 1 T	Phospholipids ≈ 0.37; diphenyl phosphate 4.1 (1.4 with gadoterate relaxation agent); tributyl phosphate 14	[17]
$CDCl_3$/MeOH/Cs-EDTA, pH 9.7 (PNA)	Not yet determined for TPP or for the phospholipid classes	Recommendation (Section 5.8)

Values are as reported; field strengths are stated where the source reports a field dependence.

Two practical consequences follow. First, the repetition time must be set by the slowest-relaxing species that is quantified, which in an internal-standard method is usually the standard rather than the analyte. Second, the T_1 of the standard must be measured in the reagent actually used, since values transferred from other solvent systems can be wrong by a factor of three. The flip angle interacts with both conditions. A 90° pulse maximizes the signal per scan when TR ≥ $5T_1$, whereas a smaller flip angle (30–45°) shortens the recovery needed for a given accuracy, and a pulse larger than 90° both reduces the signal (sin 144° ≈ 0.59) and partially inverts the magnetization, so that full recovery then requires a longer interval than with 90°[9]. The empirical approach adopted by van Rijn and co-workers, in which spectra of a representative mixture are recorded at a series of delays and the recovery of each signal relative to a fully relaxed reference is plotted, is the most direct way of establishing the repetition time for a routine method[15]; Ahmmed and co-workers, and Kato and co-workers, applied the same logic to cholate systems and arrived at considerably shorter delays for phospholipid-only quantitation[4,25].

3.5 Proton decoupling, nuclear Overhauser effect and scalar coupling

The phosphorus of a glycerophospholipid is coupled to the protons of the two adjacent methylene groups (glycerol C-3 and the first carbon of the head group) with three-bond couplings of a few hertz, and to more distant protons with smaller couplings; Gouilleux and co-workers quoted ^3J(P,H) values of 1–10 Hz for the P–O–CH_2 moieties[17]. Without decoupling, each class therefore appears as an unresolved multiplet several hertz wide, which destroys the resolution between PS, PI and the lyso-PC isomers. Broadband ^{1}H decoupling collapses the multiplets, but continuous decoupling also produces a heteronuclear nuclear Overhauser enhancement whose magnitude depends on the dipolar relaxation pathway of each phosphorus and is therefore not uniform across

classes or between the analytes and the standard. The universal solution is inverse-gated decoupling, in which the decoupler is switched on only during acquisition, so that the multiplets collapse in the detected signal. The NOE nonetheless builds up, with the T_1 time constant, while the decoupler is on, and it must be allowed to decay during the decoupler-off relaxation delay before the next pulse; when the acquisition time is long compared with T_1, as it is in the PNA method, it is the length of that delay relative to T_1 that suppresses the NOE[3,9,16]. The decoupling power required increases with field strength, and Mayar and co-workers found that at 16.4 T the decoupler heated the sample appreciably unless the acquisition time was limited, which constrained their choice of parameters[16]. At 7 T with WALTZ-16 decoupling at moderate power this is not a limitation, but the sample temperature should nonetheless be regulated, since the relative positions of several classes are temperature dependent[34].

The ^{13}C satellites of each resonance, arising from two- and three-bond ^{31}P–^{13}C couplings, contribute about 1 % of each signal per coupled carbon position and lie within a few hertz of the parent line; they are included in any window or fitted line shape of reasonable width and do not require separate treatment.

3.6 Internal standards and chemical-shift referencing

An internal standard for ^{31}P qNMR of phospholipids must be of certified purity, soluble in the phase that is measured, chemically inert under the preparation conditions, resonant in an empty region of the spectrum and, ideally, rapidly relaxing. No single compound meets every requirement, and the standards used in the literature reflect compromises that depend on the solvent system (Table 3). In organic reagents triphenyl phosphate (TPP; $(C_6H_5O)_3PO$, CAS 115-86-6) is the standard of choice: it is available as a certified qNMR reference material, it is soluble in $CDCl_3$, it does not partition into the aqueous phase, and its resonance near −17.8 ppm is far from the phospholipid envelope. Kaffarnik and co-workers used TPP at −17.95 ppm as the reference of their shift scale, whereas PNA assigns −17.80 ppm, which shows that the absolute position adopted for the standard is a convention that must be stated with every shift table[23]. In aqueous detergent systems water-soluble standards are required, and phosphonomethylglycine (glyphosate), trisodium trimetaphosphate, phosphoserine and triisopropyl phosphate have all been used[3,4,15,16,25]. Trimetaphosphate carries three equivalent phosphorus atoms, which increases its signal per mole, and Mayar and co-workers selected it for that reason even though phosphonomethylglycine relaxed faster[16].

Table 3. Internal standards used for quantitative ^{31}P NMR of phospholipids.

Standard	Formula (P per molecule)	Reported δ ^{31}P (ppm)	Medium	Remarks and references
Triphenyl phosphate (TPP)	$(C_6H_5O)_3PO$ (1)	−17.80 (PNA); −17.95[23]	$CDCl_3$ phase of organic reagents	Certified qNMR material available; T_1 in the PNA reagent not yet measured
Triphenylphosphine (also abbreviated TPP)	$(C_6H_5)_3P$ (1)	≈ −6	$CDCl_3$/CD_3OD/EDTA	Used by Ahmmed et al.[25]; air-oxidizes to the phosphine oxide; not a phosphate ester
Phosphonomethylglycine (glyphosate, PMG)	$C_3H_8NO_5P$ (1)	7.4[16]	Cholate/EDTA	SM at −7.53 ppm relative to PMG at pH 7.1[3]; T_1 0.7 s; limited solubility[16]
Trisodium trimetaphosphate (TMP)	$Na_3P_3O_9$ (3)	21.5[16]	Cholate buffer, pH 7.5	Three equivalent P per molecule; T_1 1.7 s[16]
O-Phospho-L-serine (PSER)	$C_3H_8NO_6P$ (1)	pH-dependent	Cholate/EDTA	Traceable reference material, purity 99.5 %, U = 0.9 % (k = 2); T_1 1.3–4.3 s[4]
Triisopropyl phosphate (TIP)	$C_9H_{21}O_4P$ (1)	−2.765[15]	Deoxycholate/EDTA/ TRIS, pH 9.0	T_1 3.6 s[15]
Diphenyl phosphate (DPP)	$C_{12}H_{11}O_4P$ (1)	Not stated	$CDCl_3$/MeOH/Cs-CDTA	T_1 4.1 s at 1 T; used with a gadolinium relaxation agent[17]
Tributyl phosphate (TBP)	$C_{12}H_{27}O_4P$ (1)	Not stated	$CDCl_3$/MeOH/Cs-CDTA	T_1 14 s at 1 T; used with $^1H \rightarrow {}^{31}P$ cross-polarization[17]

Chemical shifts are reported on the scale of each source and are not directly comparable between media.

The abbreviation TPP deserves a warning. It is used in the literature both for triphenyl phosphate and for triphenylphosphine, and at least one of the studies cited in this paper describes its $CDCl_3$ standard as triphenylphosphine[25]. The two compounds are not interchangeable. Triphenylphosphine is a phosphine that resonates near −6 ppm and is slowly oxidized in air to triphenylphosphine oxide (near +29 ppm), whereas triphenyl phosphate is a stable phosphate triester resonating near −18 ppm. A standard observed near −17.8 ppm is therefore triphenyl phosphate, and it should be named as such, with its CAS number, in every method description and report.

Chemical-shift referencing is a separate issue from quantitation but has a direct bearing on assignment. Where the standard serves as the shift reference, every class position is expressed relative to it, and any change in the standard's own shift with solvent composition is transferred to the whole scale. Where PC is used as the reference (set to 0 or to −0.84 ppm), the positions of the other classes become relative shifts that are more transferable between laboratories but that inherit the matrix sensitivity of PC[23,24]. In this paper shifts are quoted on the scale used by each source, and the differences are summarized as Δδ relative to PC in Section 4.6.

3.7 Quantitation equations

With an internal standard of mass m_{IS}, purity P_{IS} and molar mass M_{IS}, the amount n_X of a phospholipid class X in the measured solution follows from the ratio of integrals I corrected for the

number of phosphorus atoms per molecule N, as expressed in Eq. (2). The mass fraction w_X of the class in a sample of mass m_s is obtained by multiplying by the molar mass assigned to the class (Eq. (3)), and the phosphorus-molar distribution, which requires neither the standard nor any molar mass, is the ratio of each integral to the sum of all phospholipid integrals (Eq. (4)).

$$n_X = (I_X / I_{IS}) \cdot (N_{IS} / N_X) \cdot (m_{IS} \cdot P_{IS} / M_{IS}) \tag{2}$$

$$w_X\ (\text{wt \%}) = 100 \cdot (I_X / I_{IS}) \cdot (N_{IS} / N_X) \cdot (M_X / M_{IS}) \cdot (m_{IS} / m_s) \cdot P_{IS} \tag{3}$$

$$x_X\ (\text{P-mol \%}) = 100 \cdot I_X / \Sigma\, I_i \tag{4}$$

The total phospholipid content can be expressed either as the sum of the mass fractions (which inherits every molar-mass assumption) or as the total phospholipid phosphorus per gram of sample (µmol g⁻¹), which is free of such assumptions. For finished products the result is converted to milligrams per serving by multiplication by the mean fill weight of the dosage unit and the number of units per serving (Eq. (5)). The molar distribution of molecules, as opposed to phosphorus atoms, differs from Eq. (4) only for cardiolipin and other multiply phosphorylated species, for which the integral is divided by the number of phosphorus atoms before normalization.

$$m_X\ (\text{mg per serving}) = (w_X / 100) \cdot \bar{m}_{fill} \cdot n_{units} \tag{5}$$

Three conventions embedded in these equations are sources of disagreement between laboratories and are discussed in detail in Sections 5 and 10: the molar mass assigned to each class (and therefore the basis of every weight-percent result), the treatment of unassigned resonances, and the integration method that defines I_X. The first two are matters of documented convention; the third is a measurement issue with a systematic component that can be several percent.

3.8 Field strength and benchtop instruments

The signal-to-noise ratio obtainable in a fixed time increases approximately as $B_0^{3/2}$, while the separation between two classes, expressed in hertz, increases linearly with B_0. For phospholipids in organic reagents at moderate field, where the natural line width is small and CSA broadening modest, the resolution between neighboring classes improves with field; in micellar systems the improvement saturates, as discussed in Section 3.3[16]. Benchtop spectrometers with permanent magnets of 1–2 T have been evaluated for phospholipid analysis by Gouilleux and co-workers, who recorded ³¹P spectra of a soybean lecithin at 17.6 MHz (1 T) and 32.6 MHz (2 T)[17]. In two-hour experiments the relative PC, PI and PE contents were obtained with precisions of 1.6–7.9 % (relative standard deviation); at 2 T the accuracies relative to an 11.7 T reference were 0.5 % for PC, 8.0 % for PI and 1.7 % for PE, whereas at 1 T the PE result was in error by 19 % because of overlap, and LPC, LPE and PA were not resolved at either field[17]. Absolute quantitation against an internal standard was achieved with a relaxation agent or with ¹H → ³¹P cross-polarization, with precisions of 2–5 %[17].

These observations have been used to construct Figure 2, in which the PS capsule blend described in Section 8.4 has been simulated at four field strengths with a line-width model that combines a

term proportional to the field (0.012 ppm, representing unresolved chemical-shift dispersion among molecular species) with a field-independent term (1.8 Hz); CSA relaxation, which grows as the square of the field, is not modeled. The composition is that obtained by routine window integration, which Section 8.6 shows to overstate the minor classes adjacent to PS. At 11.7 T and 7.05 T the PE, PS, PI and PC signals are baseline-resolved. At 80 MHz (1.88 T) PE is reduced to a shoulder on the PS line, the lyso-PC signals disappear into its wings and PI survives only as a broad minor peak, and at 60 MHz (1.41 T) only the PA signal and the PS-dominated phosphodiester envelope can be integrated with confidence. The simulation is not a substitute for measurement, but it supports the conclusion drawn from the published benchtop work: a benchtop instrument can verify the total phospholipid phosphorus and the PA or PC content of a simple lecithin, but it cannot deliver the class distribution of a PS product or resolve lyso-isomers, and it is unsuitable for release testing of PS or PC concentrates without a validated deconvolution model.

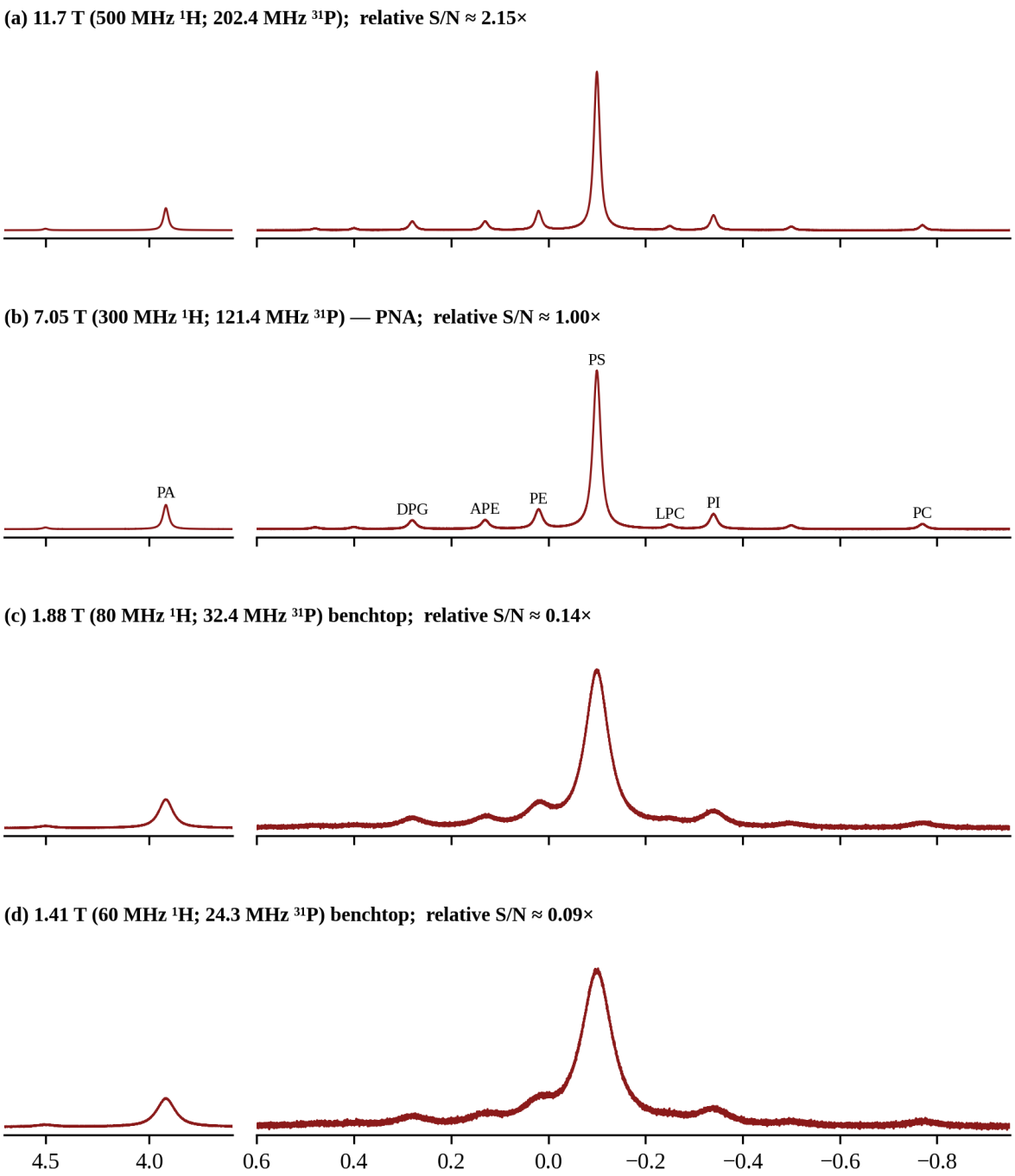


Figure 2. Synthesized $^{31}P\{^{1}H\}$ spectra of the genericized PS capsule blend (composition from Section 8.4) simulated at (a) 11.7 T, (b) 7.05 T (the field used at PNA), (c) 1.88 T and (d) 1.41 T. Left panels show the phosphomonoester (PA) region and right panels the phosphodiester region on a common ppm scale. Line widths were modeled as $\sqrt{[(0.012\ \text{ppm}\cdot\nu_0)^2 + (1.8\ \text{Hz})^2]}$ and noise was scaled as $B_0^{3/2}$ for equal acquisition time. Synthesized spectra; see Appendix A.

3.9 Two-dimensional ^{1}H–^{31}P methods for assignment

Because one-dimensional positions shift with the matrix, a number of groups have turned to heteronuclear correlation experiments to confirm assignments. In ^{1}H–^{31}P correlation spectra each phosphorus is connected to the protons of its glycerol backbone and head group through the three-bond couplings, and the resulting proton patterns are characteristic of the head group rather than of the medium. Petzold and co-workers used a semiconstant-time P,H-COSY to assign the phospholipids of *Helicobacter pylori* extracts[36]; Kaffarnik and co-workers used 2D ^{31}P,^{1}H spectra to identify ten phospholipids in cheese and nine in fish and found this step essential for correct assignment, while retaining the 1D spectrum for quantitation because transfer efficiencies in the 2D experiment differ by about ±10 % between classes[23]; Balsgart and co-workers developed a high-throughput 2D approach for complex mixtures[37]; and Gouilleux and co-workers demonstrated that a ^{1}H–^{31}P TOCSY can be run even on a benchtop instrument[17]. Bosco and co-workers had earlier applied gradient-enhanced ^{1}H-detected heteronuclear multiple-quantum experiments to lecithin phospholipids in organic solvent systems[38]. For a routine laboratory the most economical use of these techniques is not in every analysis but in the qualification of the assignment table for each new matrix type, together with spiking experiments using authentic standards, which Mayar and co-workers preferred as the pragmatic route to assignment[16].

4. Sample-Preparation Systems

4.1 What a quantitative sample must achieve

Every sample-preparation system for ^{31}P NMR of phospholipids has to accomplish five things at once. All phospholipid classes must be brought into a single measured phase without selective loss; divalent and paramagnetic cations must be removed from the phosphate groups; the phospholipids must be held in small, rapidly reorienting aggregates so that the lines are narrow; the pH and the counter-ion must be fixed so that the chemical shifts are reproducible from sample to sample; and the medium must be chemically benign, so that no hydrolysis or acyl migration occurs between preparation and measurement. The internal standard must, in addition, be dissolved in the same phase as the analytes. The systems in use differ mainly in how they balance the first requirement against the others, and in how much sample they can accommodate. Their characteristics are summarized in Table 4.

Table 4. Principal sample-preparation systems for ^{31}P NMR of phospholipids.

System	Typical composition and sample	Strengths	Limitations	References
Biphasic $CHCl_3$/MeOH/aqueous Cs-EDTA (Meneses–Glonek type; USP krill; PNA)	$CDCl_3$: MeOH (or CD_3OD) : 0.2 M Cs-EDTA ≈ 1:1:1; lower phase measured; 50–500 mg sample; pH 7.2–7.5 (literature) or 9.7 (PNA)	Extraction and measurement in one step; large samples; water-soluble P and insoluble excipients excluded; narrow lines; wide dispersion of diester classes	Partition of lyso and highly polar classes must be verified; lower-phase composition (oil load, water) shifts signals; PA/LPA shifts pH-sensitive	[11,12,14,23,24,30]
Monophasic (ternary) organic reagents	e.g. $CDCl_3$: MeOH : 0.2 M Cs-CDTA (pH 10) = 714 : 285 : 75 µL; ≤ 30–50 mg lipid	No partition losses; good resolution; suitable for extracted lipids and oils	Limited capacity; water-soluble matrix not removed; solvent ratio critical	[17,33,38–40]
Aqueous sodium cholate micelles	10 % cholate, 1 % EDTA, 20 % D_2O, pH 6.9–7.5; 15–100 mg; ultrasonication at 60 °C	Direct analysis of powders and liquids; SM/DHSM resolved; water-soluble P observed; high recovery of LPC and PS reported	PI close to PC; CSA broadening at high field; oil-rich samples need extraction	[3,4,10,16,25]
Aqueous deoxycholate–EDTA–TRIS, pH 9.0	350 mg oil extracted into 1 g detergent solution	Oils analyzed directly; PLC head groups, P_i and lyso isomers observed; validated for degumming	Slow saponification (≈ 0.05 % h^{-1}); acyl migration; long T_1 of head groups	[15]
Prior extraction (Folch, Bligh–Dyer, ethanol–hexane, repeated methanol) followed by one of the above	Matrix-dependent	Required for tissues, emulsions and low-phospholipid foods	Extraction dominates the uncertainty; lyso recovery matrix-dependent	[16,23,24,41]

4.2 Biphasic chloroform–methanol–aqueous cesium-EDTA reagents

The reagent introduced by Meneses and Glonek combines chloroform, methanol and an aqueous solution of the cesium salt of EDTA[11]. When the three are shaken with a lipid sample the mixture separates, as in a Bligh–Dyer partition, into a lower chloroform-rich phase containing the lipids and an upper methanol–water phase containing salts, sugars, amino acids, inorganic phosphate and other water-soluble material; proteins and insoluble excipients collect at the interface or sediment. EDTA removes calcium, magnesium and transition-metal ions from the phospholipid head groups, and cesium becomes the counter-ion of the anionic classes. Merchant and Glonek showed that the phospholipid resonances respond to the competition among Mg^{2+}, Ca^{2+}, Na^{+} and K^{+} for the head groups[30], which is the reason that the reagent supplies a single, large, weakly binding monovalent counter-ion in excess[11]. The lower phase retains enough methanol and water to solvate the head groups in small reverse-micellar aggregates, and Edzes and co-workers demonstrated how strongly the positions and resolution of the brain phospholipid classes depend on the chloroform–methanol–water ratio[33]. The reagent was developed further for tissue extracts by Glonek and Merchant[21] and for commercial lecithins by Diehl[12], and it underlies the automated lecithin and krill-oil method of Monakhova and Diehl and the krill-oil proficiency test organized by Zailer, Monakhova and Diehl[13,14].

The strength of the biphasic reagent is that extraction and measurement are combined in a single operation on a large sample. Several hundred milligrams of a lecithin, a softgel fill or a capsule blend can be taken, which reduces the weighing uncertainty and the sampling error for heterogeneous products, and water-soluble phosphorus compounds (inorganic phosphate, glycerophosphocholine, phosphorylated sugars) are removed with the upper phase, so that the measured spectrum contains only lipid phosphorus. The same feature is its principal weakness. The distribution of each phospholipid between the two phases depends on its polarity, its charge and the composition of the lower phase, and there is no a priori guarantee that all classes partition completely into the chloroform. Diacyl phospholipids with a cesium counter-ion partition strongly into chloroform, but the more polar lysophospholipids and the multiply charged species (LPA, lyso-PS, lyso-PI, glycerophosphodiesters) are less strongly retained. Ahmmed and co-workers compared a $CDCl_3$/CD_3OD/EDTA system with a D_2O/cholate/EDTA system on pure standards and reported recoveries in the chloroform system of 83 % for LPC and 86 % for PS when the standards were measured individually, falling to 73 % and 61 % in a mixture, whereas the corresponding recoveries in cholate were 96–109 %; they observed LPC, lysodiphosphatidylglycerol, cardiolipin, SM, lyso-PS, PI and PC in the methanol–water phase[25]. Mayar and co-workers found that egg-yolk phospholipids were recovered completely in the chloroform phase of a Folch partition, whereas a significant part of the lysophospholipids of enzyme-modified yolk ended up in the upper and interfacial phases; in mayonnaise containing 78 % oil the lysophospholipids were driven predominantly into the upper phase, which the authors attributed to the large amount of oil in the lower phase[16].

These findings do not establish that the cesium-EDTA reagent loses PS or lyso-PC in every application, since the conditions differed from those used in routine lecithin analysis (the counter-ion, pH, volume ratios and oil load all matter), but they do establish that partition completeness is a matrix-dependent property that must be demonstrated rather than assumed. The demonstration is simple in principle: the upper phase is collected, dried and redissolved in a cholate reagent, and its ^{31}P spectrum shows directly which classes, if any, have been lost; alternatively, standard additions of the classes of interest to the actual product matrix give recovery factors. This check is particularly important for oil-rich matrices (softgel fills, krill oils, fluid lecithins) and for lyso-rich materials (enzyme-modified lecithins and yolks, aged PS products).

The pH of the aqueous phase is the second critical variable. The seafood work of Bettjeman and co-workers, which followed the ^{31}P procedure of the USP krill-oil monograph, used 0.2 M EDTA adjusted to pH 7.2–7.5, at which PA and LPA resonate about 1.1 and 1.7 ppm downfield of PC, PA among the cardiolipin and PG signals and LPA just beyond them[24,42]. In the Cs-EDTA reagent of Kaffarnik and co-workers, PA and LPA were likewise recorded 1.07 ppm and 1.68 ppm downfield of PC[23]. The reagent used at PNA is adjusted to pH 9.7 with cesium carbonate, which converts PA and LPA predominantly to the dianions and moves them 4–5 ppm downfield of PC, into an otherwise empty region (Section 5). This is an advantage for PA-rich materials such as PS products, in which PA is the principal by-product, because it removes PA from the crowded phosphodiester region entirely. It has two costs. The PA and LPA positions still vary from sample to sample (by about 0.25 ppm for PA in the four analyses of Section 8), which shows that ionization and cation binding in the lower phase are not fully controlled; and the alkaline medium accelerates acyl migration in lysophospholipids, so that the ratio of lyso-isomers measured at pH 9.7 tends toward the equilibrium composition rather than reflecting the enzymatic origin of the lyso fraction[15,26].

4.3 Monophasic organic reagents

When the volumes of chloroform, methanol and aqueous salt solution are chosen so that the mixture remains homogeneous, the partition problem disappears, because every phosphorus-containing solute is in the measured phase. Branca and co-workers described a ternary homogeneous system for ^{31}P analysis of phospholipids[39], Bosco and co-workers compared organic solvent systems for lecithin phospholipids and used them for gradient-enhanced ^{1}H-detected heteronuclear experiments[38], and Murgia, Mele and Monduzzi applied a monophasic mixture to the quantitative characterization of milk-fat phospholipids[40]. The benchtop study of Gouilleux and co-workers used $CDCl_3$, methanol and 0.2 M aqueous cesium CDTA (trans-1,2-diaminocyclohexanetetraacetate, adjusted to pH 10 with CsOH) in the volume ratio 714 : 285 : 75, and emphasized that the ternary composition was a determining factor for resolution[17]. The price of a monophasic reagent is capacity: the amount of lipid that can be dissolved without disturbing the solvent composition, and therefore the shifts, is limited to a few tens of milligrams, and Kaffarnik and co-workers placed the practical limit at about 30 mg of lipid per tube[23]. Water-soluble matrix components are not removed, so a monophasic reagent is best suited to extracted

lipids, pure lecithins and oils, and poorly suited to finished products containing carbohydrate carriers or mineral excipients.

4.4 Aqueous detergent micelles: cholate and deoxycholate

The detergent approach, introduced by London and Feigenson and developed by Dennis and Plückthun, disperses the phospholipid in mixed micelles with a detergent in D_2O-containing buffer; nonionic detergents such as Triton X-100 were used in much of the early work, whereas the current food methods use bile salts, principally cholate and deoxycholate[10,27]. MacKenzie, Vyssotski and Nekrasov prepared samples in 10 % (w/w) sodium cholate and 1 % EDTA containing 20 % D_2O at pH 7.1, with phosphonomethylglycine as internal standard, and dispersed the sample by ultrasonication at 60 °C; spectra were acquired at 121.5 MHz and 30 °C with 192 transients, a 3.5 s delay and a 5.4 s acquisition time, for an analysis time of 36 min[3]. A notable advantage of this system was that high-phospholipid dairy materials (buttermilk protein concentrate powder, beta-serum liquid) could be analyzed directly without prior lipid extraction, and the SM and DHSM signals, which co-elute in most chromatographic systems, were resolved[3]. Kato and co-workers optimized a cholate–EDTA system with phosphoserine as an SI-traceable internal standard, controlled the pH to separate the maximum number of classes, verified the purity of their DOPC calibrant by ^{1}H qNMR against a certified reference material, and applied the method to soybean lecithins and PS supplements[4]. Van Rijn and co-workers adapted the approach to crude and degummed oils by extracting 350 mg of oil into an aqueous deoxycholate–EDTA solution buffered with TRIS at pH 9.0, a pH chosen as the compromise between the resolution of lysophosphatidylinositol from PE (which improved with pH) and the slow saponification of phospholipids (approximately 0.05 % h^{-1} at room temperature)[15]. Mayar and co-workers optimized the cholate system for egg yolk by factorial design, finding pH and sample concentration, but not temperature between 290 and 300 K, to be the significant factors for line width and signal separation[16].

The aqueous systems have three distinctive properties. They observe water-soluble phosphorus compounds (inorganic phosphate, glycerophosphocholine, glycerophosphoethanolamine and the choline, ethanolamine and inositol phosphates released by phospholipase C), which is essential in degumming studies but must be excluded when total phospholipid is reported[15,25]. They place PI close to PC ($\Delta\delta \approx 0.10$–$0.14$ ppm), much closer than in the organic reagents ($\Delta\delta \approx 0.43$–$0.51$ ppm), which makes the PI/PC pair the critical separation for soybean and sunflower lecithins. And they suffer the CSA line broadening discussed in Section 3.3, which limits the benefit of high-field instruments[16]. Ahmmed and co-workers found that cholate gave better resolution and more detected species than a $CDCl_3$-based system for soybean lecithin, krill oil and egg-yolk lipid, and recommended it as the preferred detection solvent[25]; their optimized conditions (pH 7.3, 192 scans and a 3.5 s relaxation delay) gave a run time of 16 min per sample.

4.5 Extraction before analysis: whole foods, emulsions and tissues

For materials in which phospholipid is a minor constituent, or in which it is bound in membranes or at emulsion interfaces, a separate extraction precedes the NMR measurement, and the extraction rather than the spectroscopy becomes the dominant source of error. Bettjeman and co-workers used ^{31}P NMR to compare the Folch, Bligh–Dyer and Jensen extractions of five seafoods and found that the Folch method extracted the most oil and the most phospholipid from every tissue; because the differences between methods were larger for phospholipid than for total oil, inefficient phospholipid extraction was identified as the cause of the lower oil yields[24]. Mayar and co-workers showed for mayonnaise that direct cholate solubilization of the freeze-dried emulsion recovered only 56–74 % of the phospholipids, that a Folch partition recovered lysophospholipids only when both phases were analyzed, and that four sequential methanol extractions followed by solubilization in cholate recovered 100–108 % of the phospholipids and 96–104 % of the lysophospholipids[16]. Ahmmed and co-workers developed a one-pot ethanol–hexane extraction for phospholipidomic profiling of total oil and lecithin[41], and Kaffarnik and co-workers enriched the phospholipids of cheese fat and fish oil by liquid–liquid partition between hexane and aqueous methanol before 1D and 2D analysis[23]. None of these extractions is universal, and each must be validated for recovery in the matrix to which it is applied.

4.6 Comparative chemical shifts and the pH dependence of PA and LPA

The positions of the principal classes relative to PC in the seven systems considered in this paper are collected in Table 5 and plotted in Figure 3. Three features stand out. First, the order of the phosphodiester classes is broadly conserved (PC most shielded, followed by PI, the lyso-PC isomers and PS, then SM and PE, then DHSM, cardiolipin, LPE and PG), but the spacings differ by a factor of three or more between the organic and aqueous systems; PI in particular moves from 0.43–0.51 ppm above PC in the organic reagents to 0.10–0.14 ppm above it in cholate or deoxycholate. Second, the relative order of adjacent classes is not conserved: SM lies upfield of PE in the Cs-EDTA reagents but downfield of it in the cholate systems, PS and LPC likewise exchange positions between the two families of reagent, and PE and its ether analogue have been observed in either order within a single system[3,16,23]. Third, PA and LPA behave as a separate family whose position is governed by their ionization state, which depends on both the pH and the medium: they lie 1.1–1.7 ppm from PC in the organic Cs-EDTA reagents at pH 7.2–7.5, but 3.9–5.3 ppm from it in the alkaline PNA reagent and in the cholate and deoxycholate micelles, including the cholate system of Kato and co-workers at pH 6.9. Ahmmed and co-workers observed the PA resonance in cholate move from about 2.5 to 3.9 ppm as the pH was raised from 7.4 to 12[25], and van Rijn and co-workers mapped the pH dependence of all relevant classes between pH 7.2 and 10 before selecting pH 9.0[15].

Table 5. Chemical shifts of phospholipid classes relative to PC (Δδ = δ(class) − δ(PC), ppm) in seven sample-preparation systems.

Class	PNA, Cs-EDTA pH 9.7	Kaffarnik 2013, Cs-EDTA	Bettjeman 2018, Cs-EDTA pH 7.2–7.5	MacKenzie 2009, cholate pH 7.1	Kato 2018, cholate pH 6.9	Mayar 2020, cholate pH 7.5	van Rijn 2020, deoxycholate pH 9.0
PI	0.43	0.48	0.51	0.14	0.14	0.10	0.14
LPC (major)	0.52	0.56	0.58	0.45	—	0.53	0.45
PS	0.67	—	0.74	0.36	0.33	—	—
SM	—	0.77	0.66	0.61	0.62	0.61	—
PE	0.79	0.88	0.88	0.56	0.56	0.56	0.59
DHSM	—	0.98	0.98	0.70	0.72	—	—
CL (DPG)	1.05	0.97	1.05	—	—	—	—
LPE	1.17	1.30	—	0.99	0.87	1.09	1.01
PG	1.25	1.31	1.39	—	1.07	—	—
PA	4.69	1.07	1.14	—	3.92	—	4.45
LPA	5.27	1.68	1.72	—	4.54	—	4.89

PNA values are typical positions from the four datasets of Section 8 (nominal scale of Appendix A). Literature values were converted from the published tables[3,4,15,16,23,24]; — indicates not reported. LPC (major) is the 1-acyl isomer.

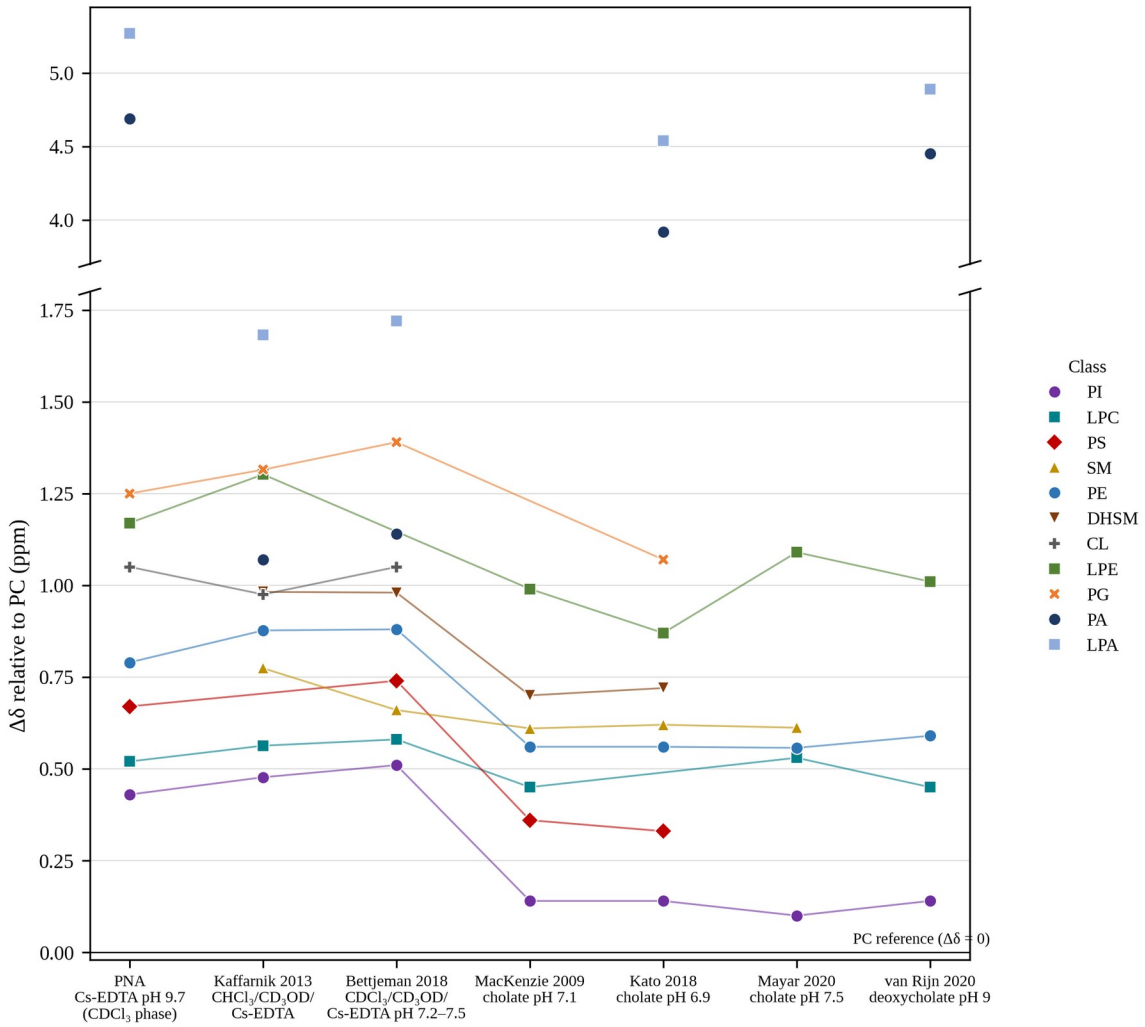


Figure 3. Chemical shifts of the principal phospholipid classes relative to PC (Δδ = δ(class) − δ(PC)) in seven sample-preparation systems. The upper panel shows the phosphomonoester classes PA and LPA, whose positions are governed by pH; the lower panel shows the phosphodiester classes. PNA values are typical positions observed in the four datasets of Section 8 on the routine PNA scale. Literature values are from the sources indicated and were converted to Δδ from the published tables.

The practical lesson is that shift tables cannot be transferred between systems, and that even within one system they should be regarded as starting points to be confirmed for each matrix by standard addition or by 2D correlation. A shift table that lists PA at 1.5 ppm and LPA at 2.8 ppm, for example, matches none of the systems in Table 5 and would misassign both signals if it were applied to spectra recorded after equilibration at pH 9.7.

4.7 Selecting a system

The choice of system is governed by the matrix and by the question. For lecithins, PC and PS concentrates, softgel fills and capsule blends intended for release testing, the biphasic cesium-EDTA reagent is attractive because it tolerates large samples, removes water-soluble excipients and gives the widest dispersion of the phosphodiester classes; its partition behavior for lyso and highly polar classes must, however, be verified in each product type. For dairy powders and

liquids, for which direct analysis without extraction is valuable and SM/DHSM resolution is required, the cholate system is preferred[3]. For crude and degummed oils in which the products of phospholipase C and A must be followed together with the intact phospholipids, the buffered deoxycholate system, validated for oils, is the appropriate choice because it observes the water-soluble head groups together with the intact phospholipids[15]. For emulsions and tissues, a validated extraction must precede either reagent[16,24]. Wherever the lyso-isomer ratio is itself the analytical question, as in distinguishing phospholipase A_1 from A_2 products, the preparation should be conducted at the lowest practicable pH and temperature and measured promptly, and the isomer ratio should be interpreted with the acyl-migration equilibrium in mind[15,26].

5. The Current PNA Method

5.1 Scope and principle

The procedure applied at PNA is a biphasic cesium-EDTA method of the Meneses–Glonek type, adapted for large samples of lecithins, phospholipid concentrates and finished products and run at an alkaline pH. It is used to determine the class distribution of phospholipid phosphorus and the absolute content of each class in vegetable lecithins, PS and PC powders, softgel fills, capsule blends and krill oils. A weighed portion of the sample and a weighed amount of triphenyl phosphate are partitioned between deuterochloroform, methanol and aqueous cesium-EDTA; the lower chloroform-rich phase, which contains the phospholipids as cesium salts together with the neutral lipids and the internal standard, is filtered into an NMR tube and measured at 121.4 MHz with inverse-gated proton decoupling. Integrals are referenced to the standard, and the results are calculated with Eq. (3), Eq. (4) and Eq. (5). The chemical shifts used for assignment were originally taken from published tables, in particular those of the automated lecithin and krill-oil analysis of Monakhova and Diehl and the krill-oil proficiency test of Zailer and co-workers[13,14], and the procedure is related to the ^{31}P procedure of the USP krill-oil monograph[42]. The description that follows is based on the reports and spectrometer parameter files of four analyses performed in 2026 (Section 8); where the parameters actually recorded differ from nominal values, the recorded values are given.

5.2 Reagents, standard and instrument

The internal standard is triphenyl phosphate of 99.95 % certified purity supplied as a qNMR reference material (CAS 115-86-6), with a molar mass of 326.28 g mol^{-1} and one phosphorus atom per molecule. Deuterochloroform (99.8 atom % D, containing 0.03 % v/v tetramethylsilane) provides the field-frequency lock; methanol of analytical grade and deionized water are used without deuteration. The aqueous reagent is 0.2 M EDTA brought to pH 9.7 with cesium carbonate; at this pH EDTA is present predominantly as HY^{3-} with a minor fraction of Y^{4-}, so the reagent carries roughly 0.6 M cesium, an excess of about 2.5- to 11-fold over the estimated anionic phospholipid charge of the samples considered here. It should be noted that 1 mL of the reagent contains 0.2 mmol of EDTA and can therefore complex at most about 8.0 mg of calcium or 4.9 mg of magnesium; this capacity is ample for lecithins and most concentrates but can be exceeded by finished products formulated with mineral salts (Section 5.8). Spectra are acquired on a Varian Mercury-300 spectrometer (7.05 T) equipped with a 5 mm broadband (ATB) probe, at a ^{31}P frequency of 121.44 MHz with ^{1}H decoupling at 299.99 MHz.

5.3 Sample preparation

The sample and the internal standard are weighed into a glass vial. In the four analyses considered here, sample masses of 302.66–475.10 mg and TPP masses of 5.32–12.04 mg were used, corresponding to TPP:sample mass ratios of 1:57 to 1:39 and to TPP:phospholipid-phosphorus molar ratios of about 1:17 (lecithin and PS powder), 1:4 (softgel fill) and 1:3 (capsule blend). For

finished products the contents of the dosage units are removed and the mean fill weight is recorded, since it is required for conversion to milligrams per serving (Eq. (5)); a composite of the contents of several units is the appropriate test portion for capsule blends, whose homogeneity cannot be assumed. One milliliter of $CDCl_3$ is added and the vial is shaken for 2 min, after which 1 mL of methanol is added with a further 2 min of agitation, followed by 1 mL of the 0.2 M Cs-EDTA reagent and a final 2 min of agitation. The mixture is centrifuged to separate the chloroform layer from the upper aqueous–methanolic phase and from suspended solids derived from carriers, capsule contents or other product ingredients. After settling, the lower organic layer is drawn into a syringe, passed through a syringe filter and transferred to a 5 mm NMR tube.

The order of addition has a purpose. The sample is first dispersed in chloroform, in which phospholipids, neutral lipids and the standard dissolve; the addition of methanol then produces a single-phase solvent that wets and penetrates solid particles; and the aqueous reagent, added last, both exchanges the counter-ions and induces the phase separation. The 1:1:1 volume ratio places the system in the biphasic region of the chloroform–methanol–water diagram, close to the final composition of a Bligh–Dyer extraction. The lower phase therefore contains, in addition to chloroform, a few percent of methanol and a small amount of water, which solvate the head groups and set the chemical shifts; any change in the volume ratio, in the water content of the sample (for example a wet gum) or in the amount of oil carried into the lower phase alters that composition and therefore the positions of the signals[31,33].

5.4 Acquisition

The acquisition parameters recorded in the spectrometer files of the four analyses are listed in Table 6. A standard single-pulse sequence (s2pul) is used with the decoupler gated off during the relaxation delay and on during acquisition, with WALTZ-16 modulation, which is the inverse-gated scheme described in Section 3.5. The spectral width is 6997.9 Hz (57.6 ppm), sufficient to include the TPP resonance at −17.8 ppm and the phosphomonoester region to beyond +5 ppm with a margin for digital-filter roll-off; 48 000 complex points are acquired over 6.86 s, and a relaxation delay of 15 s gives a repetition time of 21.86 s. The temperature is regulated at 30 °C and the sample is spun (20 Hz in the recorded parameter set). Between 156 and 680 scans were accumulated in the four analyses, according to the phospholipid concentration and the signal-to-noise ratio required, giving experiment times from 57 min to 4.1 h. Before Fourier transformation the free-induction decay is zero-filled to 262 144 points and multiplied by an exponential function (0.8 Hz line broadening in the stored parameter set).

Table 6. Acquisition parameters recorded in the spectrometer files of the four PNA analyses discussed in Section 8.

Parameter	Sunflower lecithin	PS-enriched sunflower powder	PS softgel fill	PS capsule blend
Spectrometer; probe	Varian Mercury-300 (7.05 T); 5 mm ATB	same	same	same
^{31}P / ^{1}H frequency (MHz)	121.435 / 299.987	same	same	same
Pulse sequence; decoupling	s2pul; inverse-gated ^{1}H (WALTZ-16)	same	same	same
Temperature (°C)	30	30	30	30
Spectral width (Hz)	6997.9	6997.9	6997.9	6997.9
Complex points acquired	48 000	48 000	48 000	48 000
Acquisition time (s)	6.859	6.859	6.859	6.859
Relaxation delay (s)	15	15	15	15
Repetition time (s)	21.86	21.86	21.86	21.86
Pulse width (µs); nominal flip angle*	20; ≈ 144°	20; ≈ 144°	12; ≈ 86°	11; ≈ 79°
Number of scans	160	604	156	680
Experiment time	58 min	3.7 h	57 min	4.1 h
Receiver gain	28	28	26	38
Processed size (points)	262 144	262 144	262 144	262 144
Sample mass (mg)	338.75	302.66	475.10	343.90
TPP mass (mg)	5.93	5.32	12.04	6.02

*Calculated from the 90° pulse width of 12.5 µs stored in the capsule-blend parameter set; the calibration applicable to the other analyses was not recorded in the material examined.

Two features of Table 6 deserve comment. First, the repetition time of 21.86 s is long compared with the T_1 values of 0.6–1.7 s reported for phospholipids in micellar systems and with the 3.6 s reported for the phosphate-triester standard triisopropyl phosphate[4,15]; if the T_1 of TPP in the PNA reagent is of the same order, relaxation losses are below 0.3 % for a 90° pulse. Because the repetition time is fixed, however, the bias grows rapidly if the T_1 of TPP is longer. For a 90° pulse the TPP signal would be under-recovered, and every absolute result biased high, by 1.3 % at T_1 = 5 s, 4.6 % at 7 s and 16 % at 11 s; for a 144° pulse the corresponding biases are 2.3 %, 8.3 % and 29 %. Phosphate triesters can relax slowly in organic media (tributyl phosphate had a T_1 of 14 s in a $CDCl_3$/methanol reagent[17]), and T_1 values transferred from other media can be wrong by a factor of three (Section 3.4). The T_1 of TPP has not been measured in the PNA reagent, and its measurement is the most important single check of the trueness of the absolute results (Section 5.8). Second, the recorded pulse widths ranged from 11 to 20 µs. The parameter set of the capsule-blend analysis stores a 90° pulse width of 12.5 µs at the transmitter power used, against which 11 µs, 12 µs and 20 µs correspond to nominal flip angles of about 79°, 86° and 144°. If that calibration applied to all four analyses, the two lecithin-type samples were excited with pulses well beyond 90°, which lowers the signal per scan by about 40 % and lengthens the recovery needed for full

relaxation, although with a 21.86 s repetition time the effect on the integral ratios is still expected to be small for species with T_1 below about 3.5 s (Eq. (1)). The pulse width should be calibrated for each probe tuning and fixed in the method.

5.5 Processing, referencing and integration

The transformed spectrum is phased and baseline-corrected, and the chemical-shift scale is set so that the TPP resonance lies at −17.80 ppm. Integral regions are then defined across the phospholipid region, thirteen to sixteen of them in the four analyses in addition to the TPP region, each labeled with a class or with a question mark when no assignment is made, and the TPP integral is normalized to 100. The integral table, with the region limits, the normalized integrals and the absolute integrals, is transferred to a calculation workbook. The windows used in the four analyses are listed in Table 7.

Table 7. Integration windows (ppm, routine PNA scale) used in the four analyses.

Window label	Sunflower lecithin	PS-enriched sunflower powder	PS softgel fill	PS capsule blend
LPA	5.09 … 4.94	5.23 … 4.89	4.58 … 4.42	4.61 … 4.34
? (monoester)	4.66 … 4.48	4.65 … 4.38	—	—
PA	4.10 … 3.66	4.08 … 3.45	4.08 … 3.83	3.98 … 3.47
? (3 ppm region)	—	3.26 … 2.65	—	—
? (downfield diesters)	1.15 … 0.56	1.14 … 0.53	0.74 … 0.37	1.17 … 0.61
PG	0.56 … 0.46	0.53 … 0.40	0.37 … 0.31	0.61 … 0.52
LPE	0.46 … 0.34	0.40 … 0.35	0.31 … 0.23	0.52 … 0.46
DPG	0.34 … 0.23	0.35 … 0.25	0.23 … 0.14 [a]	0.47 … 0.29
? (between DPG and PE)	0.23 … 0.16	0.25 … 0.13	—	0.13 … 0.03
APE	0.16 … 0.10	0.13 … 0.01	0.14 … 0.07	0.29 … 0.13
PE	0.10 … −0.04	0.01 … −0.06	0.06 … −0.03	0.03 … −0.03
PS	−0.04 … −0.10	−0.06 … −0.21	−0.03 … −0.27	−0.03 … −0.20
2-LPC	−0.10 … −0.30	−0.21 … −0.25	−0.27 … −0.36	−0.20 … −0.23
PI	−0.30 … −0.42	−0.25 … −0.40	−0.36 … −0.47	−0.23 … −0.33
1-LPC	−0.42 … −0.62	−0.40 … −0.60	−0.47 … −0.66	−0.33 … −0.40
PC	−0.62 … −1.14	−0.60 … −0.93	−0.74 … −0.81	−0.69 … −0.81
TPP (= 100)	−17.54 … −18.06	−17.70 … −17.90	−17.66 … −18.00	−17.51 … −18.23

[a] Labeled "?" in the integral table but calculated and reported as DPG. — indicates that no window was defined.

The windows are placed by the analyst for each spectrum, around the observed peaks, and Table 7 shows how much they vary. The PS window was 0.06 ppm wide in the sunflower lecithin, where PS is minor, and 0.15–0.24 ppm wide in the three PS products; the TPP window ranged from 0.20 to 0.72 ppm; and the windows labeled 2-LPC and 1-LPC were placed wherever the corresponding intervals between PS, PI and PC fell in each spectrum. Because a Lorentzian line of full width at

half-height W places only the fraction (2/π)·arctan(2Δ/W) of its area within a symmetric window of half-width Δ (Eq. (6)), a window of ±5W captures 93.7 % of the line, ±10W 96.8 %, ±20W 98.4 % and ±50W 99.4 %. For the TPP line of about 1.3 Hz measured in the capsule-blend spectrum (Section 5.7), the 0.20 ppm window used for the PS powder would capture 96.6 % of the standard and the 0.72 ppm window used for the capsule blend 99.0 %, so that the choice of TPP window alone moves every absolute result by about 2.6 %. The same arithmetic applies, with larger consequences, to the broader and more crowded analyte lines, and it is examined quantitatively in Section 8.6.

$$f(\Delta) = (2/\pi) \cdot \arctan(2\Delta/W) \tag{6}$$

5.6 Calculation and reporting conventions

The workbook computes, for each window, the normalized integral, the phosphorus mole percentage (Eq. (4), reported as "mole %"), the weight percentage in the sample (Eq. (3)) and, for finished products, the milligrams per serving (Eq. (5)). The molar masses are those of Table 1. Resonances that are not assigned are summed and reported as "other phospholipid" with a nominal molar mass of 770 g mol^{-1}. Cardiolipin (DPG) is calculated per phosphorus atom. The total phospholipid content is reported as the sum of the class weight percentages. The report states the principal class, its position, its weight percentage, the total phospholipid content and, where a specification has been provided, whether the result meets it.

Several conventions implicit in this scheme should be stated explicitly on every report, because they change the numbers. The molar mass basis of each class (the tabulated values correspond approximately to C_{18} species in the free-acid or zwitterionic form), the treatment of unassigned signals, the per-phosphorus treatment of cardiolipin, and the distinction between "mole %" of phospholipid phosphorus and weight percent of the sample are the most important. A PS content of "32 %" can describe either basis for the same material (Section 8.2), and the two differ by a factor that depends on the total phospholipid content and on the mean molar mass of the other classes.

5.7 Observations from four recent datasets

The four datasets reproduced in Section 8 provide a realistic picture of how the method behaves across matrices. The line widths are narrow: in the re-processed capsule-blend spectrum the TPP resonance had a full width at half-height of 1.3 Hz, PC 2.1 Hz, PE 2.7 Hz, PS 3.4 Hz and PA 5.0 Hz, and the ratio of the TPP peak height to the root-mean-square noise was about 1900 after 680 scans. The broader PS and PA lines are consistent with the greater sensitivity of the anionic classes to residual cation binding, exchange and aggregation, and the PA line may be broadened further by heterogeneity of ionization and cation binding among the PA molecules in the lower phase.

The positions of the classes, by contrast, move appreciably between samples. On the routine scale PS was observed between about −0.07 and −0.13 ppm, PE between −0.03 and +0.02 ppm and PC between −0.72 and −0.78 ppm, but PI appeared at −0.35 ppm in the sunflower lecithin, near −0.30 ppm in the PS-enriched sunflower powder, near −0.42 ppm in the softgel fill and near −0.27 ppm in

the capsule blend. PA moved between about 3.73 and 3.97 ppm. These displacements, of up to 0.15 ppm relative to PS for PI, are of the same size as the matrix effects reported by Kaffarnik and co-workers[23], and they arise from differences in the composition of the lower phase: the amount of oil and other lipid carried into it, its water and methanol content, and the final pH of the aqueous phase, which is set by the limited buffer capacity of the reagent and can be pulled down by acidic sample components. They are the reason the windows must be adjusted by the analyst, and they are also the reason that fixed shift tables can mislead. The shift table printed in the routine report template lists PA at 1.50 ppm and LPA at 2.78 ppm, positions that match neither the PNA reagent nor any of the literature systems compared in Section 4.6; in the alkaline PNA reagent PA is observed near 3.7–4.0 ppm and the signals labeled LPA near 4.5 or 5.0 ppm, and the template table should be replaced by one measured in the PNA reagent itself.

Two further observations bear on the assignment table. In the two sunflower materials, two minor phosphomonoester resonances were present, near 4.5 and 5.0 ppm; the 5.0 ppm signal was labeled LPA and the 4.5 ppm signal left unassigned, whereas in the two PS products a single signal near 4.5 ppm was labeled LPA. Both signals are plausibly lysophosphatidic acid or other phosphomonoesters (the two acyl-positional isomers of LPA, for example), but the assignments have not been confirmed with authentic standards in this reagent and should be harmonized. Similarly, the lyso-PC labels follow the convention described in Section 2.3, in which the major isomer between PS and PI is called 2-LPC; spiking with 1-acyl-2-lyso-PC (the common commercial form) would confirm the assignment and allow the unambiguous names to be adopted in reports. Finally, when the raw free-induction decay of the capsule blend was re-processed with the TPP resonance set exactly at −17.80 ppm, the routine integration windows reproduced the reported integrals only when the whole scale was displaced by −0.066 ppm (the root-mean-square difference across fourteen windows fell from 6.1 to 0.43 integral units), which implies that on the routine scale the TPP resonance lay near −17.87 ppm rather than at the stated −17.80 ppm. An offset of this size is harmless for integration, because the windows are set on the displayed spectrum, but it is not harmless for comparison with shift tables or for automated processing, and it explains part of the apparent disagreement between the tabulated and observed positions.

5.8 Refinements for operation under ISO/IEC 17025

The method as described is fit for its principal purpose, which is the class distribution and PS or PC content of lecithins and phospholipid products, but a number of refinements would strengthen it considerably as it is brought under an ISO/IEC 17025 quality system, in which the validation of non-standard methods, the evaluation of measurement uncertainty and the statement of decision rules are all explicit requirements[18]. They are set out here in the order in which an assessor would be likely to raise them.

The method description and the report template should be generated from, or checked against, the acquisition parameters actually recorded in the spectrometer files, so that the spectral width, acquisition time, relaxation delay, pulse width and number of scans stated in a report are always those used. The internal standard should be named as triphenyl phosphate with its CAS number

throughout, and the label "PPh_3", which denotes triphenylphosphine, should be removed from templates and spectrum annotations. The shift table should be replaced by one measured in the PNA reagent at pH 9.7, with ranges rather than single values, and every assignment in it should be confirmed by standard addition of authentic material; lyso-PS, lyso-PI and the two LPA isomers are the most important additions for PS products.

The ^{31}P 90° pulse should be calibrated for each probe tuning (or fixed at a verified value for a standard sample volume), and T_1 should be measured by inversion recovery for TPP and for the principal classes in representative matrices, so that the repetition time can be justified as at least seven times the longest T_1 of any quantified species or, alternatively, the flip angle reduced. The spectrum should be referenced so that TPP lies at exactly −17.80 ppm, and the integration windows should be defined by a written rule (for example, a minimum half-width expressed in multiples of the observed line width, with a common, wide window for TPP), or the overlapped PS–lyso-PC–PI region should be integrated by line fitting with a documented line-shape and baseline model (Section 8.6). The mass of TPP should be increased to at least 15 mg, or a gravimetrically prepared stock solution used, so that its weighing uncertainty falls to about 0.1 % or less.

The completeness of partition into the lower phase should be demonstrated for each product type by analyzing the dried upper phase in a cholate reagent and by standard additions of PS, LPC and PA to representative matrices (lecithin, PS powder, oil-based softgel fill, capsule blend), since the published evidence summarized in Section 4.2 shows that lysophospholipids and PS can be partially lost in chloroform–methanol–water partitions, particularly when the lower phase carries a large load of oil[16,25]. The EDTA capacity of the reagent should be compared with the calcium and magnesium content of mineral-containing formulations, and the reagent volume or concentration increased where necessary. The stability of the prepared sample should be checked by repeating the measurement after 24 h, since the reagent is alkaline and the longest acquisitions exceed 4 h.

Duplicate preparations should be analyzed routinely for release testing, and an in-house reference lecithin and an in-house PS reference material should be analyzed with each batch and plotted on control charts. Participation in interlaboratory comparisons, such as the krill-oil proficiency scheme described by Zailer and co-workers[14], and comparison against commercially certified lecithin standards of the type used by MacKenzie and co-workers[3], would provide external evidence of trueness. The calculation workbook should be validated as software, with locked formulas, an independent check of every cell reference against the equations of Section 3.7 and a record of the molar-mass table used. Finally, the measurement uncertainty should be evaluated (Section 10.2) and a decision rule agreed with each client before results close to specification limits are reported, as ISO/IEC 17025 requires[18].

6. Phospholipid Sources and Their ^{31}P Profiles

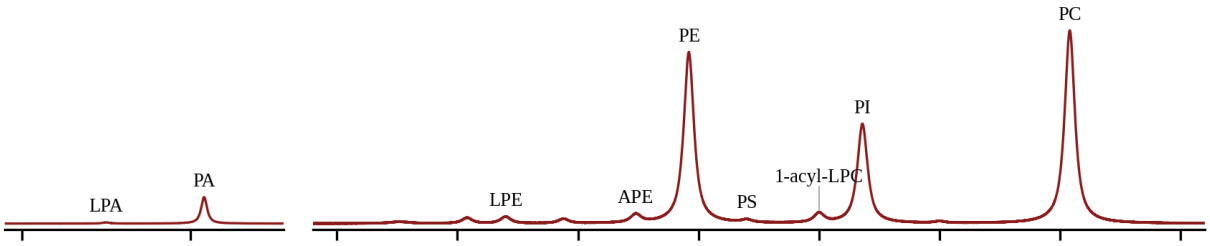
6.1 Soybean lecithin

Soybean lecithin remains the reference material of the industry. Crude soybean oil contains roughly 1.5–3.0 % phospholipid[2], most of which is recovered as wet gums by water degumming and dried to a fluid lecithin containing about 62 % acetone-insoluble matter, the balance being soybean oil, free fatty acids, sterols, glycolipids and carbohydrates[1,43]. Deoiling with acetone raises the acetone-insoluble fraction to 95 % or more and yields powders and granules whose phospholipid content is typically 65–75 % on a dry basis[2], the remainder being mainly glycolipids and oligosaccharides that are acetone-insoluble but invisible to ^{31}P NMR. The discrepancy between acetone insolubles and ^{31}P phospholipid is itself a useful quality indicator, since acetone insolubles are a gravimetric, non-specific measure.

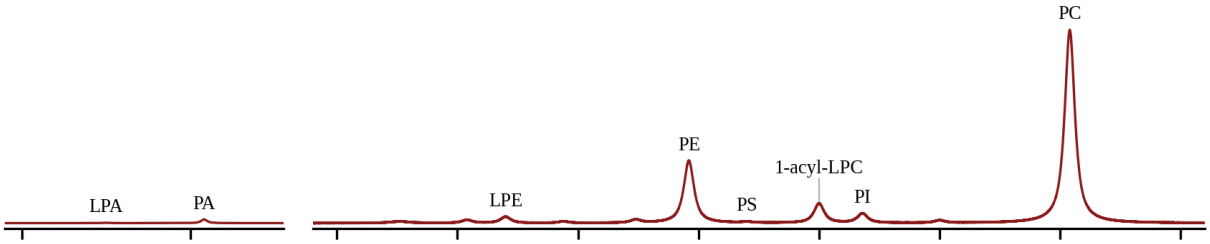
In ^{31}P terms a native soybean lecithin is characterized by PC and PE of comparable size, each about one third of the phospholipid phosphorus, by PI at 12–18 % and by PA at 2–12 %, with lyso-PC, lyso-PE, N-acyl-PE, PG, cardiolipin and a trace of PS making up the remainder. This pattern is seen consistently across the literature despite the differences in preparation system: MacKenzie and co-workers analyzed a certified crude soybean lecithin standard in cholate[3], Kato and co-workers a soybean polar-lipid extract[4], Gouilleux and co-workers a certified α-lecithin in a monophasic Cs-CDTA reagent[17], and Ahmmed and co-workers a commercial fluid soybean lecithin in both cholate and $CDCl_3$[25]. Diehl reviewed the application of high-resolution NMR, including ^{31}P, to commercial lecithins[12], and Yang, Hiserodt and Li described a rapid ^{31}P procedure for the identification and relative quantification of the phospholipids in commercial lecithins[44]. Helmerich and Koehler compared ^{31}P NMR with chromatographic methods for lecithins and flour improvers and showed how strongly the chromatographic results depend on calibration[5].

The PE:PC ratio and the PI content of a lecithin are fixed largely by the seed, whereas PA and the lyso species reflect its history. Phosphatidic acid is present in the seed but is also formed by endogenous phospholipase D during seed storage, conditioning and flaking, particularly when damaged or moist seed is processed, and its calcium and magnesium salts make up much of the non-hydratable fraction that remains in the oil after water degumming[1,45]. Lyso species are formed by phospholipase A activity in the seed and by chemical hydrolysis during drying and storage of wet gums. A high PA fraction in a lecithin therefore signals seed quality or process problems, and a rising LPC fraction in a stored lecithin signals hydrolysis. Synthesized spectra representing a fluid soybean lecithin and four products derived from it are shown in Figure 4; the compositions are representative values chosen to illustrate the product types discussed in Section 7 and are not measurements.

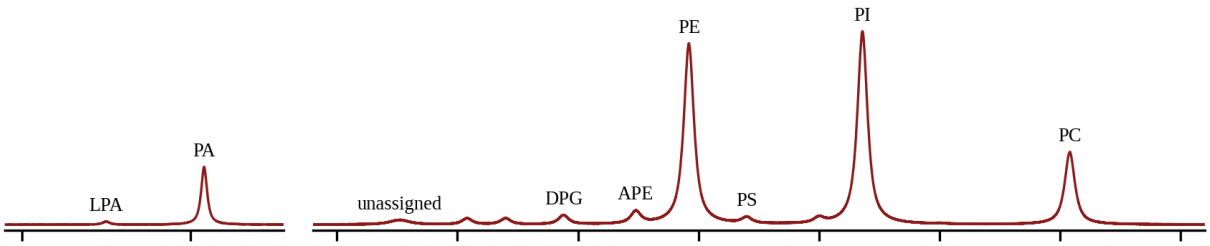
(a) Fluid soybean lecithin



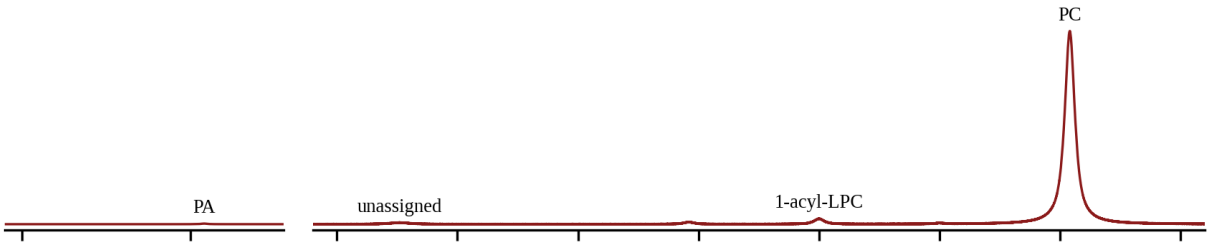
(b) PC-enriched (ethanol-soluble) fraction



(c) PI/PE-enriched (ethanol-insoluble) fraction



(d) Chromatographic high-purity PC



(e) PLA$_2$-hydrolyzed soybean lecithin

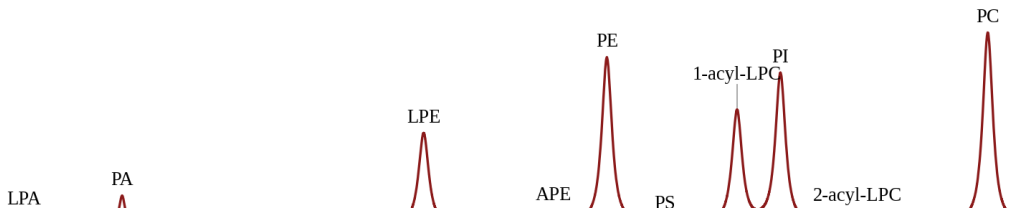


Figure 4. Synthesized $^{31}P\{^{1}H\}$ spectra (121.4 MHz, positions of the routine PNA Cs-EDTA scale) of (a) a fluid soybean lecithin, (b) a PC-enriched ethanol-soluble fraction, (c) the complementary PI/PE-enriched ethanol-insoluble fraction, (d) a chromatographically purified high-PC product and (e) a phospholipase A_2-hydrolyzed lecithin. Left panels show the phosphomonoester region (PA, LPA) and right panels the phosphodiester region. Compositions are representative and are listed in Appendix A; 1-acyl-LPC and 2-acyl-LPC denote the two lyso-PC isomers. Synthesized spectra.

6.2 Sunflower lecithin

Sunflower lecithin has become the principal alternative to soybean lecithin where a non-genetically-modified or allergen-free label is required, and its price premium creates an incentive for adulteration with soybean lecithin. Its phospholipid profile differs from that of soybean in a way that ^{31}P NMR resolves directly: PE is markedly lower, PI higher, and PC is the largest single class. Monakhova and Diehl exploited these differences, together with finer spectral features, to build PLS regression models that estimate the proportion of soybean lecithin in sunflower lecithin from NMR spectra, and they subsequently showed that such models can be transferred between spectrometers[46,47].

A sunflower lecithin analyzed by the PNA method is shown in Figure 5 and Figure 6. The phosphodiester region is dominated by PC (37.1 % of phospholipid phosphorus), PI (25.9 %) and PE (15.7 %), with PA (5.4 %) clearly separated at 3.95 ppm; lyso-PC accounts for 7.6 % of the phosphorus, and cardiolipin, N-acyl-PE, lyso-PE, PS, PG and LPA are each present at 0.3–1.6 %. The total phospholipid content was 69.8 % by weight (926 µmol of phospholipid phosphorus per gram), and the PC content 26.5 %. The PE/PC ratio of 0.42 on a phosphorus basis compares with values of 0.9–1.1 for the soybean lecithins cited in Section 6.1, which illustrates why the PE/PC and PI/PC ratios are the first-line indicators of the botanical origin of a lecithin. The relatively high lyso-PC fraction suggests that this material had experienced some hydrolysis. The shoulder on the low-field side of the PI signal and the 0.20 ppm width of the window labeled 2-LPC in this spectrum (Table 7) mean, however, that part of the lyso-PC integral may belong to the wing of the PI line; this is discussed further in Section 8.1.

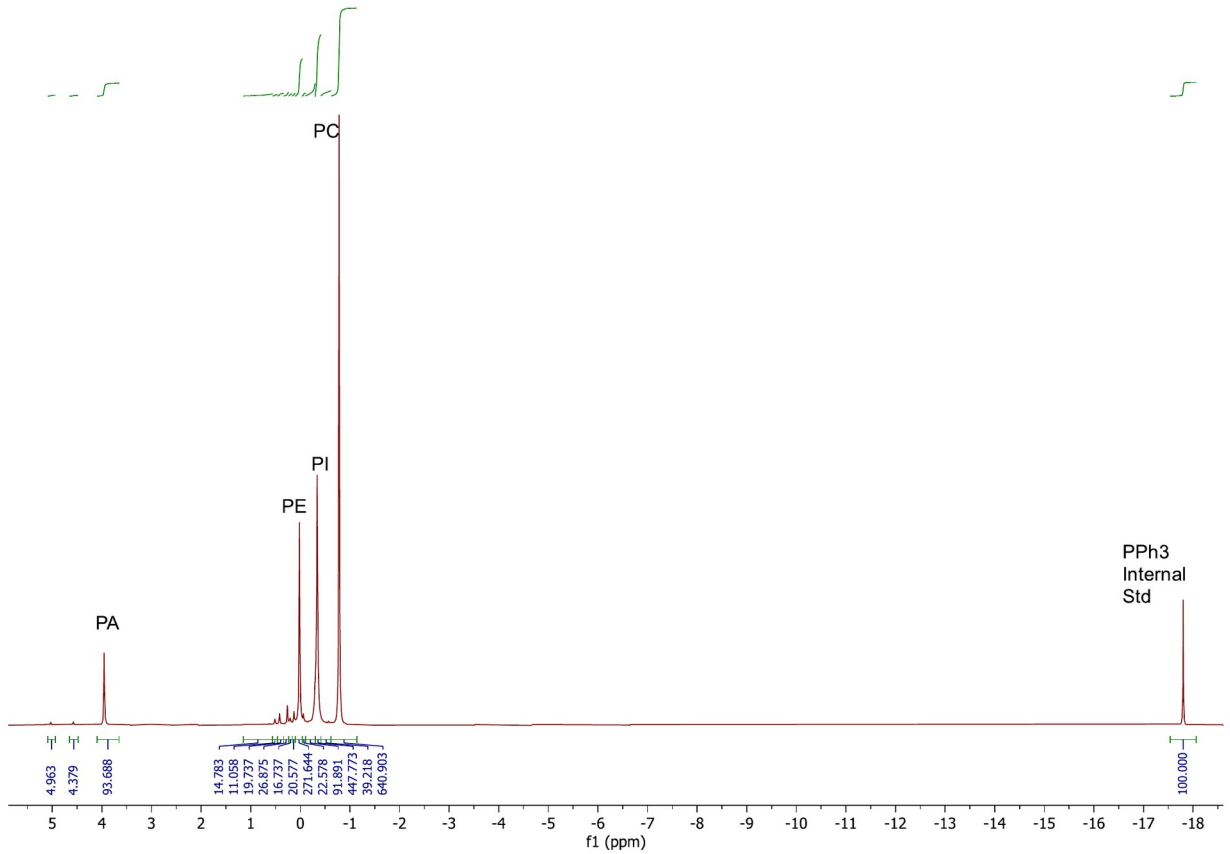
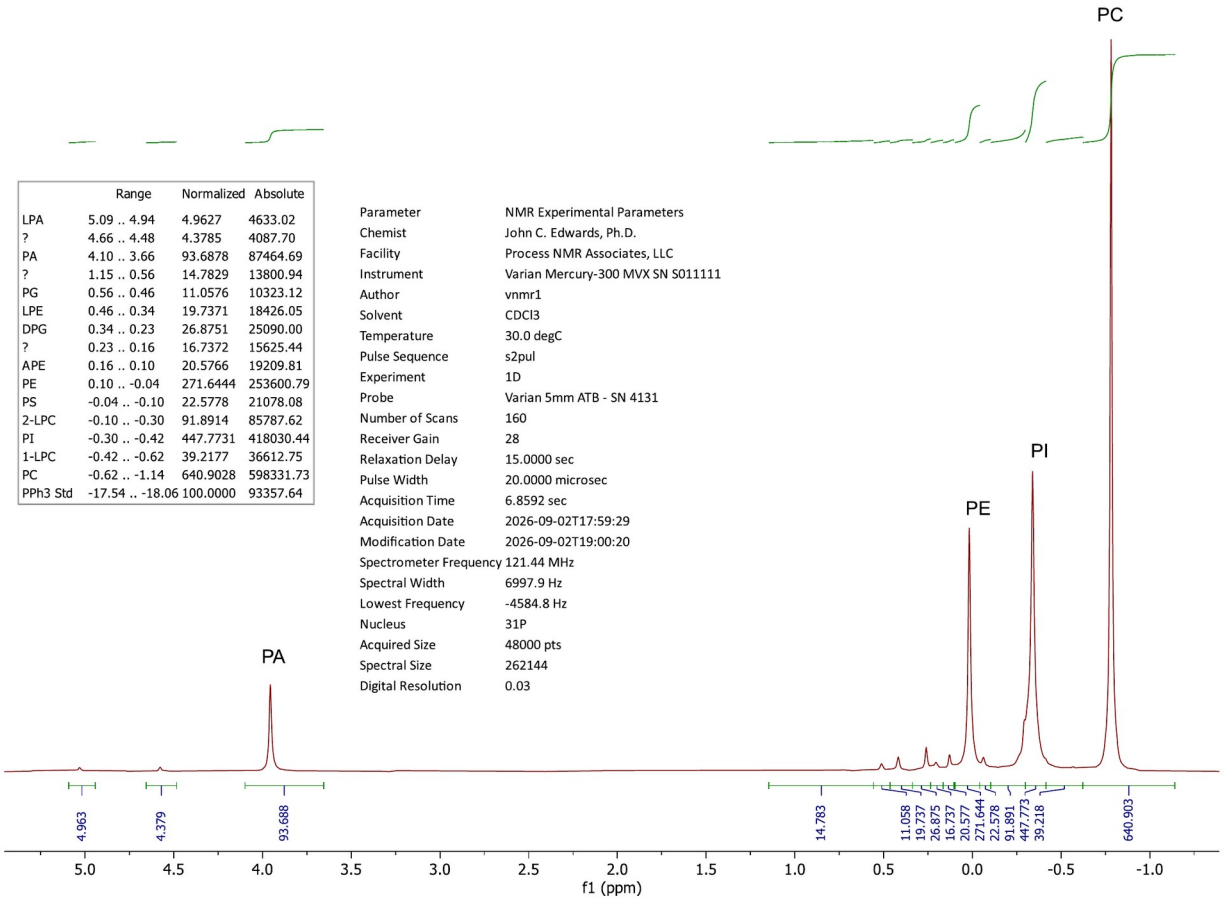


Figure 5. ^{31}P{^{1}H} NMR spectrum (121.4 MHz, 30 °C; lower phase of the PNA $CDCl_3$/methanol/Cs-EDTA reagent, pH 9.7) of a sunflower lecithin, showing the phospholipid region and the triphenyl phosphate internal standard at −17.8 ppm (labeled "PPh3 Internal Std" by the routine processing template; the compound is triphenyl phosphate). Genericized PNA data.



	Range	Normalized	Absolute
LPA	5.09 .. 4.94	4.9627	4633.02
?	4.66 .. 4.48	4.3785	4087.70
PA	4.10 .. 3.66	93.6878	87464.69
?	1.15 .. 0.56	14.7829	13800.94
PG	0.56 .. 0.46	11.0576	10323.12
LPE	0.46 .. 0.34	19.7371	18426.05
DPG	0.34 .. 0.23	26.8751	25090.00
?	0.23 .. 0.16	16.7372	15625.44
APE	0.16 .. 0.10	20.5766	19209.81
PE	0.10 .. -0.04	271.6444	253600.79
PS	-0.04 .. -0.10	22.5778	21078.08
2-LPC	-0.10 .. -0.30	91.8914	85787.62
PI	-0.30 .. -0.42	447.7731	418030.44
1-LPC	-0.42 .. -0.62	39.2177	36612.75
PC	-0.62 .. -1.14	640.9028	598331.73
PPh3 Std	-17.54 .. -18.06	100.0000	93357.64

Parameter	NMR Experimental Parameters
Chemist	John C. Edwards, Ph.D.
Facility	Process NMR Associates, LLC
Instrument	Varian Mercury-300 MVX SN S011111
Author	vnmr1
Solvent	CDCl3
Temperature	30.0 degC
Pulse Sequence	s2pul
Experiment	1D
Probe	Varian 5mm ATB - SN 4131
Number of Scans	160
Receiver Gain	28
Relaxation Delay	15.0000 sec
Pulse Width	20.0000 microsec
Acquisition Time	6.8592 sec
Acquisition Date	2026-09-02T17:59:29
Modification Date	2026-09-02T19:00:20
Spectrometer Frequency	121.44 MHz
Spectral Width	6997.9 Hz
Lowest Frequency	-4584.8 Hz
Nucleus	31P
Acquired Size	48000 pts
Spectral Size	262144
Digital Resolution	0.03

Figure 6. Expansion of the phospholipid region of the sunflower lecithin in Figure 5. The inset table lists the routine integration windows (ppm), the integrals normalized to TPP = 100 and the absolute integrals, and the second table the acquisition parameters as recorded. Genericized PNA data.

6.3 Rapeseed, canola and niche-oilseed lecithins

Rapeseed and canola lecithins resemble sunflower lecithin more than soybean lecithin, with PC as the dominant class, and they share its non-genetically-modified positioning in Europe. A recent review of lecithins from niche oils compiled class distributions for flax, hemp, corn germ, pumpkin and camelina and compared them with soybean, rapeseed and sunflower[2]. On the HPLC basis used in the underlying study, flax lecithin contained 45.0 % PC, 28.7 % PI, 22.7 % PE and 3.6 % PA, an unusually high PE share, whereas rapeseed and sunflower lecithins contained about 56 % PC; the phospholipid fraction of flax was distinguished further by a polyunsaturated fatty-acid content of 53.3 %[2]. The review also recorded the scarcity of class-resolved data for pumpkin, corn and camelina, for which only total phospholipid contents or tissue-level lipidomic data were available, and it identified standardized class-resolved characterization as the principal gap preventing their rational use[2]. ^{31}P NMR is well placed to fill that gap, because it gives class distributions on a phosphorus basis without calibration standards matched to the unusual fatty-acid compositions of these oils. It should be noted, however, that HPLC-derived percentages of the kind compiled in the review are not directly comparable with ^{31}P phosphorus mole percentages; the

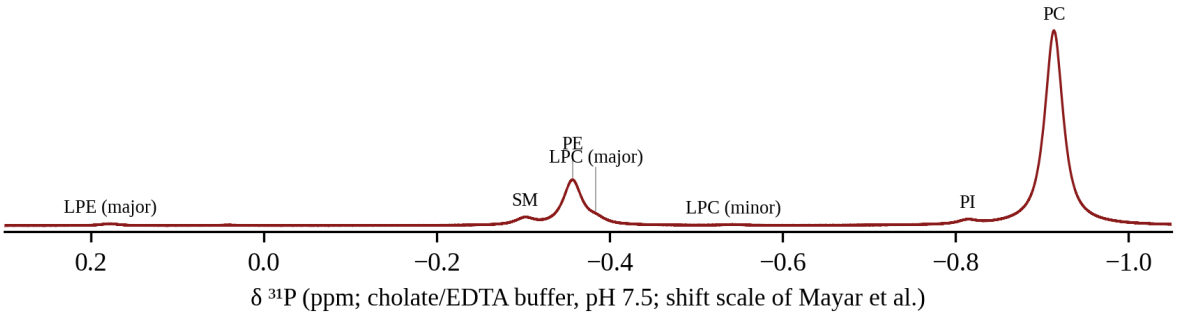
sunflower lecithin in Figure 6, for example, contains 37 % of its phospholipid phosphorus as PC but would appear considerably richer in PC on a detector-response basis calibrated with soybean standards. Comparisons of botanical origin should always be made within one analytical basis.

6.4 Egg yolk and enzyme-modified egg yolk

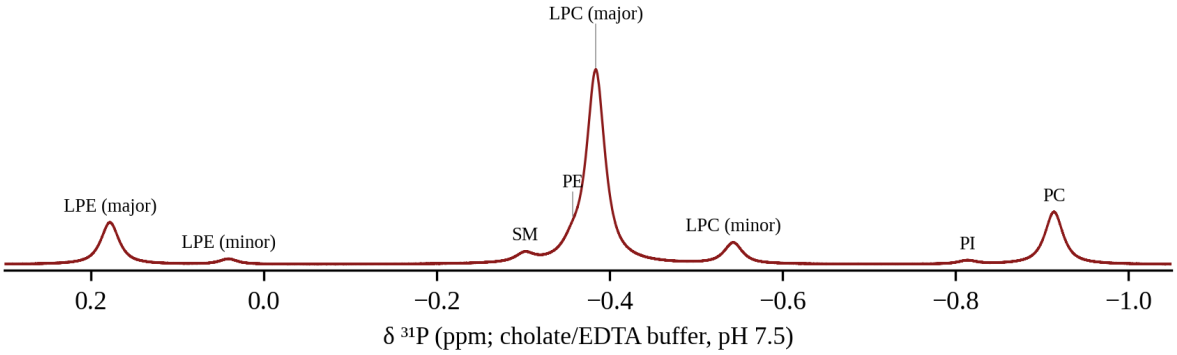
Egg-yolk phospholipids are dominated by PC, which accounts for roughly three quarters of the phospholipid phosphorus, with PE at about one sixth and minor amounts of SM, lyso-PC, lyso-PE and PI; the acyl chains are more saturated than those of soybean PC, and plasmalogen and ether-linked species are present at low levels. Egg-yolk lecithin is prepared by ethanol extraction of dried yolk followed by acetone precipitation of the phospholipids[48]. Its principal industrial use outside pharmaceuticals is in food emulsions, and the enzyme-modified yolk (EMEY) produced by phospholipase A_2 treatment, in which much of the PC and PE is converted to the 1-acyl lyso forms, gives mayonnaise and sauces greater heat stability. The degree of modification, expressed as the lyso fraction of the total choline and ethanolamine phospholipids, is the key specification for EMEY, and it is also the basis on which the use of EMEY can be verified in a finished emulsion.

Mayar and co-workers developed and validated a ^{31}P method for exactly this purpose[16]. In cholate buffer at pH 7.5 and 290 K they resolved PC (−0.914 ppm), PI (−0.814), PE (−0.357), SM (−0.302), the two LPC isomers (−0.384 and −0.543 ppm) and the two LPE isomers (0.178 and 0.041 ppm), confirmed the assignments by spiking, and quantified the classes in mayonnaises prepared with egg yolk and with EMEY at three levels. Synthesized spectra based on their shift table are shown in Figure 7(a) and (b). The ^{31}P method achieved limits of quantification of 0.10–0.15 mg g^{-1} and extraction recoveries of 96–108 %, and its within-laboratory reproducibility (7–24 %) was dominated by the extraction rather than the NMR measurement[16]. Ahmmed and co-workers reported the phospholipid profile of an egg-yolk lipid extract in both cholate and $CDCl_3$ reagents and found that SM and PI were detected only in the cholate system, while the recoveries of cardiolipin, PE, LPC and PC were lower in $CDCl_3$[25].

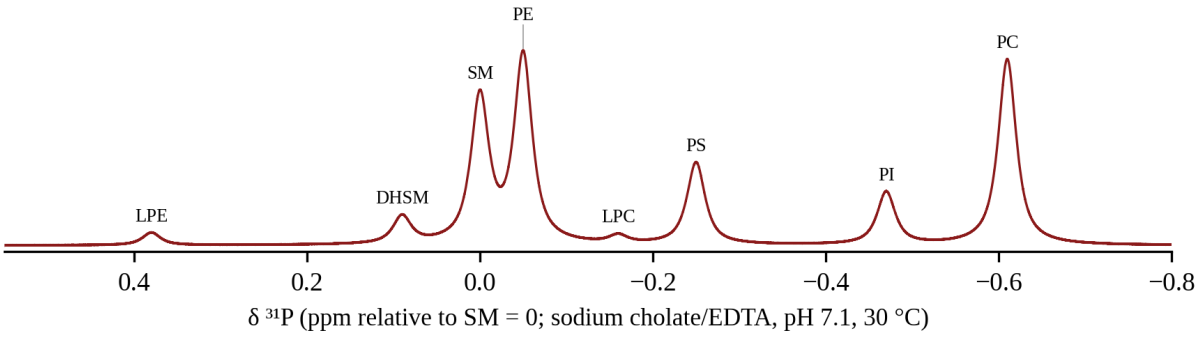
(a) Egg-yolk phospholipids (representative composition) — cholate buffer, pH 7.5



(b) Enzyme-modified (PLA_2) egg yolk (illustrative composition)



(c) Cream polar lipids — composition of MacKenzie et al. (^{31}P, pH 7.1)



(d) Buttermilk protein concentrate powder, direct analysis — composition of MacKenzie et al.

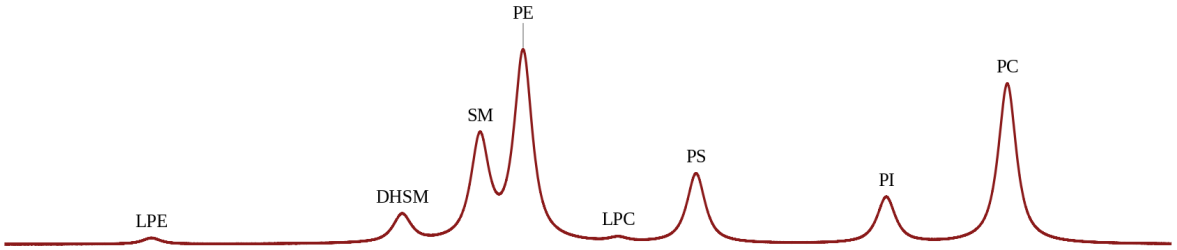


Figure 7. Synthesized ^{31}P{^{1}H} spectra of (a) egg-yolk phospholipids (representative composition) and (b) enzyme-modified (phospholipase A_2) egg yolk (illustrative composition), both on the shift scale of Mayar et al. for cholate buffer at pH 7.5 and 290 K; and (c) cream polar lipids and (d) buttermilk protein concentrate powder analyzed directly, using the compositions and relative shifts (SM = 0) reported by MacKenzie et al. for sodium cholate/EDTA at pH 7.1 and 30 °C. In (d) the PE signal includes lactosyl-PE. Synthesized spectra.

6.5 Dairy phospholipids and milk-fat-globule-membrane ingredients

The phospholipids of milk are concentrated in the milk-fat-globule membrane (MFGM), and they differ from vegetable and egg phospholipids in three respects: sphingomyelin is a major class, PS is present at about a tenth of the phospholipid, and the acyl chains are largely saturated and monounsaturated[49]. MacKenzie and co-workers reported, for cream polar lipids, 26.5 % PC, 26.7 % PE, 20.8 % SM, 3.9 % DHSM, 11.7 % PS, 7.5 % PI, 1.1 % LPC and 1.8 % LPE, and very similar distributions for a buttermilk protein concentrate, a phospholipid-enriched dairy powder and beta serum[3]. Their cholate method resolved DHSM from SM, which chromatographic methods usually fail to do, and it could be applied directly to powders and liquids without extraction, which makes it the method of choice for process control in MFGM ingredient manufacture. They also synthesized the Amadori product of lactose and PE (lactosyl-PE), which is formed when dairy materials containing both lactose and PE are heated or stored, and showed that at pH 7.1 its signal coincides with that of PE[3]; its formation during spray drying and storage is therefore a hidden source of error in PE results for dairy powders unless the pH is chosen to separate the two signals.

Other ^{31}P studies have addressed milk fat and dairy products from complementary angles. Murgia, Mele and Monduzzi quantified milk-fat phospholipids in a monophasic organic reagent[40], Garcia and co-workers compared the phospholipid fingerprints of milks from different mammals by ^{31}P NMR with a view to human nutrition[50], and Kaffarnik and co-workers identified ten phospholipids in cheese by 2D ^{31}P,^{1}H NMR, finding SM to be the second most abundant class (over 20 %) and total phospholipid to account for only 0.3–0.4 % of cheese fat[23]. The low concentration in whole dairy fat means that an enrichment step is required before ^{31}P analysis of cheese, butter or infant formula, whereas MFGM ingredients and buttermilk powders can be measured directly. Synthesized spectra based on the data of MacKenzie and co-workers are shown in Figure 7(c) and (d).

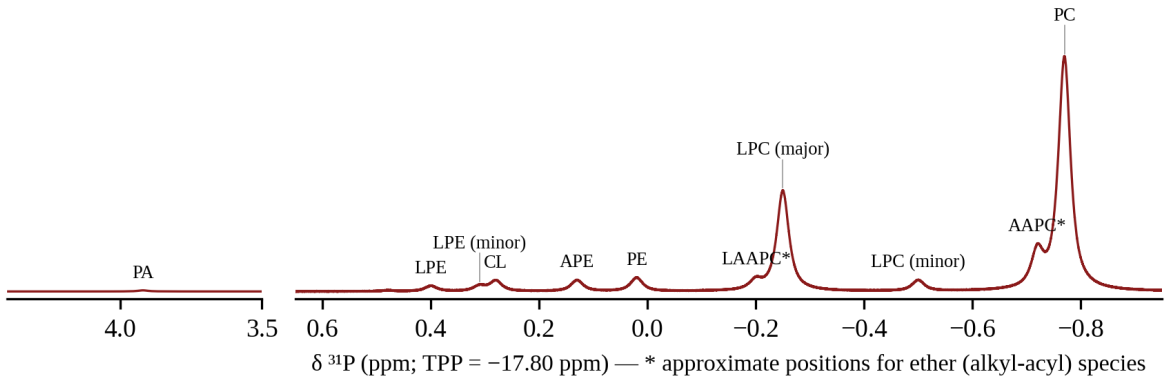
6.6 Krill oil

Krill oil, extracted from Antarctic krill (*Euphausia superba*), is the most important marine phospholipid product. Its commercial premise is that a large part of its eicosapentaenoic and docosahexaenoic acids is carried in phospholipid, principally PC, rather than in triacylglycerol[51,52]. The phospholipid content depends on the extraction: supercritical carbon dioxide alone extracts almost no phospholipid, whereas carbon dioxide with an ethanol co-solvent yields oils containing roughly 28–52 % phospholipid[24], and conventional solvent extraction with ethanol or acetone is also practiced[51]. The phospholipid fraction is dominated by PC, accompanied by alkylacyl-PC (AAPC), which is characteristic of krill, and by lyso-PC and lyso-alkylacyl-PC formed by the very active endogenous phospholipases of krill after capture; PE, lyso-PE, N-acyl-

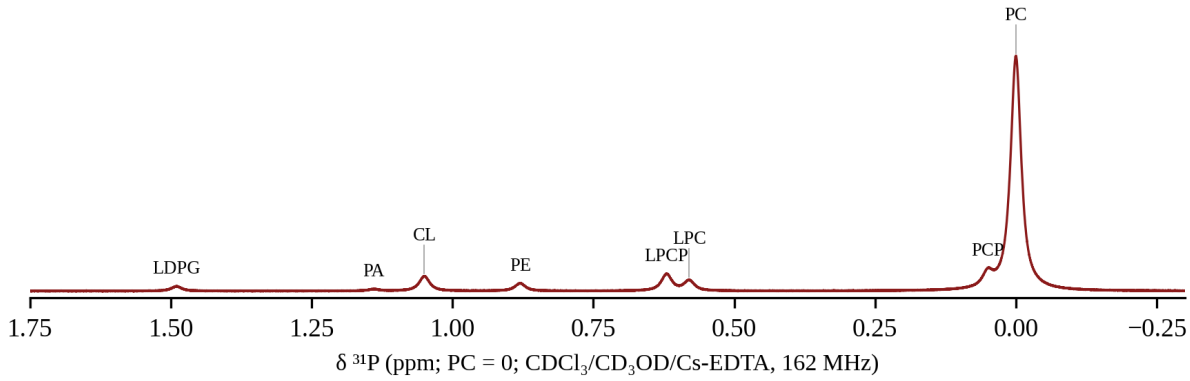
PE and cardiolipin are minor, and PI and PS are minor or absent[25,29,52]. Free fatty acids, formed by the same hydrolytic processes, are also present in commercial krill oils at levels that affect oxidative stability[53].

The ³¹P method for krill oil has progressed further toward official status than any other phospholipid application. A ³¹P NMR procedure is included in the USP krill-oil monograph[42], Burri and co-workers fingerprinted krill oils by ³¹P, ¹H and ¹³C NMR[52], Monakhova and Diehl automated the multicomponent analysis[13], and Zailer, Monakhova and Diehl organized a proficiency test as a step toward an official method[14]. Akanbi and Barrow examined which compositional parameters, including phospholipid profiles, are useful to authenticate krill oil and to detect adulterants[29]. Because PI and PS are minor or absent in krill oil and PI is abundant in vegetable lecithins, and because AAPC is absent from vegetable lecithins, the ³¹P spectrum offers a direct test for fortification of fish oil with plant lecithin, which would introduce PI, PE in soybean proportions and PA while lacking AAPC. A synthesized spectrum of a krill oil on the PNA scale, using the composition reported by Ahmmed and co-workers, is shown in Figure 8(a); the positions of the ether species are approximate.

(a) Krill oil — composition of Ahmmed et al. (cholate data); positions on the PNA Cs-EDTA scale



(b) Hoki roe oil (Folch extract) — composition and positions of Bettjeman et al.



(c) Squid mantle oil (Folch extract) — composition and positions of Bettjeman et al.; phosphonolipids at left

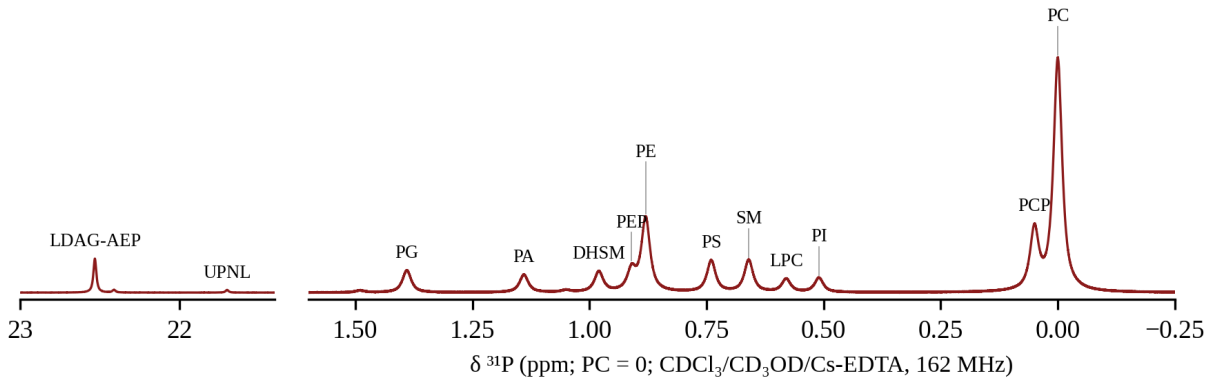


Figure 8. Synthesized ³¹P{¹H} spectra of marine phospholipids: (a) krill oil, composition of Ahmmed et al. (cholate data) placed on the PNA Cs-EDTA scale, with approximate positions (*) for alkylacyl-PC (AAPC) and lyso-alkylacyl-PC (LAAPC); (b) hoki roe oil and (c) squid mantle oil, compositions (μmol g^{-1} wet tissue) and positions (PC = 0; $CDCl_3$/CD_3OD/Cs-EDTA, 162 MHz) of Bettjeman et al., with the phosphonolipid region near 22 ppm shown at left in (c). PCP and LPCP, PC and lyso-PC plasmalogens; PEP, PE plasmalogen; LDPG, lyso-diphosphatidylglycerol; DAG-AEP and LDAG-AEP, diacylglyceryl- and lysodiacylglyceryl-(2-aminoethyl)phosphonates; UPL and UPNL, unknown phospholipid and phosphonolipid. Synthesized spectra.

6.7 Fish roe, shellfish and fish oils

Beyond krill, phospholipid-rich marine ingredients are prepared from fish roe, from shellfish such as mussels and squid, and from fish-processing by-products. Bettjeman and co-workers determined the absolute phospholipid contents of five New Zealand seafoods by ³¹P NMR and found hoki roe to be a high-phospholipid material, with 15.97 μmol g^{-1} of wet tissue, two to four times the contents of monkfish fillet (4.26), squid mantle (8.95), Greenshell mussel (7.04) and hoki liver (6.03)[24]. PC was the principal class in every tissue except one mussel extract, which was unusually rich in PE plasmalogen; hoki roe oil was rich in cardiolipin, while squid and mussel oils contained high proportions of SM and DHSM and substantial amounts of phosphonolipids, the diacylglyceryl- and lysodiacylglyceryl-(2-aminoethyl)phosphonates, which resonate near 22.4–22.5 ppm and are therefore unmistakable[24]. Synthesized spectra based on their data for hoki roe and squid mantle are shown in Figure 8(b) and (c). Kaffarnik and co-workers found total phospholipid contents of 5–12 % in fish oils extracted from fillets and a high proportion of lyso-PC, which they attributed to post-mortem lipolysis during storage[23]. Refined fish oils sold as triacylglycerol or ethyl-ester concentrates, by contrast, contain essentially no phospholipid[24], so that a ³¹P spectrum of a product labeled as phospholipid-bound omega-3 immediately distinguishes it from a fish-oil concentrate.

6.8 High-PC concentrates

PC-enriched products are prepared from deoiled lecithin by ethanol fractionation, in which PC and lyso-PC partition preferentially into the ethanol-soluble fraction while PI, PA and much of the PE remain in the insoluble residue, and by adsorption chromatography of the ethanol-soluble fraction on silica or alumina, which gives products containing roughly 70–95 % PC[1,54]. The ³¹P spectrum of such a product is dominated by a single line, and the analytical interest shifts to the minor components: residual PE and PI, which measure the efficiency of the separation; lyso-PC, which increases with hydrolysis during processing and storage and is often specified as a maximum; PA; and cardiolipin. Because the PC signal is so large, the minor classes must be integrated with care against its wings, and the linear dynamic range of the measurement becomes relevant; a 95 % PC product with 1 % PE requires a signal-to-noise ratio of at least several hundred on the PE signal if the PE result is to have a relative precision of a few percent. Synthesized spectra of a PC-enriched fraction, the complementary PI/PE-enriched fraction and a high-purity PC are shown in Figure 4(b)–(d).

6.9 Phosphatidylserine products

Commercial PS is now produced almost entirely by phospholipase D (PLD)-catalyzed transphosphatidylation of PC from soybean or sunflower lecithin with L-serine, the brain-derived PS used historically having been abandoned[55,56]. PLD cleaves the phosphodiester bond between the phosphate and choline to form a phosphatidyl–enzyme intermediate that is captured either by serine, giving PS, or by water, giving PA. The ratio of the two products depends on the enzyme source, the serine concentration, the water activity and the reaction medium, and Juneja and co-workers showed early on that PLDs of different origin differ greatly in their ability to form PS rather than PA[57]. Iwasaki and co-workers described an aqueous suspension system that avoids organic solvents[58], and Damnjanović and Iwasaki reviewed the enzymology and engineering of PLD for phospholipid synthesis[56]. PE is also a substrate but reacts more slowly, and PI is essentially unreactive, which gives the ³¹P analyst an internal reference for the reaction (Section 8.5). Commercial products are sold as powders standardized to nominal PS contents such as 20, 50 or 70 %, as oil-based softgels and as capsule blends in which the PS concentrate is diluted with other ingredients.

The ³¹P spectrum of a PS product therefore contains, in addition to PS, the hydrolysis product PA (usually the second most abundant class), residual PC and PE, the unreactive PI carried over from the substrate, lyso-PC from the substrate and the lyso-PS and LPA formed from it, and minor species such as PG formed by transphosphatidylation to glycerol when glycerol is present. Because PA is the principal by-product, the alkaline PNA reagent, which moves PA far from the phosphodiester region, is particularly well suited to these materials. Three genericized PNA analyses of PS products are presented in Section 8: a PS-enriched sunflower phospholipid powder (32.2 % of phospholipid phosphorus as PS, 23.7 % PS by weight), a high-purity PS softgel fill (73.1 % and 17.0 %, the latter diluted by the carrier oil) and a PS capsule blend (56.5 % and 7.4 %). Kato and co-workers applied their cholate method to two commercial PS supplements, labeled at 50 mg of PS with 30 mg of PC per hard capsule and at 100 mg of PS per softgel[4]. The molar mass assigned to PS is a significant source of systematic error for these products, because PS may be supplied as the free acid or as the sodium or calcium salt and the acyl composition depends on the substrate; this is discussed in Section 10.2.

6.10 Other modified lecithins and phosphatides

Several further lecithin products have characteristic ³¹P signatures. Phospholipase A_2-hydrolyzed lecithins (lyso-lecithins), used as emulsifiers in bakery and feed, contain large LPC and LPE signals and an increased LPA signal, and their degree of hydrolysis is read directly from the lyso fractions (Figure 4(e))[1,55]. Acetylated lecithins, prepared with acetic anhydride to improve dispersibility in water, carry N-acetyl-PE, whose signal falls in the N-acyl-PE region; the ratio of APE to APE + PE is a direct measure of the degree of acetylation, although native N-acyl-PE must be allowed for[1,28]. Hydroxylated lecithins, produced by treatment with hydrogen peroxide and lactic or acetic acid, and hydrogenated lecithins show little change in their class distribution, since the modification is confined to the acyl chains, and ¹H or ¹³C NMR is more informative for them;

^{31}P NMR nevertheless verifies that the class distribution and the lyso content have not been altered by the process. Phosphatidylglycerol can be produced from PC by PLD in the presence of glycerol and appears as a large PG signal. Ammonium phosphatide emulsifiers (E 442), which are synthetic mixtures of phosphorylated partial glycerides used in chocolate, have been quantified in chocolate by ^{31}P NMR by Malmos and co-workers[59]. Finally, it should be recorded that water-soluble phosphorus compounds sold as ingredients in their own right, such as glycerophosphocholine, partition into the upper phase of the biphasic reagent and must be analyzed in an aqueous system.

Table 8. Phospholipid sources and products: typical phospholipid content, class profile and distinctive ^{31}P features.

Source or product	Phospholipid content	Principal classes (% of phospholipid P unless stated)	Distinctive species and ^{31}P notes	References
Soybean lecithin (fluid, deoiled)	≈ 62 % AI (fluid); 65–75 % PL (deoiled, dry basis)	PC 23–38; PE 26–37; PI 12–18; PA 2–12	PE/PC ≈ 0.9–1.1; APE, PG, DPG, LPC minor	[2–4,17,25]
Sunflower lecithin	≈ 70 wt % (PNA example)	PC 37; PI 26; PE 16; PA 5; LPC 8 (PNA example)	PE/PC ≈ 0.4; PI/PC ≈ 0.7; authenticity vs soybean	This work;[46]
Rapeseed and flax lecithins	Crude oils 1–3 % PL	Rapeseed PC ≈ 56; flax PC 45.0, PI 28.7, PE 22.7, PA 3.6 (HPLC basis)	Class data from HPLC; ^{31}P data scarce	[2]
Egg-yolk lecithin	PL ≈ 30 % of yolk lipid	PC ≈ 75; PE ≈ 17; minor SM, LPC, PI	SM present; saturated PC species	[16,25,48]
Enzyme-modified egg yolk (EMEY)	As yolk	Large 1-acyl LPC and LPE fractions	Degree of PLA_2 conversion; lyso isomers	[16]
Dairy MFGM materials (cream polar lipids, BPC, beta serum)	PL 15–70 % in enriched powders	PC 26.5; PE 26.7; SM 20.8; DHSM 3.9; PS 11.7; PI 7.5 (cream)	SM/DHSM resolved; lactosyl-PE coincides with PE at pH 7.1	[3,49,50]
Krill oil	≈ 28–52 % PL	PC (with AAPC) ≈ 60; LPC up to ≈ 27 (example of Ahmmed et al.)	AAPC, LAAPC; PI and PS minor or absent	[24,25,42,52]
Fish roe (hoki)	15.97 µmol g^{-1} wet tissue	PC dominant (≈ 77 %)	Cardiolipin rich; PC and LPC plasmalogens	[24]
Squid and mussel oils	7.0–9.0 µmol g^{-1} wet tissue	PC 40 % (squid); diverse profiles	SM, DHSM, PE plasmalogen; phosphonolipids at 21.7–22.5 ppm	[24]
High-PC concentrates	PC 70–95 %	PC dominant; residual PE, PI, PA, LPC	Minor classes on the wings of PC	[1,54]
PS products (powders, softgels, blends)	PS 20–70 % in concentrates	PS 32–73; PA 11–15; PE 3–10; PI 1–25 (PNA examples)	PA main by-product; PI from substrate; possible lyso-PS	This work;[4,56]
Hydrolyzed, acetylated and other modified lecithins	As parent	LPC and LPE (hydrolyzed); N-acetyl-PE (acetylated)	Degree of modification from lyso or APE fractions	[1,55,59]

AI, acetone insolubles; BPC, buttermilk protein concentrate. Ranges are indicative and depend on the preparation system and reporting basis.

7. Processing Routes and ^{31}P NMR Checkpoints

7.1 Overview

The manufacture of every phospholipid product considered in this paper is a sequence of separations and chemical or enzymatic transformations, and at nearly every step the phospholipid class distribution changes in a way that is either intended (fractionation, enzymatic conversion) or diagnostic of a problem (hydrolysis, incomplete separation, cation contamination). Because ^{31}P NMR measures all classes simultaneously on a molar basis, it is suited to mass balances across a process step in a way that single-class methods are not. The oilseed route is summarized in Figure 9, with eleven checkpoints (C1–C11) at which a ^{31}P measurement answers a specific process question; the corresponding analytical questions, measurements and complementary methods are listed in Table 9. The PS route and the egg, dairy and marine routes, with a further sixteen checkpoints, are treated in Sections 7.6 and 7.8 and in Table 10.

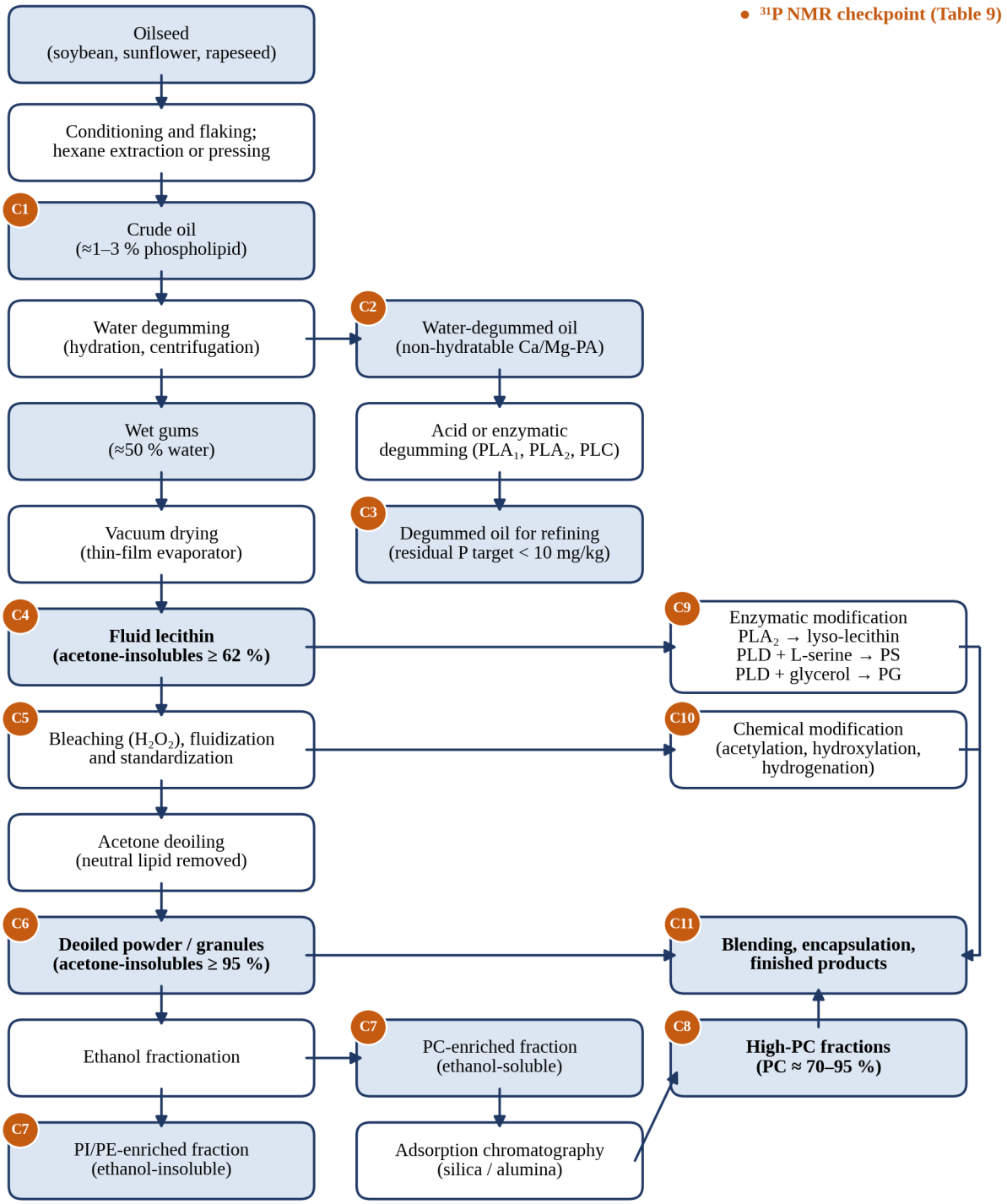


Figure 9. Simplified processing route for oilseed lecithins and products derived from them, from crude oil through degumming, drying, standardization, deoiling, fractionation and modification to finished products. Numbered badges (C1–C11) mark the ^{31}P NMR checkpoints described in Table 9. Acetone-insoluble and phosphorus values are typical specification targets.

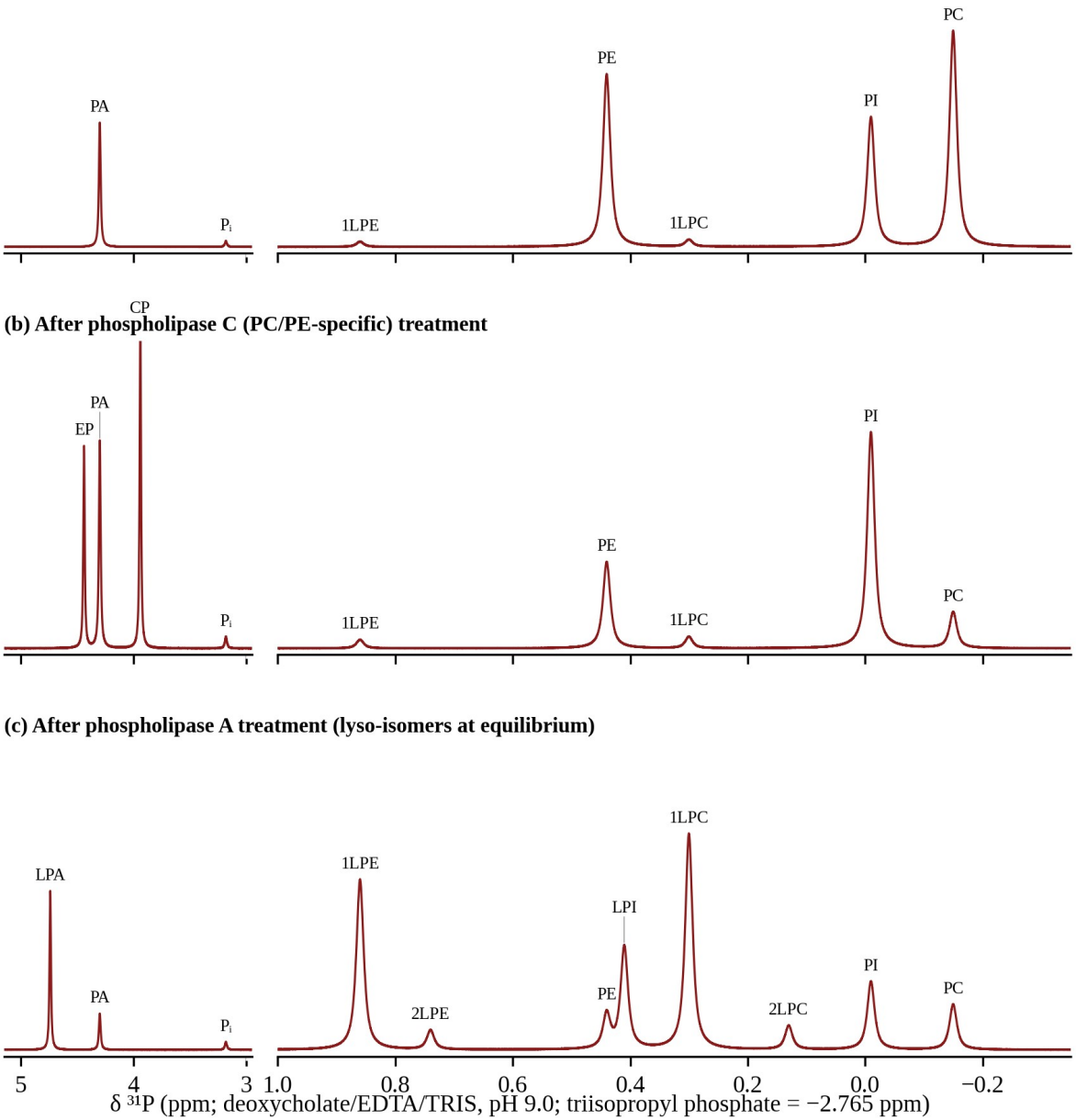
Table 9. ^{31}P NMR checkpoints in the processing of oilseed lecithins (C1–C11, Figure 9).

No.	Process stage	Analytical question	^{31}P NMR measurement and indicators	Complementary methods
C1	Crude oil	How much phospholipid is present and in which classes?	Class profile in µmol per 100 g; PA + PE as non-hydratable indicator	Total P (ICP-OES); FFA
C2	Water-degummed oil	Which classes remain after hydration?	Residual PA, PE (Ca/Mg salts); choice of acid or enzymatic degumming	Total P; Ca, Mg
C3	Degummed oil for refining	Has the phosphorus target been reached; what did the enzyme do?	Process development only (practical LoQ ≈ 31 mg P kg^{-1}); PLC head groups, lyso isomers, DAG gain	Total P for release (target ≤ 10 mg kg^{-1})
C4	Fluid lecithin	Class distribution, PL content, hydrolysis history	PL wt %, µmol P g^{-1}; LPC, LPA; PE/PC and PI/PC ratios	Acetone insolubles; acid value; moisture
C5	Bleaching, fluidization, standardization	Has the process changed the class profile?	Before/after comparison; LPC and PA growth	Color; viscosity; peroxide value
C6	Deoiled powder or granules	PL content; carry-over of class profile	PL wt % vs acetone insolubles; class ratios vs fluid lecithin	Acetone insolubles; residual acetone
C7	Ethanol fractionation (both fractions)	Partition of PC vs PI/PE; mass balance	Composition and µmol P g^{-1} of each fraction; reconstructed feed	Fraction yields
C8	High-PC fractions	PC purity and impurities	PC; residual PE, PI, PA; LPC specification	HPLC-ELSD/CAD
C9	Enzymatic modification (PLA_2, PLD)	Degree of hydrolysis; conversion and selectivity	LPC/(LPC + PC); PS/(PS + PA); PI-anchored balance; PG formation	FFA; residual serine
C10	Chemical modification	Degree of acetylation; absence of hydrolysis	APE/(APE + PE); lyso fractions	Iodine and hydroxyl values; ^{1}H/^{13}C NMR
C11	Finished products	Label claim; stability	mg per serving; degradation products	Fill weight; uncertainty; decision rule

7.2 Crude oil, degumming and the non-hydratable phosphatides

Crude soybean, sunflower and rapeseed oils contain roughly 1–3 % phospholipid[2]. In water degumming the oil is mixed with a small amount of water at 60–80 °C, the hydratable phospholipids swell into a separate gum phase and are removed by centrifugation, and the gums are the raw material for lecithin[1,43]. Not all phospholipid is removed in this way. The calcium and magnesium salts of PA, and to a lesser extent of PE, are poorly hydratable and remain in the oil, where they constitute the non-hydratable phosphatides; their proportion rises with seed damage and with phospholipase D activity before extraction[45]. The ^{31}P profile of a crude oil (checkpoint C1) and of the water-degummed oil (C2) therefore identifies the fraction of the phosphorus that will require acid or enzymatic treatment and the classes that carry it, which is information that total-phosphorus methods cannot provide.

Enzymatic degumming, reviewed by Dijkstra[45], converts the non-hydratable species into forms that can be removed with water or that remain in the oil as neutral lipid. Phospholipase A_1 or A_2 removes one acyl chain to give lysophospholipids, which are hydratable; phospholipase C cleaves the glycerol–phosphate bond to release 1,2-diacylglycerol, which stays in the oil and adds to the yield, and a water-soluble phosphate monoester (choline, ethanolamine or inositol phosphate) that leaves with the water phase. Van Rijn and co-workers developed their deoxycholate method specifically to follow these reactions, and because the aqueous detergent phase retains the released head groups, a single spectrum shows the residual intact phospholipids, the lyso species and their isomers, the head-group phosphates and inorganic phosphate (Figure 10)[15]. Their validation gave intermediate precisions of 2.2–3.5 % for the phospholipid and head-group species, a limit of detection of 5 µmol per 100 g of oil and a practical limit of quantification of 100 µmol per 100 g for results with less than 5 % variation[15].



Synthesized; chemical shifts from van Rijn et al.; compositions are illustrative. Head groups appear only when the aqueous phase is included.

Figure 10. Synthesized $^{31}P\{^{1}H\}$ spectra illustrating enzymatic degumming as observed in a deoxycholate/EDTA/TRIS reagent at pH 9.0 (shift scale of van Rijn et al., triisopropyl phosphate = −2.765 ppm, 162 MHz): (a) a water-degummed oil, (b) the same oil after treatment with a PC/PE-specific phospholipase C, showing the released choline phosphate (CP) and ethanolamine phosphate (EP) and the unreacted PI and PA, and (c) after phospholipase A treatment, with the lyso species at their isomer equilibrium. P_i, inorganic phosphate. Compositions are illustrative. Synthesized spectra.

These figures define the role of ³¹P NMR at checkpoint C3. The phosphorus specification of a degummed oil destined for physical refining is usually of the order of 10 mg kg^{-1} or less, which corresponds to about 32 µmol of phosphorus per 100 g, well below the practical quantification limit of 100 µmol per 100 g (31 mg P kg^{-1}) established by van Rijn and co-workers and close to

their detection limit of 5 µmol per 100 g (1.5 mg P kg⁻¹). Release testing of degummed oil at C3 therefore remains a task for total-phosphorus methods such as inductively coupled plasma emission spectroscopy, and the value of ³¹P NMR at this point lies in process development and troubleshooting: identifying which classes survive a given degumming regime, measuring the conversion achieved by each enzyme, and closing the phosphorus mass balance between oil, gum and water phases.

7.3 From gums to fluid lecithin

Wet gums contain about half their weight of water and are dried, usually in a thin-film evaporator under vacuum, to a fluid lecithin of about 62 % acetone insolubles[1]. Hydrolysis during storage of the wet gums, before drying, produces lyso-phospholipids and free fatty acids, and excessive temperature during drying causes darkening through Maillard and oxidative reactions. At checkpoint C4 the ³¹P spectrum confirms the class distribution of the lecithin, gives its phospholipid content independently of the gravimetric acetone-insoluble test, and records the lyso-PC and LPA levels that indicate hydrolytic history. Bleaching with hydrogen peroxide, fluidization with fatty acids or oil and standardization to a viscosity or acetone-insoluble target (C5) should not change the class distribution, but they dilute the phospholipid and, when excess peroxide or acid is used, can promote hydrolysis; a ³¹P comparison before and after standardization verifies both effects. Because the method is molar and needs no class-specific calibration, it is also the most direct check that a lecithin labeled by botanical origin has the class profile expected for that origin (Section 6.2).

7.4 Deoiling

Deoiling with acetone removes triacylglycerol, free fatty acids and most other neutral lipids and leaves a powder or granulate of 95 % or more acetone insolubles[1,43]. The ³¹P measurement at checkpoint C6 shows the increase in phospholipid content per gram, which should be consistent with the amount of acetone-soluble material removed, and it confirms that the class distribution has been carried through unchanged. Minor species with appreciable solubility in acetone may be partly lost, and the ³¹P profile of the powder compared with that of its fluid precursor is the most direct way to detect such losses. The difference between the acetone-insoluble fraction and the ³¹P phospholipid content of a deoiled lecithin, typically 20–30 percentage points, represents glycolipids, oligosaccharides and other acetone-insoluble matter that is not phospholipid, and a change in that difference from lot to lot is itself worth monitoring.

7.5 Ethanol fractionation and high-PC products

When deoiled lecithin is extracted with ethanol, PC and lyso-PC dissolve preferentially, PI, PA and the calcium and magnesium salts remain largely insoluble, and PE distributes between the two fractions[1,54]. The ethanol-soluble fraction is a PC-enriched lecithin with improved oil-in-water emulsifying properties, and the insoluble residue a PI- and PE-enriched lecithin with water-in-oil properties; synthesized spectra of the two fractions are shown in Figure 4(b) and (c). ³¹P NMR at

checkpoint C7 serves two purposes. It gives the composition of each fraction, and, because it also gives the phospholipid phosphorus per gram, the compositions and masses of the two fractions can be combined to reconstruct the composition of the feed, which provides a closure check on both the fractionation and the analytical method. Chromatographic purification of the ethanol-soluble fraction on silica or alumina then yields high-PC products (C8), whose specifications concern the residual PE, PI, PA and lyso-PC as much as the PC content itself.

7.6 Enzymatic modification: phospholipase A_2 and phospholipase D

Phospholipase A_2 modification (checkpoint C9) converts part of the PC and PE of a lecithin to 1-acyl lyso forms, and its extent is expressed by the degree of hydrolysis, the ratio of LPC to LPC + PC (and of LPE to LPE + PE) on a phosphorus basis[55]. The ³¹P spectrum measures both terms in the same experiment. The lyso-isomer ratio could in principle distinguish PLA_2 from PLA_1 products, since the former give 1-acyl and the latter 2-acyl lyso-phospholipids, but acyl migration toward the equilibrium mixture of about 90 % 1-acyl isomer occurs readily and is accelerated at alkaline pH[15,26], so the ratio measured in the alkaline PNA reagent should not be interpreted as an enzymatic signature without evidence that migration was suppressed.

The phospholipase D route to PS is shown in Figure 11. The substrate (checkpoint P1) is a PC-rich lecithin or PC-enriched fraction, whose PC content sets the maximum attainable PS content and whose lyso-PC is carried forward as a precursor of lyso-PS. In the reaction mixture (P2) the conversion of PC and PE and the formation of PS and PA are followed together; the selectivity, S = PS/(PS + PA) for the products formed, is the key process variable and depends on the enzyme, the serine concentration and the water activity[56–58]. Because PI is not a substrate, its phosphorus serves as an internal reference that allows the conversion to be computed even when the sample has been partially purified or diluted, as shown in Section 8.5. After inactivation of the enzyme and removal of the aqueous phase containing serine and choline, the PS concentrate is dried and standardized, often on a carrier (P3), and then formulated into softgels, capsules or tablets (P4), whose stability is followed during storage (P5).

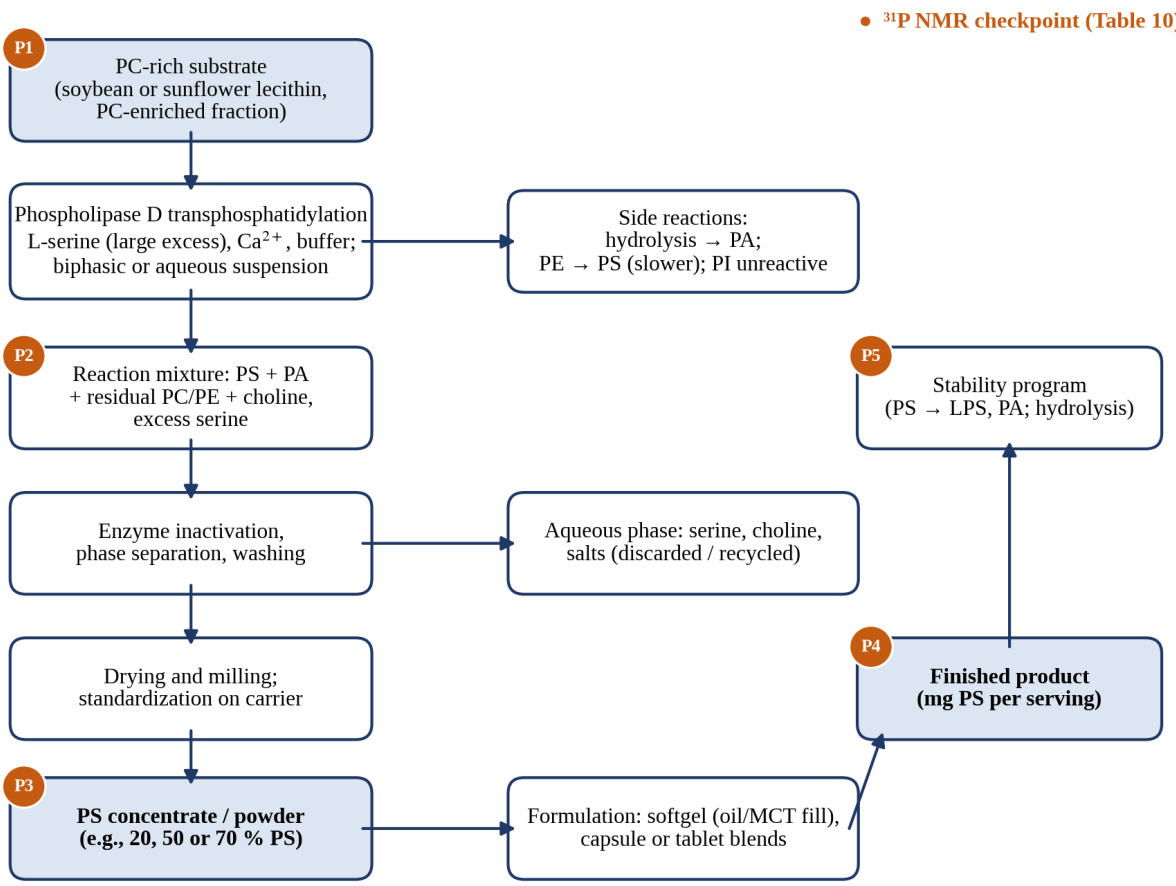
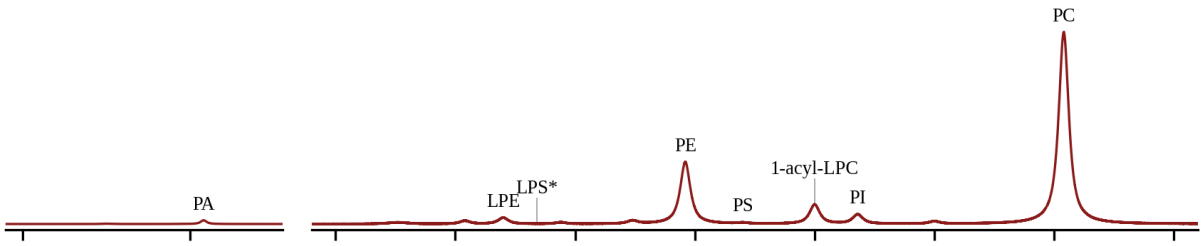


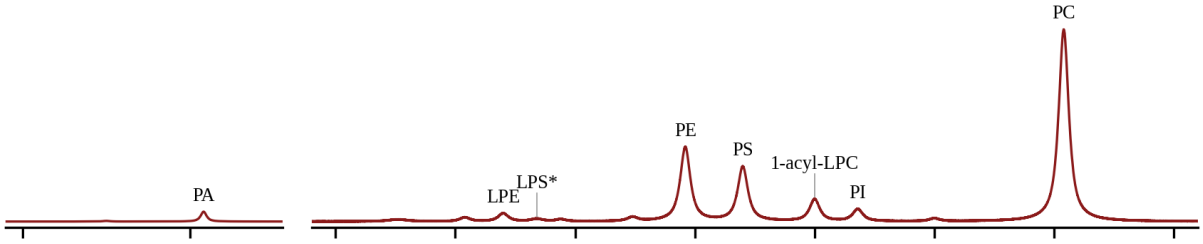
Figure 11. Production of PS by phospholipase D transphosphatidylation and formulation of finished products. Badges P1–P5 mark the ^{31}P NMR checkpoints described in Table 10.

A synthesized time course of a PLD reaction, generated with a simple pseudo-first-order model, is shown in Figure 12. PC is converted with a transphosphatidylation rate constant of 0.22 h^{-1} and a competing hydrolysis rate constant of 0.035 h^{-1}, which gives a selectivity of 0.86; PE reacts at 0.3 times the rate of PC and lyso-PC at 0.5 times, with the same selectivity, and PI is inert. The model is not intended to reproduce any particular process, but it illustrates the features that a ^{31}P time course reveals: the monotonic growth of PA alongside PS, the persistence of PE after PC has been consumed, the appearance of lyso-PS from lyso-PC, and the unchanging PI signal. The position assigned to lyso-PS in the figure is an estimate by analogy with the lyso/diacyl pairs PC/LPC and PE/LPE in the same reagent, and it is discussed in Section 8.5.

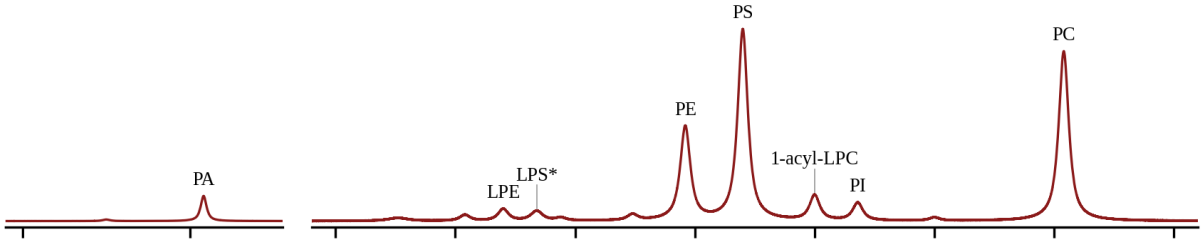
(a) t = 0 h



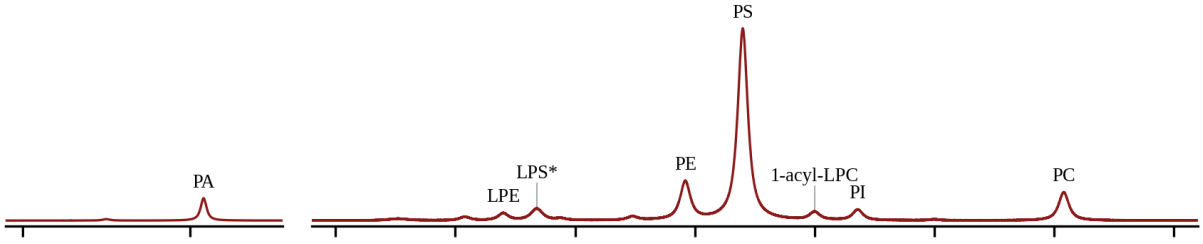
(b) t = 1 h



(c) t = 3 h



(d) t = 8 h



(e) t = 24 h

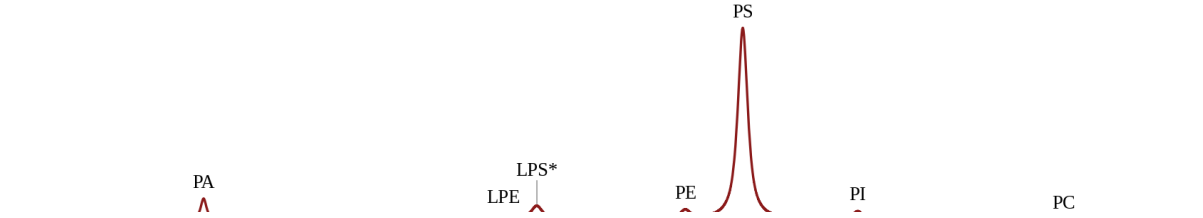


Figure 12. Synthesized $^{31}P\{^{1}H\}$ spectra of a phospholipase D transphosphatidylation of a PC-enriched soybean fraction with L-serine at (a) 0 h, (b) 1 h, (c) 3 h, (d) 8 h and (e) 24 h, calculated with an illustrative pseudo-first-order model (transphosphatidylation 0.22 h^{-1}, hydrolysis 0.035 h^{-1}; PE reacting at 0.3 times and lyso-PC at 0.5 times the rate of PC; PI inert), plotted on the routine PNA scale. The lyso-PS position (*) is estimated. Synthesized spectra; compositions in Appendix A.

7.7 Chemical modification

Chemical modifications (checkpoint C10) are of three kinds. Acetylation with acetic anhydride converts part of the PE to N-acetyl-PE, and the degree of acetylation follows directly from the N-acyl-PE and PE integrals, after correction for the native N-acyl-PE of the starting lecithin[1,28]. Hydroxylation with hydrogen peroxide and a carboxylic acid, and catalytic hydrogenation, modify the acyl chains and leave the head groups intact, so that the ^{31}P class distribution should be unchanged; a ^{31}P measurement is then used to confirm that no hydrolysis or head-group degradation has accompanied the treatment, while the extent of modification is measured by ^{1}H or ^{13}C NMR, iodine value or hydroxyl value. Chemical interesterification and the synthesis of ammonium phosphatides produce new phosphorus species whose signals must be assigned from reference materials[59].

7.8 Egg, dairy and marine routes

The egg, dairy and marine routes are summarized in Figure 13. For egg yolk the checkpoints are the raw or dried yolk (E1), whose lyso-PC content reflects freshness and handling; the egg-yolk lecithin obtained by ethanol extraction and acetone precipitation (E2)[48]; the enzyme-modified yolk (E3), whose specification is its degree of PLA_2 conversion; and the finished emulsion (E4), in which the presence of EMEY and the yolk content can be verified by ^{31}P NMR after a validated extraction[16]. For dairy materials the checkpoints are the buttermilk, butter serum and whey-cream streams from which the MFGM is recovered (D1), the phospholipid-enriched powders produced by membrane filtration and supercritical-fluid removal of neutral lipid (D2), the MFGM and whey-protein–phospholipid concentrates sold for their SM and PS contents (D3), and the infant-formula and nutritional products into which they are formulated (D4)[3,49]. At D1–D3 the cholate method can be applied directly to the powder or liquid, and the SM/DHSM ratio, the PS content and the lactosyl-PE formed by Maillard reaction are the most informative results; at D4, where phospholipid is a minor component of a complex matrix, an extraction is required and the ^{31}P measurement complements chromatographic and mass-spectrometric methods rather than replacing them.

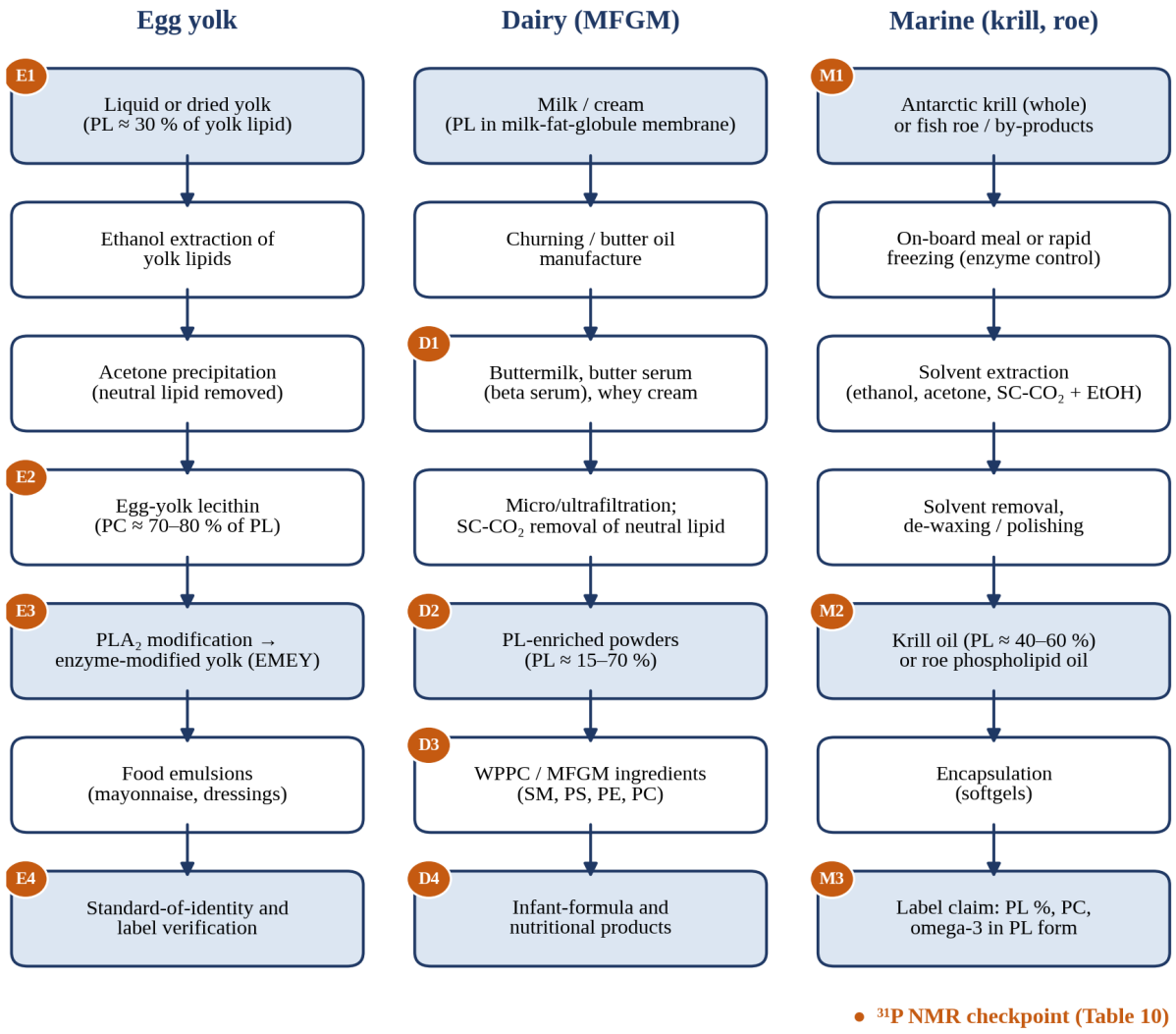


Figure 13. Processing routes for egg-yolk, dairy (milk-fat-globule-membrane) and marine phospholipid products. Badges E1–E4, D1–D4 and M1–M3 mark the ^{31}P NMR checkpoints described in Table 10. Phospholipid contents are typical values.

For krill and roe the decisive step is the first one (M1). The endogenous phospholipases of krill act rapidly after capture[51], and post-mortem lipolysis produces lyso-PC in fish muscle as well[23], so the lyso-PC fraction of the raw material and of the oil records the time and temperature history between capture and extraction. The oil itself (M2) is characterized by its total phospholipid content, its PC and alkylacyl-PC content, its lyso fraction and its free-fatty-acid content[52,53], and the label claim (M3), usually expressed as phospholipid percentage and sometimes as PC or as omega-3 fatty acids in phospholipid form, is verified by ^{31}P NMR in combination with ^{1}H or ^{13}C NMR. The same measurements distinguish a genuine krill oil from a fish oil fortified with vegetable lecithin, as discussed in Section 6.6[29].

Table 10. ^{31}P NMR checkpoints in the production of PS (P1–P5, Figure 11) and of egg, dairy and marine phospholipids (E1–E4, D1–D4, M1–M3, Figure 13).

No.	Process stage	Analytical question	^{31}P NMR measurement and indicators	Notes
P1	PC-rich substrate	Maximum attainable PS; precursors of by-products	PC, PE, PI, LPC content	LPC → lyso-PS
P2	PLD reaction mixture	Conversion, selectivity, end point	PS/(PS + PA); PI-normalized ratios	Quench before analysis
P3	PS concentrate or powder	Grade (e.g. 20, 50, 70 % PS)	PS P-mol % and wt %; PA, residual PC/PE, PI	State salt form and basis
P4	Finished product	mg PS per serving	PS wt % × mean fill weight	Uncertainty; decision rule
P5	Stability program	Loss of PS; degradation route	Lyso-PS, PA, LPA growth; class balance	Include initial time point
E1	Liquid or dried yolk	Freshness, handling	LPC fraction; class profile	Extraction required
E2	Egg-yolk lecithin	PC content, purity	PC ≈ 70–80 % of PL; PE, SM, LPC	[48]
E3	Enzyme-modified yolk	Degree of PLA_2 conversion	LPC/(LPC + PC); LPE/(LPE + PE)	Isomer ratio with caution
E4	Emulsions (mayonnaise)	Yolk content; use of EMEY	PL and LPL in mg g^{-1} after 4 × MeOH extraction	[16]
D1	Buttermilk, butter serum, whey cream	PL content of process streams	Direct cholate analysis; SM, PS, PI	[3]
D2	PL-enriched powders	PL content, class profile	PL wt %; SM/DHSM; lactosyl-PE	Maillard products
D3	MFGM ingredients (WPPC)	SM and PS claims	SM, DHSM, PS; LPC, LPE	Direct analysis
D4	Infant formula and nutritionals	MFGM phospholipid claims	After extraction; low levels	Complements LC–MS
M1	Raw krill or roe	Enzymatic history after capture	LPC/(LPC + PC); total PL	Rapid processing
M2	Krill or roe oil	PL %, PC, AAPC, lyso fraction	Class profile; total PL; with ^{1}H/^{13}C for omega-3 in PL form	[42,52]
M3	Label claim and authenticity	PL %; fortification with lecithin	PI, PA, PE/PC pattern; AAPC present/absent	[29]

7.9 Finished products and stability

At the end of each route (checkpoints C11, P4, E4, D4 and M3) the analytical question changes from process control to conformity with a label claim or specification, and the uncertainty of the result, the fill weight of the dosage unit and the decision rule become as important as the spectrum itself (Section 10). Stability programs (P5) add a time dimension. The principal degradation route of PS in finished products is ester hydrolysis, to lyso-PS and free fatty acid and eventually to glycerophosphoserine; PA is formed from PS only if residual phospholipase D activity has survived inactivation. PC products degrade analogously to lyso-PC. Because the degradation products are themselves phospholipids that ^{31}P NMR resolves, a stability time point gives not only

the loss of the active class but also the identity of the products formed, which distinguishes hydrolysis from other apparent loss mechanisms such as incomplete recovery from an aged matrix. A stability sample of a PS capsule blend is examined in Section 8.4.

8. Worked Examples from PNA Analyses

8.1 Recalculation basis and the sunflower lecithin

Four recent PNA analyses are used in this section to show how the method performs on real materials and how the results should be read. The materials are a sunflower lecithin, a PS-enriched sunflower phospholipid powder, a high-purity PS softgel fill and a PS capsule blend taken from a stability program; product names, lot numbers and client details have been removed. For each analysis the integrals recorded in the routine integration table were recalculated with Eq. (3) and Eq. (4), using the molar masses of Table 1, the certified purity of the standard and the per-phosphorus treatment of cardiolipin. The recalculated results are collected in Table 11. They reproduce the principal-class results originally reported to within 0.01 percentage point; the totals differ slightly (by 0.03–0.35 percentage points of total phospholipid) because the molar masses applied to LPA, LPE and cardiolipin were harmonized with Table 1 for this paper.

Table 11. Recalculated results of the four PNA analyses: phosphorus mole percentage of total phospholipid (P-mol %) and weight percentage of the sample (wt %) for each class.

Class	Sunflower lecithin P-mol %	wt %	PS-enriched powder P-mol %	wt %	PS softgel fill P-mol %	wt %	PS capsule blend P-mol %	wt %
PC	37.11	26.46	6.13	4.36	0.09	0.02	1.77	0.22
LPC, major ("2-LPC")	5.32	2.54	2.00	0.95	0.56	0.08	1.38	0.12
LPC, minor ("1-LPC")	2.27	1.08	2.00	0.95	0.40	0.06	1.28	0.11
PI	25.93	20.05	24.53	18.94	1.36	0.33	5.35	0.73
PS	1.31	0.96	32.21	23.74	73.07	16.99	56.52	7.39
PE	15.73	10.56	10.17	6.82	3.48	0.74	6.71	0.80
APE	1.19	1.09	3.18	2.91	1.45	0.42	3.15	0.51
DPG	1.56	1.04	2.03	1.36	1.97	0.42	3.09	0.37
LPE	1.14	0.50	0.39	0.17	2.52	0.35	0.71	0.05
PG	0.64	0.45	1.40	0.98	0.48	0.11	0.57	0.07
PA	5.43	3.44	11.01	6.97	12.19	2.44	14.66	1.65
LPA	0.29	0.11	0.32	0.13	1.07	0.13	0.99	0.07
Unassigned	2.08	1.48	4.63	3.30	1.35	0.30	3.82	0.48
Total phospholipid	100.00	69.78	100.00	71.58	100.00	22.39	100.00	12.57
Phospholipid P (µmol g⁻¹)		926		925		292		164
PS per serving (mg)		—		—		105.3 (1 softgel)		103.7 (2 capsules)

Calculated from the recorded integrals with the molar masses of Table 1, TPP purity 99.95 %, DPG per phosphorus and unassigned signals at 770 g mol⁻¹. Mean fill weights: softgel 619.68 mg; capsule 701.92 mg. Single determinations.

The sunflower lecithin (Figure 5, Figure 6; Section 6.2) contained 69.8 % phospholipid by weight, or 926 µmol of phospholipid phosphorus per gram, with PC (37.1 P-mol %, 26.5 wt %), PI (25.9 %, 20.0 wt %) and PE (15.7 %, 10.6 wt %) as the principal classes and PA at 5.4 P-mol %. The class

profile is typical of sunflower rather than soybean origin, with a PE/PC ratio of 0.42 and a PI/PC ratio of 0.70 on a phosphorus basis. Three features merit comment. The lyso-PC total of 7.6 P-mol % is high for a standard fluid lecithin; the window labeled 2-LPC in this spectrum spans −0.10 to −0.30 ppm, adjoining the PS window on one side and the PI resonance on the other, and it therefore collects the upfield wing of PS and the low-field wing and shoulder of PI, so that the true lyso-PC content is probably lower and the PI content correspondingly higher. Unassigned resonances account for 2.1 P-mol %, distributed over three regions (4.48–4.66, 0.56–1.15 and 0.16–0.23 ppm). And two small phosphomonoester signals are present, the one near 5.0 ppm labeled LPA and the one near 4.57 ppm left unassigned; as noted in Section 5.7, these assignments need confirmation. The ratio of the two lyso-PC windows (5.3 : 2.3) is, moreover, far from the roughly 90 : 10 equilibrium that acyl migration establishes at alkaline pH[15,26], which again suggests that these windows collect intensity from neighboring lines rather than from the lyso-PC isomers alone; the same is true of the other three samples (Table 11). None of these points affects the principal results (PC and total phospholipid) by more than a few tenths of a percentage point, but each would matter if the lecithin were being tested against a lyso-PC or PA specification.

8.2 PS-enriched sunflower phospholipid powder

The expanded spectrum of the PS-enriched sunflower powder is shown in Figure 14. PS is the largest class, at 32.2 % of the phospholipid phosphorus and 23.7 % by weight of the powder, followed by PI (24.5 %; 18.9 wt %), PA (11.0 %; 7.0 wt %), PE (10.2 %; 6.8 wt %) and PC (6.1 %; 4.4 wt %). The total phospholipid content is 71.6 % by weight (925 µmol P g^{-1}), essentially the same as that of the sunflower lecithin, which is consistent with a material produced by PLD treatment of a whole lecithin and dried without a carrier. The high PI content confirms that a whole sunflower lecithin, not a PC-enriched fraction, was the substrate, since PI is not converted by PLD and would have been largely removed by ethanol fractionation (Section 7.5).

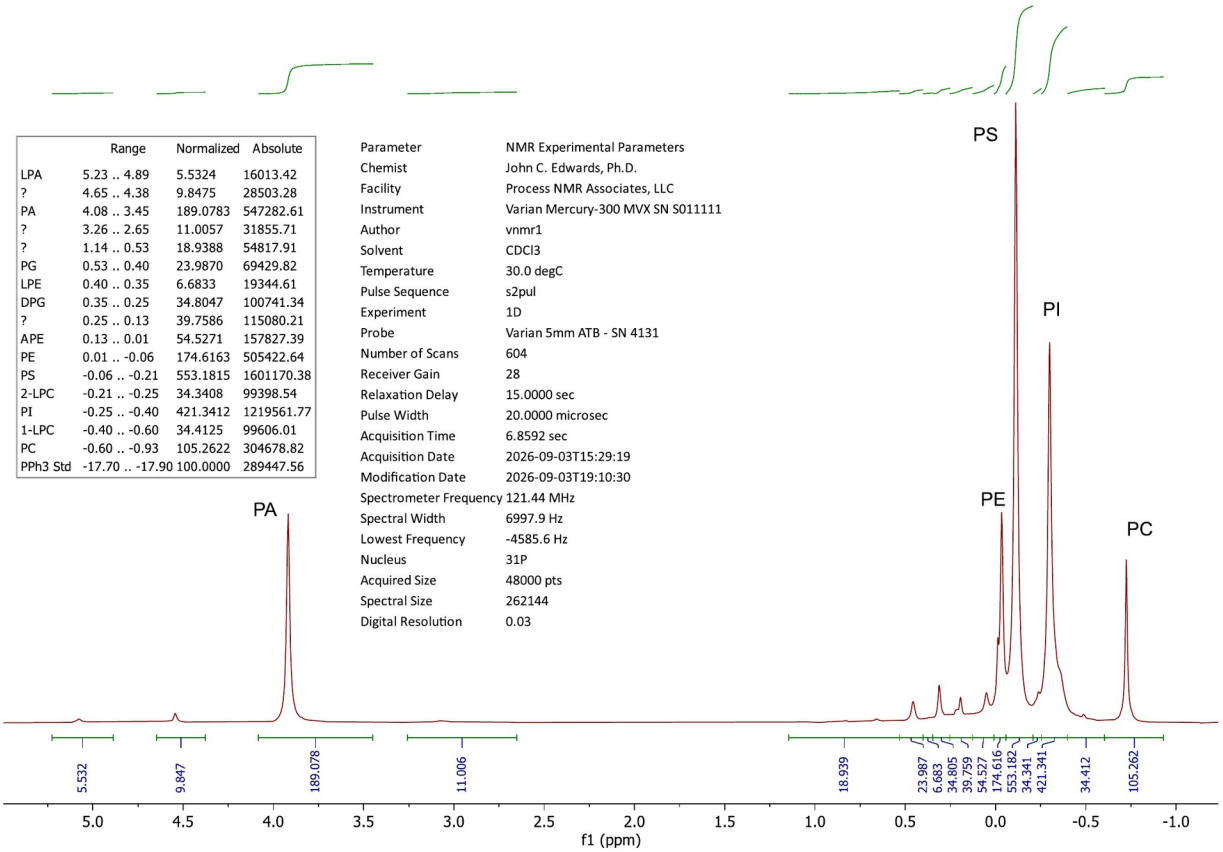


Figure 14. $^{31}P\{^{1}H\}$ NMR spectrum (121.4 MHz, PNA Cs-EDTA reagent) of a PS-enriched sunflower phospholipid powder, expanded to show the phosphomonoester and phosphodiester regions, with the routine integration windows (inset table) and the acquisition parameters as recorded. Genericized PNA data.

This example also illustrates why the basis of a percentage must always be stated. The same PS signal corresponds to 32.2 % of the phospholipid phosphorus and to 23.7 % of the weight of the powder. The first figure answers the question "what fraction of the phospholipid is PS?" and the second "how much PS does the powder contain?", and a specification written as "PS > 18 %" can be tested only against the basis in which it was written. For this powder the specification is met on either basis, but for a product nearer its limit, or for one with a lower total phospholipid content, confusion between the two bases would change the conformity decision. The weight-percent result carries, in addition, the molar-mass assumptions discussed in Section 10.2, whereas the phosphorus-molar result does not.

The minor signals of the powder are more numerous than those of the lecithin: four unassigned regions totaling 4.6 P-mol %, including a broad feature between 2.65 and 3.26 ppm that lies between PA and the phosphodiester envelope, together with increased N-acyl-PE, cardiolipin and PG regions. The signals between PE and PG are interpreted in Section 8.5. The broad feature near 3 ppm lies between the PA dianion and the phosphodiesters and could correspond to PA in a different ionization or cation environment, but it has not been assigned.

8.3 High-purity PS softgel fill

The softgel fill (Figure 15, Figure 16) is a concentrated PS preparation dispersed in an oil carrier. PS accounts for 73.1 % of the phospholipid phosphorus, with PA at 12.2 %, PE at 3.5 %, PI at 1.4 % and PC at only 0.09 %. The low PI and the near-complete absence of PC indicate a PC-enriched substrate converted almost to completion, and the ratio PS/(PS + PA) of 0.86 records the overall selectivity of transphosphatidylation over hydrolysis for the material as supplied. Because the fill is largely oil, total phospholipid is only 22.4 % of the fill weight (292 µmol P g⁻¹), and PS is 17.0 wt %; with a mean fill weight of 619.7 mg this corresponds to 105.3 mg of PS per softgel, against a specification of not less than 100 mg.

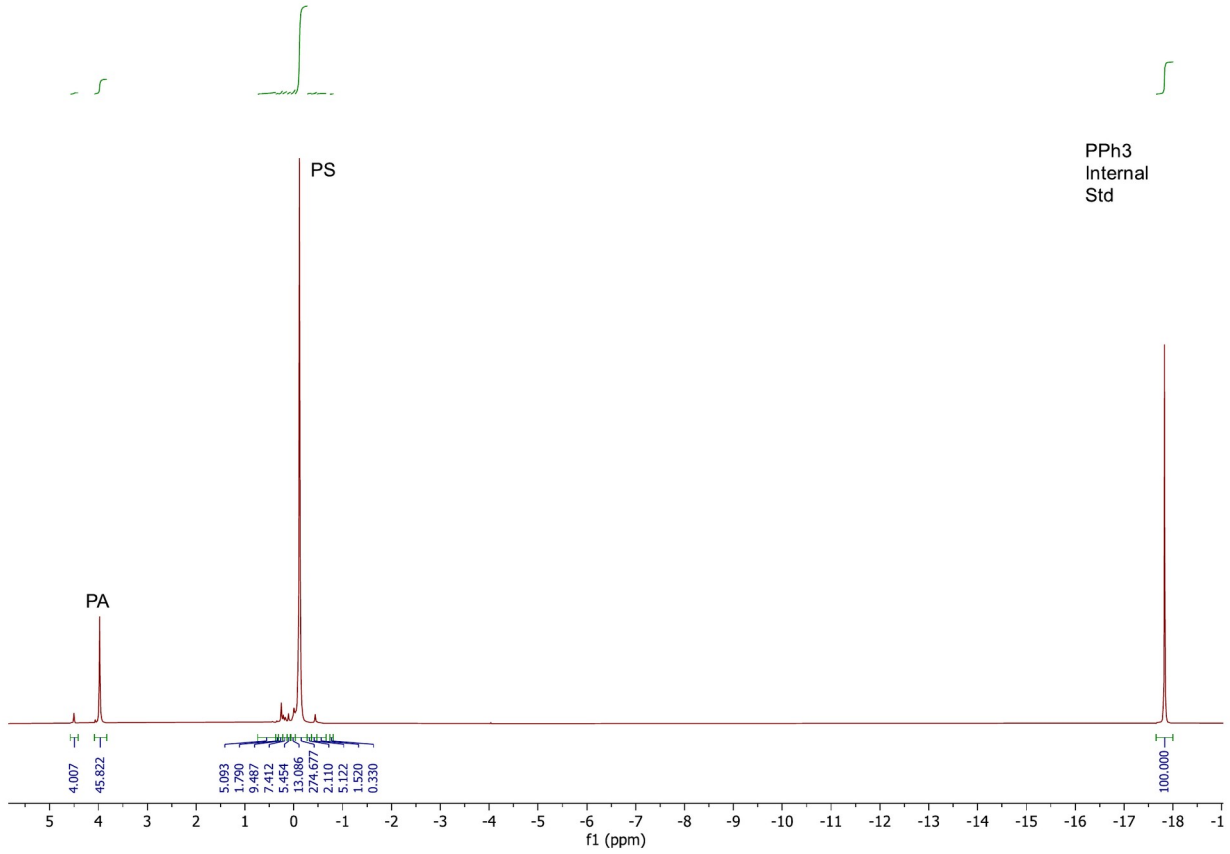


Figure 15. 31P{1H} NMR spectrum (121.4 MHz, PNA Cs-EDTA reagent) of a high-purity PS softgel fill, showing the dominant PS resonance, the PA and LPA signals near 4 ppm and the triphenyl phosphate internal standard at −17.8 ppm (labeled "PPh3 Internal Std" by the routine template). Genericized PNA data.

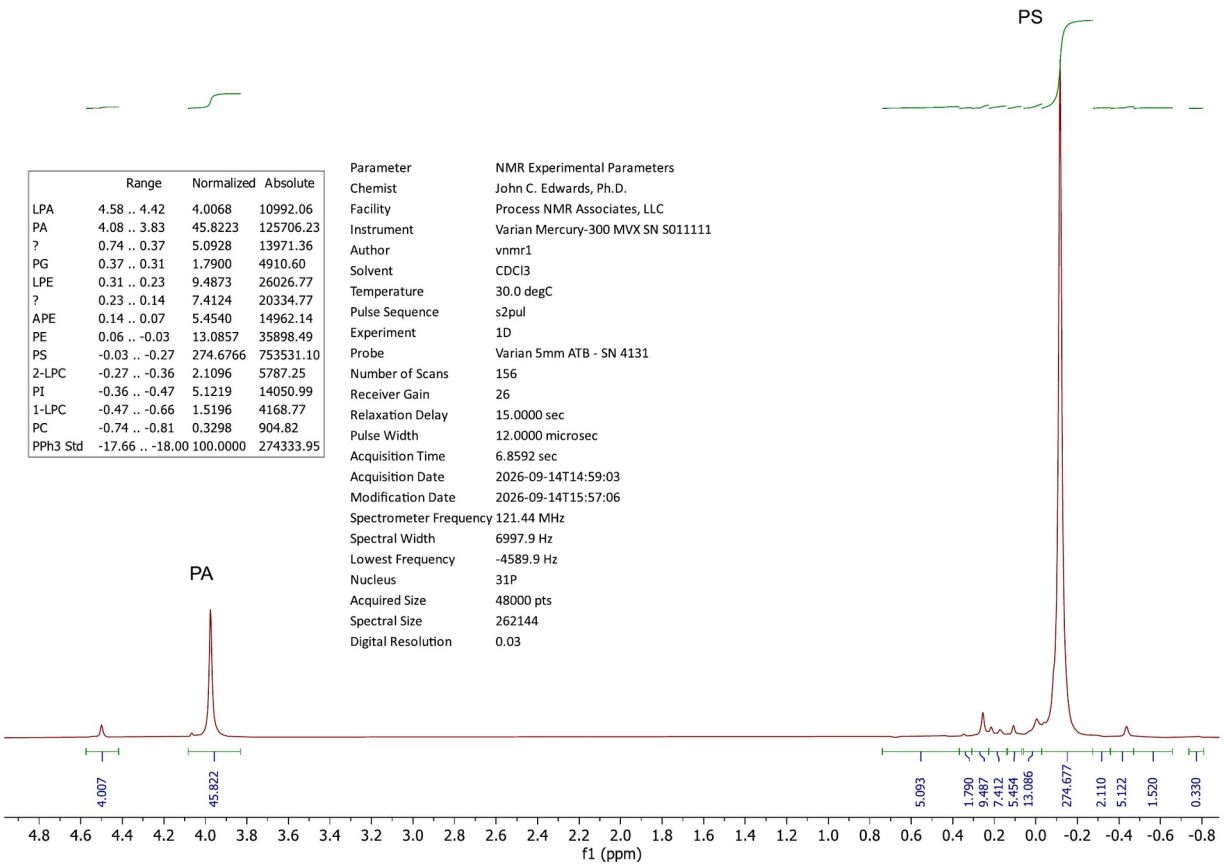


Figure 16. Expansion of the spectrum in Figure 15 with the routine integration windows (inset table) and the acquisition parameters as recorded. Genericized PNA data.

Two features of this spectrum deserve attention. The first is a sharp signal near 0.26 ppm, integrated in the window labeled LPE and amounting to 2.5 P-mol %, together with smaller signals between 0.14 and 0.23 ppm (2.0 %, calculated as cardiolipin) and near 0.10 ppm (1.5 %, N-acyl-PE). In a product whose PE content is only 3.5 %, an LPE content of 2.5 % would imply that about two fifths of the residual ethanolamine phospholipid had been hydrolyzed, which is possible but unusual; the alternative, that these signals include lyso-PS, is discussed in Section 8.5. The second is the result itself. At 105.3 mg per softgel the product exceeds its lower specification limit by 5.3 %, which is comparable to the expanded uncertainty of a single determination by the current procedure (Section 10.2). The conformity statement therefore depends on the decision rule, a point developed in Section 10.3.

8.4 PS capsule blend (stability sample)

The capsule blend (Figure 17) contains a PS concentrate diluted with other ingredients, and the sample analyzed was a stability time point. PS accounts for 56.5 % of the phospholipid phosphorus, PA for 14.7 %, PE for 6.7 %, PI for 5.3 %, and PC for 1.8 %; total phospholipid is 12.6 % of the blend (164 µmol P g^{-1}). PS is 7.39 wt %, which with the stated mean fill weight of 701.9 mg corresponds to 103.7 mg of PS per two-capsule serving against a specification of 100–120 mg

per serving. The ratio PS/(PS + PA) of 0.79 is lower than that of the softgel fill, and the ratio PA/PS of 0.26 is correspondingly higher. Whether this reflects a different PS concentrate or conversion of PS to PA by residual phospholipase D activity during storage cannot be decided from a single time point (ester hydrolysis would produce lyso-PS rather than PA), and it illustrates why stability programs should include the initial time point analyzed by the same procedure and why the 31P class distribution, not only the PS content, should be trended.

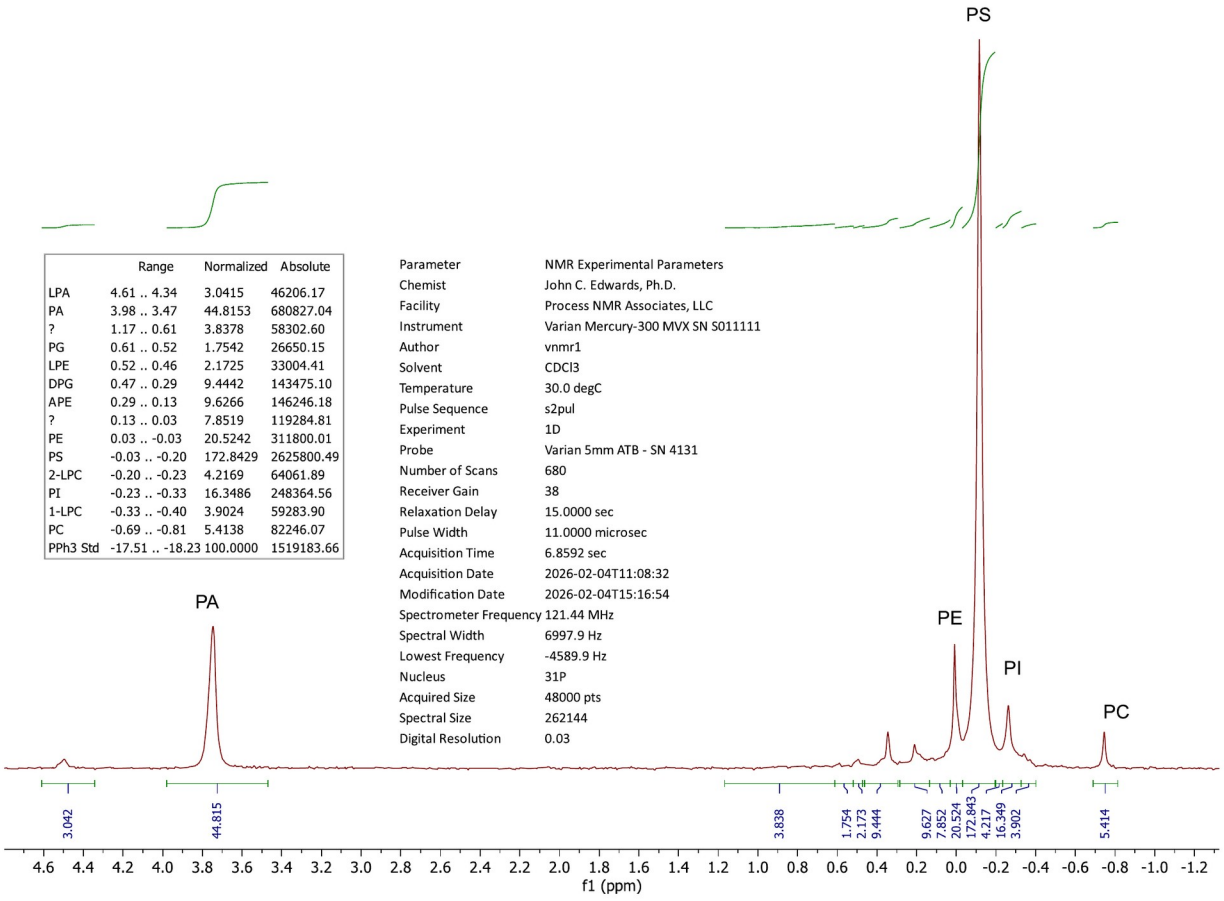


Figure 17. 31P{1H} NMR spectrum (121.4 MHz, PNA Cs-EDTA reagent) of a PS capsule blend (stability sample), expanded to show the phosphomonoester and phosphodiester regions, with the routine integration windows (inset table) and the acquisition parameters as recorded. Genericized PNA data.

The capsule blend is also the sample for which the raw free-induction decay was available, and it was re-processed independently for this paper (Section 8.6). The re-processed spectrum reproduced the routine integrals with a root-mean-square difference of 0.43 integral units (maximum 0.8 units, on the TPP = 100 scale) across the fourteen windows once the referencing offset described in Section 5.7 was applied, which confirms that the routine processing is internally consistent; the differences discussed below arise from the integration model, not from phasing or baseline errors.

8.5 A PI-anchored balance for phospholipase D conversion

Because PI is not a substrate for the phospholipase D used in PS manufacture, the ratio of any class to PI is unaffected by dilution, drying or the removal of water-soluble reagents, and changes in those ratios between a substrate and its product measure the conversion directly. The sunflower lecithin and the PS-enriched sunflower powder of Sections 8.1 and 8.2 were supplied by the same source, and while there is no documentation that the one was made from the other, the pair is used here to illustrate the calculation. Table 12 lists the phosphorus-molar compositions, the PI-normalized ratios and their differences.

Table 12. PI-anchored balance between the sunflower lecithin and the PS-enriched sunflower powder, calculated on the assumption that the powder was produced from a lecithin of the measured composition.

Class	Lecithin (P-mol %)	Powder (P-mol %)	Lecithin X/PI	Powder X/PI	Δ(X/PI)
PC	37.11	6.13	1.431	0.250	−1.181
PE	15.73	10.17	0.607	0.414	−0.192
LPC (both isomers)	7.59	4.00	0.293	0.163	−0.130
PS	1.31	32.21	0.050	1.313	+1.262
PA	5.43	11.01	0.209	0.449	+0.240
LPA	0.29	0.32	0.011	0.013	+0.002
PG	0.64	1.40	0.025	0.057	+0.032
Signals between PE and PG (LPE + DPG + APE + unassigned window)	4.86	7.91	0.187	0.322	+0.135
Other unassigned	1.11	2.32	0.043	0.094	+0.052
PI (anchor)	25.93	24.53	1.000	1.000	+0.000
Total phospholipid P	100.00	100.00	3.856	4.076	+0.219

Apparent conversions: PC 82.5 %, PE 31.7 %; PS formed 1.26 and PA formed 0.24 mol per mol PI; selectivity PS/(PS + PA) of products formed 0.84. The rise in total P per PI (5.7 %) shows that the balance does not close (see text).

If the powder is assumed to have been produced from a lecithin of the composition measured, 82.5 % of the PC and 31.7 % of the PE were consumed, and 1.26 mol of PS and 0.24 mol of PA were formed per mole of PI, which corresponds to a transphosphatidylation selectivity of 0.84 for the products formed; this is similar to the overall PS/(PS + PA) ratio of 0.86 found for the softgel fill in Section 8.3. The lyso-PC decreased by 0.13 mol per mole of PI, and the signals between PE and PG (integrated as LPE, cardiolipin, N-acyl-PE and an unassigned window) increased by the same amount, 0.13 mol per mole of PI. The near-equality of these two changes is consistent with the conversion of lyso-PC to lyso-PS (and a little LPA) by PLD, with lyso-PS resonating in that region. The position is plausible on structural grounds, since in the same reagent the lyso forms of PC and PE resonate 0.52 and 0.38 ppm downfield of their diacyl parents (Section 4.6), which would place lyso-PS roughly 0.3–0.5 ppm downfield of PS, that is between about +0.2 and +0.4 ppm on the routine scale. The same argument applies to the softgel fill (Section 8.3) and to the capsule blend, in which the windows labeled cardiolipin and N-acyl-PE together account for 6.2 % of the phosphorus, an implausibly high value for materials made from PC-enriched substrates. The

hypothesis is testable by standard addition of lyso-PS, and it should be tested before lyso-PS is added to, or excluded from, the assignment table.

The balance does not close exactly. The total phospholipid phosphorus per mole of PI rose from 3.86 in the lecithin to 4.08 in the powder, 5.7 % more than conservation would allow, and the PS + PA formed exceed the PC + PE consumed by about 9 % of the latter. Two explanations are consistent with the direction of the discrepancy and are not mutually exclusive: the powder was not made from this lot, or not from an identical lecithin; or some PI was lost during manufacture, for example with an aqueous wash, which would raise every PI-normalized ratio of the powder. The integration problems noted in Section 8.1 also distort the ratios, but correcting the lecithin's PI for the intensity in its 2-LPC window would lower its total phosphorus per mole of PI and so widen, rather than close, the gap. The exercise is therefore not a proof of provenance, but it shows how the PI anchor turns two independent class profiles into a quantitative process balance, and how the lack of closure itself points to specific analytical and process questions. Applied to samples taken before and after a PLD reaction within one plant, where the substrate is known, the same calculation gives conversion and selectivity without any need for mass recovery data.

8.6 Integration strategy: windows versus line fitting

The raw free-induction decay of the capsule blend was re-processed independently (0.8 Hz exponential line broadening, zero filling to 512 k points, automated phasing, TPP set to −17.80 ppm), and the phosphodiester region between −0.47 and +0.16 ppm was fitted with ten pseudo-Voigt components and a linear baseline (Figure 18). The PS line was found to be essentially Lorentzian (fitted full width at half-height 3.4 Hz), as was the PE line (2.6 Hz), whereas the principal PI-region line (3.2 Hz) required a partly Gaussian shape, and the fitted components were then integrated within the routine windows to show where the intensity of each line is counted (Table 13).

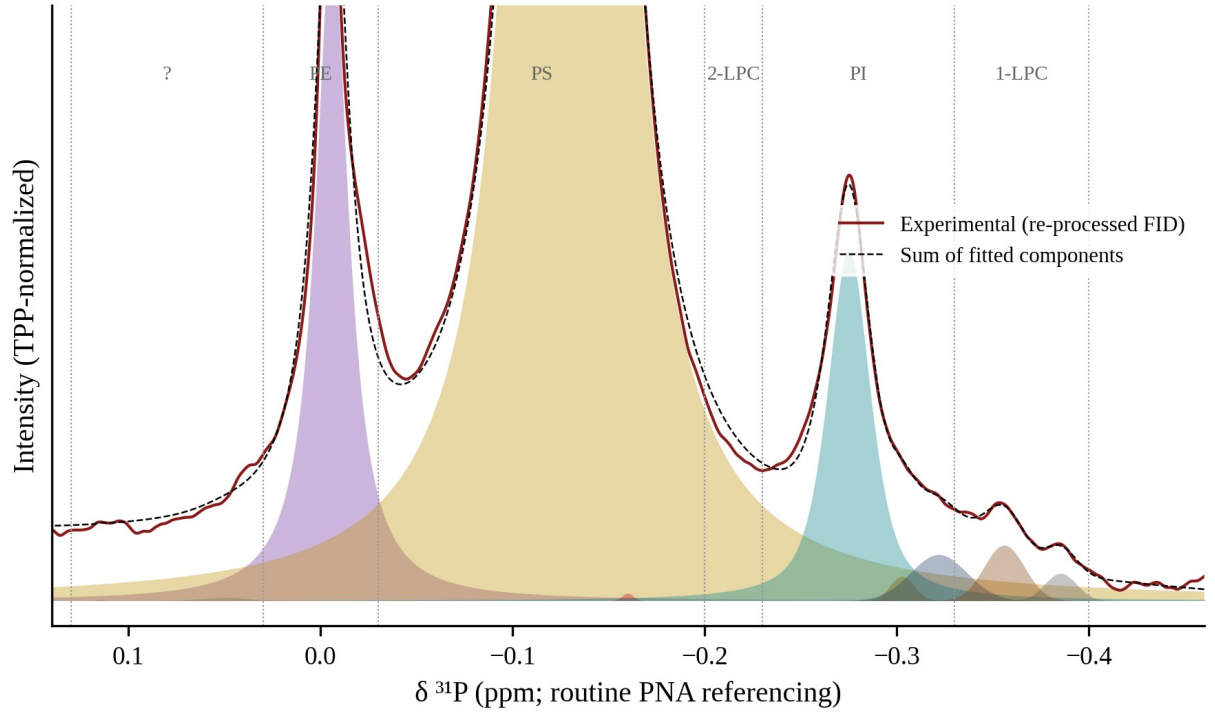


Figure 18. Line fitting of the phosphodiester region of the PS capsule blend (re-processed raw data; routine PNA referencing). The experimental spectrum is shown with the individual fitted components (shaded) and their sum including a linear baseline term (dashed); the vertical dotted lines mark the routine integration windows. The PS line (Lorentzian, 3.4 Hz full width at half-height) extends into the PE, 2-LPC and PI windows. Intensities are normalized to the TPP integral.

Table 13. Distribution of the fitted line intensities of the PS capsule blend among the routine integration windows (TPP window integral = 100).

Window (ppm)	Routine integral (reported)	Re-processed window integral	Principal fitted contributions within the window	Baseline term	Interpretation
Unassigned (0.13 … 0.03)	7.85	7.52	PS wing 1.99; PE wing 1.23; small components 0.08	4.44	No resolved resonance
PE (0.03 … −0.03)	20.52	19.89	PE line 14.45 (of 18.55); PS wing 3.09	2.28	PE ≈ 18.6 by fitting
PS (−0.03 … −0.20)	172.84	173.22	PS line 165.56 (of 185.54); PE wing 2.10; others 0.35	4.87	PS ≈ 185.5 by fitting (+7.3 %)
2-LPC (−0.20 … −0.23)	4.22	3.95	PS wing 3.44; PI-region line 0.28; PE wing 0.04	0.62	No resolved lyso-PC
PI (−0.23 … −0.33)	16.35	16.90	Line at −0.275 ppm 9.70 (of 11.02); PS wing 4.10; lines at −0.303 and −0.322 ppm 1.35	1.53	PI ≈ 12.8 by fitting
1-LPC (−0.33 … −0.40)	3.90	4.14	Lines at −0.356 and −0.385 ppm 1.70; PS wing 1.07	0.59	Minor lines ≈ 1.7

Fitted region −0.47 to +0.16 ppm; ten pseudo-Voigt components and a linear baseline (Appendix A.2). Residual root-mean-square ≈ 4.5 × noise. The APE window (0.29 … 0.13 ppm) lies largely outside the fitted region and is omitted.

The result is instructive. Of the total fitted PS area of 185.5 (on the TPP = 100 scale), only 165.6 falls within the PS window. The remaining 20.0 units, or 10.8 % of the PS line, fall outside it: 14.9 units in the neighboring windows, principally PI (4.1), 2-LPC (3.4), PE (3.1), the unassigned window at 0.03–0.13 ppm (2.0), N-acyl-PE (1.2) and 1-LPC (1.1), and the rest in the far wings beyond the integrated regions. Conversely, the routine PS window collects 2.1 units from the PE line and about 4.9 units from the broad baseline component. The net effect is that the routine PS integral (172.8) is 6.8 % lower than the fitted PS area. The window labeled 2-LPC contains almost nothing but the PS wing, so that no resolved 1-acyl-LPC signal is present in this sample, and the unassigned window between 0.03 and 0.13 ppm contains the wings of PE and PS and a share of the broad component rather than any resolved resonance. If the fitted areas replace the window integrals in this region, PS rises from 56.5 to 62.1 P-mol % and from 7.39 to 7.93 wt % (111.3 mg per serving, or 110.2 mg if the TPP line is also integrated to its full Lorentzian area), PI falls from 5.3 to 4.3 P-mol %, and the lyso-PC and unassigned fractions fall from 2.7 and 3.8 % to 0.6 and 1.3 %.

Two cautions attach to these numbers. The fit required a sloping linear baseline under the whole envelope, whose integral over the fitted region amounts to about 5 % of the phospholipid signal, and the residual after fitting remained about 4.5 times the noise level, which indicates that the line shapes are not perfectly Lorentzian and that some intensity has been assigned to the baseline that may belong to the wings of the lines or to a broad phospholipid component. The fitted areas are therefore themselves model-dependent. What the comparison establishes beyond doubt is that the integration model moves the PS result for this sample by about 7 %, and that fixed narrow windows systematically transfer intensity from a large line to its minor neighbors, inflating the lyso, PI and unassigned fractions of PS products. Because the same truncation affects the internal standard to a lesser degree (its window is wider in relation to its line width), the effect does not cancel in the ratio. The remedy is not to prefer one model on theoretical grounds but to establish, with gravimetrically prepared mixtures of PS and TPP in the actual reagent, which integration procedure returns the true ratio, and then to fix that procedure in the method.

9. Chemometrics and Authentication

9.1 Class profiles as authentication variables

The phospholipid class profile is determined in the first instance by biology: the enzymes of phospholipid synthesis in a soybean, a sunflower seed, a hen's egg, a mammary gland or a krill differ, and so do the resulting class distributions. The profile is then modified by processing, as described in Section 7. Several single features are diagnostic on their own. SM and DHSM are absent from plant lecithins and present at about a quarter of the phospholipid in dairy materials[3]; alkylacyl-PC and its lyso analogue are characteristic of krill[25,52]; phosphonolipids indicate molluscan or other invertebrate origin[24]; and PI is abundant in vegetable lecithins but minor or absent in krill oil and egg yolk. Ratios among the major classes, principally PE/PC and PI/PC, separate soybean from sunflower lecithin (Sections 6.1 and 6.2). Because these variables are measured on a molar basis without calibration, they are more transferable between laboratories than chromatographic peak-area ratios, provided the assignments are correct and the same integration conventions are used.

9.2 Full-spectrum multivariate models

Monakhova and Diehl took the next step and built partial least squares regression models on NMR spectra to quantify the adulteration of sunflower lecithin with soybean lecithin, rather than relying on a few class ratios[46]. They subsequently showed that such models could be transferred between high-resolution spectrometers, which is the prerequisite for using a model developed in one laboratory to control materials in another[47], and they automated the multicomponent integration of lecithin and krill-oil spectra so that the class distribution itself is produced without operator-dependent window placement[13]. Full-spectrum models exploit fine structure, such as the fatty-acid-dependent shoulders on the class resonances[22,23], that class integrals discard, and they can therefore detect differences that a class table would miss. The price is that they are sensitive to exactly the chemical-shift variability described in Sections 3.2 and 5.7. A displacement of 0.05–0.15 ppm in the PI signal between two lecithin matrices is, to a bucketed or full-resolution regression model, a large spectral change unrelated to composition, and it must be removed by alignment or absorbed into the calibration set before the model can be trusted.

9.3 Krill oil fingerprinting and adulteration

Krill oil is the product for which multivariate NMR authentication has been most thoroughly examined. Burri and co-workers fingerprinted krill oils by ^{31}P, ^{1}H and ^{13}C NMR[52], Akanbi and Barrow evaluated the compositional information useful for authentication and for the detection of adulterants[29], and the ^{31}P content test of the USP monograph and the proficiency test of Zailer and co-workers provide the framework within which such results are compared between laboratories[14,42]. The adulteration scenarios of practical concern are the dilution of krill oil with fish oil, which lowers the phospholipid content without changing the class profile, and the fortification of fish oil with vegetable or egg lecithin, which introduces classes (PI, PA and a

soybean or sunflower PE/PC ratio) and lacks others (alkylacyl-PC) in ways that the ³¹P spectrum shows directly. Free fatty acid content and lyso-PC fraction provide further information about the handling history of the oil[53].

9.4 Exploratory principal component analysis of class profiles

To illustrate how class profiles separate by source, a principal component analysis was carried out on nineteen class profiles, four from the PNA analyses of Section 8 and fifteen from the literature: four soybean lecithins or lecithin extracts[3,4,17,25], four dairy materials[3], an egg-yolk lipid and a krill oil[25], and five seafood oils[24]. Ten variables were used (PC, LPC, PE, LPE, PI, PS, PA, SM including DHSM, PG plus cardiolipin, and other), each profile was closed to 100 % on a phosphorus basis, and the data were mean-centered and Pareto-scaled. The first three components explained 44.8, 25.3 and 13.9 % of the variance (Figure 19). The first component is a contrast between PC (loading +0.72) and PS (−0.59) with PA (−0.25), and it separates the three PS products (scores −9 to −14) from the PC-dominated egg and fish or krill materials (+7 to +9); the squid and mussel oils, whose profiles are more diverse, lie nearer the origin. The second component is dominated by PE (+0.67), SM (+0.36) and PI (+0.29) against PS (−0.41), LPC (−0.28) and PC (−0.22), and it places the vegetable lecithins and dairy materials above the marine oils and PS products. The third component contrasts PI (+0.57), PA and the unassigned fraction with SM (−0.63), and it separates the vegetable lecithins (scores +3.5 to +5.5) cleanly from the dairy materials (−4.4 to −5.8). The PNA sunflower lecithin falls within the vegetable-lecithin group, and the PS-enriched sunflower powder lies between that group and the high-purity PS products, as its composition would suggest.

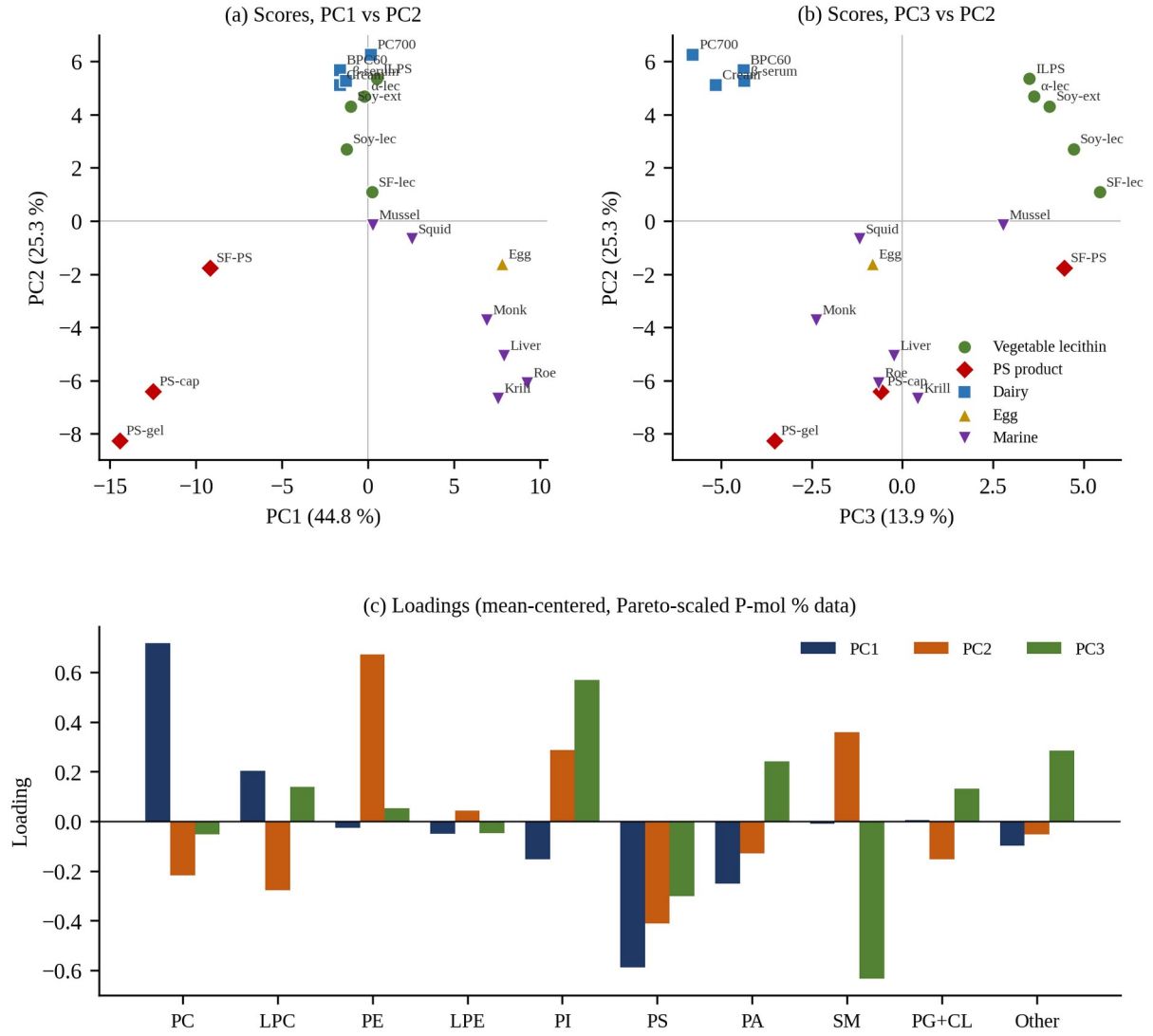


Figure 19. Exploratory principal component analysis of nineteen phospholipid class profiles (four PNA analyses and fifteen literature profiles; ten class variables closed to 100 % on a phosphorus basis, mean-centered and Pareto-scaled). (a) Scores on PC1 and PC2; (b) scores on PC3 and PC2; (c) loadings of the first three components. SF-lec, sunflower lecithin; SF-PS, PS-enriched sunflower powder; PS-gel, PS softgel fill; PS-cap, PS capsule blend; ILPS, certified soybean lecithin standard; other abbreviations identify the literature samples listed in the companion workbook.

The analysis was repeated with a centered log-ratio transformation after replacing zero entries by 0.05 %, which is the more appropriate treatment for closed compositional data. The same four groups emerged, but the order of the components changed: the SM-dominated contrast that separates the dairy materials became the first component (36.3 % of the variance), because the log-ratios of the replaced zeros for SM in the non-dairy samples are large, while the PC/PS and PI contrasts became the second and third. The grouping is therefore robust to the preprocessing, but the apparent importance of individual components is not, and it would be a mistake to attach chemical significance to the variance fractions.

9.5 Limitations and the risk of overinterpretation

This exercise is exploratory and should not be mistaken for a classification model. The nineteen profiles were obtained in five different solvent systems and on three different bases (phosphorus mole percent, weight percent converted to mole percent, and micromoles per gram of wet tissue normalized to 100), the "other" variable has a different meaning in every source, and several of the literature profiles are single samples. The separation of dairy from vegetable lecithins, or of PS products from lecithins, is trivial and would be found by inspection of the class tables; the value of the PCA is in showing that the structure of the data is dominated by a few well-understood chemical contrasts, not in discovering new ones. Closure introduces spurious negative correlations among the class fractions, which is why log-ratio methods are preferred for compositional data. Any authentication model intended for use, whether built on class profiles or on full spectra, requires a calibration set of authentic materials that spans the natural and processing variability of each class of product, an independent validation set, and a test of chance correlation (for example by permutation of the class labels), and it must be rebuilt or re-validated when the sample-preparation system or the integration procedure changes. The number of latent variables in a PLS model should be justified by cross-validation and should remain small relative to the number of independent samples; a model with many buckets and few samples will fit any difference, including differences in shift referencing, and will fail on new materials.

10. Validation, Uncertainty and Decision Rules

10.1 Published performance data

Published validation data for ³¹P phospholipid methods are collected in Table 14. The pattern is consistent. Where the sample is a lecithin or oil dissolved directly in the reagent, repeatabilities and intermediate precisions of 1–4 % are obtained for the major classes: Kato and co-workers reported relative standard deviations below 1 % for PC, PI and PE across a range of pulse intervals[4], van Rijn and co-workers intermediate precisions of 2.2–3.5 % for phospholipids and head groups in oils[15], and Gouilleux and co-workers precisions of 1.6–2.7 % for the major classes at 2 T[17]. Where an extraction precedes the measurement, the within-laboratory reproducibility is larger, 7–24 % for egg phospholipids in mayonnaise, and the ratio of reproducibility to repeatability of 2–5 shows that the sample preparation dominates[16]. Trueness has been assessed by spike recovery (84–92 % for phospholipids and head groups spiked into oil[15]; 96–108 % after extraction from mayonnaise[16]), by comparison with certified values or with ¹H qNMR traceable to a certified reference material[3,4], and by comparison with 2D-TLC and molybdenum-blue phosphorus determination, which agreed for total phospholipid but differed by up to 10 % or more for individual classes[4]. The recovery data of Ahmmed and co-workers for PS and LPC in a chloroform-based reagent (61–86 %) stand out as a warning that recovery cannot be assumed for every reagent and class[25].

Table 14. Published performance characteristics of quantitative ^{31}P NMR methods for phospholipids.

Study	System and internal standard	Matrix	Performance reported	Reference
Kato et al. 2018	Cholate–EDTA; phosphoserine (traceable)	Soybean lecithins; PS supplements	RSD < 1 % for PC, PI, PE across pulse intervals; DOPC purity by SI-traceable ^{1}H qNMR; total PL agrees with molybdenum blue; classes differ from 2D-TLC by up to ≥ 10 %	[4]
MacKenzie et al. 2009	Cholate–EDTA pH 7.1; PMG	Certified soybean lecithin; dairy materials	Agreement with certified values for major classes; TLC values lower; 36 min per analysis	[3]
van Rijn et al. 2020	Deoxycholate–EDTA–TRIS pH 9.0; TIP	Crude and degummed oils	Intermediate precision 2.2–3.5 % (phosphate 11 %); spike recovery 84–92 %; LoD 5 µmol/100 g; practical LoQ 100 µmol/100 g	[15]
Mayar et al. 2020	4 × MeOH extraction + cholate pH 7.5; TMP; 9.4 and 16.4 T	Mayonnaise (egg yolk, EMEY)	RSDr 1.9–15.2 %; RSDR 7.2–24.0 %; recovery 100–108 % (PL), 96–104 % (LPL); LoQ 0.10–0.15 mg g^{-1}	[16]
Gouilleux et al. 2019	$CDCl_3$/MeOH/Cs-CDTA; 1 and 2 T benchtop	Soybean α-lecithin	Relative: precision 1.6–7.9 %, accuracy 0.5–19 % vs 11.7 T; absolute: precision 2.2–5.0 %, accuracy 0.5–8.7 %	[17]
Ahmmed et al. 2021	D_2O/cholate/EDTA vs $CDCl_3$/CD_3OD/EDTA	Standards; lecithin; krill oil; egg lipid	Recovery 83–109 % (cholate) vs 61–101 % ($CDCl_3$); PS 61–86 % and LPC 73–83 % in $CDCl_3$	[25]
Zailer et al. 2018	Cs-EDTA reagent (lecithin/krill method)	Krill oil	Interlaboratory proficiency test toward an official method	[14]
Kaffarnik et al. 2013	Cs-EDTA reagent; 1D and 2D ^{31}P,^{1}H	Cheese and fish lipids	LoD ≈ 4 nmol (2D, 8 h); 2D transfer efficiencies differ by ≈ ± 10 %	[23]
PNA (this work)	Cs-EDTA pH 9.7; TPP	Lecithin; PS powder, softgel, blend	Single determinations; no replicate or recovery data yet; integration model changes PS by about 7 %; T_1 of TPP not measured	Section 8

The PNA method has not yet been validated in this formal sense. The four datasets of Section 8 give single determinations, without replicate preparations, and there are no recovery or intermediate-precision data specific to the PNA reagent. The uncertainty budget developed below therefore combines estimates derived from the literature and from the observations of Sections 5 and 8 with standard metrological treatment of the weighing and reference-material terms. It is a provisional budget whose purpose is to identify the dominant components and to show how the proposed refinements would reduce them; it must be replaced by one based on PNA validation data.

10.2 An uncertainty budget for PS in a finished product

The measurand is the mass of PS per serving of a finished product, calculated with Eq. (3) and Eq. (5). Its relative combined standard uncertainty is the root sum of squares of the relative standard uncertainties of the input quantities and of the method-performance terms, on the assumption that they are independent. The components, their bases and their magnitudes are listed in Table 15 for

two scenarios: the current procedure as it was applied to the samples of Section 8, and the procedure after the refinements proposed in Section 5.8.

Table 15. Provisional uncertainty budget for the mass of PS per serving (relative standard uncertainties, %), for the current procedure and after the refinements of Section 5.8.

Component	Distribution	Current u (%)	Refined u (%)	Refinement
Mass of TPP (≈ 6 mg; u(m) ≈ 0.015 mg)	Normal	0.25	0.10	≥ 15 mg TPP or gravimetric stock
Mass of sample (≈ 340 mg)	Normal	0.01	0.01	—
Purity of TPP (99.95 %; certificate)	Normal	0.05	0.05	—
Molar mass of PS (797 vs ≈ 775–810 g mol^{-1})	Rectangular, ± 2.2 %	1.27	0.30	Declared salt form; MW from fatty-acid profile
Integration model (windows vs line fitting)	Rectangular, ± 3.5 %	2.02	0.80	Validated procedure with gravimetric mixtures
Incomplete relaxation (TPP T_1 not measured; assumed ≤ 4 s)	Rectangular	0.30	0.10	T_1 measured; TR ≥ 7 T_1
Recovery into lower phase (not verified)	Rectangular, ± 3 %	1.73	0.60	Upper-phase analysis; spike recovery
Repeatability of preparation and measurement	Normal	2.00	1.20	Duplicate preparations (mean reported)
Sampling and homogeneity of composite	Normal	1.00	0.70	Composite of ≥ 10 units
Mean fill weight	Normal	0.50	0.30	Fill weight of ≥ 10 units
Signal-to-noise (PS S/N > 1000)	Normal	0.10	0.10	—
Combined relative standard uncertainty, u_c		3.76	1.77	
Expanded relative uncertainty, U (k = 2)		7.51	3.55	
U for the softgel (105.3 mg PS)		± 7.9 mg	± 3.7 mg	
U for the capsule blend (103.7 mg PS)		± 7.8 mg	± 3.7 mg	

Type B estimates based on the literature and on Sections 5 and 8; to be replaced by PNA validation data. Components are assumed independent. The budget is valid only if the T_1 of TPP in the reagent is ≤ about 4 s; at 7 s the results would be biased high by 4.6 % (90° pulse) or 8.3 % (144° pulse).

Four components dominate the current budget. Repeatability of a single preparation and measurement is taken as 2.0 %, consistent with the literature values for directly dissolved samples. The integration model is represented by a rectangular distribution of half-width 3.5 %, half of the 7 % difference between window integration and line fitting found in Section 8.6, which gives a standard uncertainty of 2.0 %. Recovery from the matrix into the lower phase is represented by a rectangular distribution of half-width 3 %, pending recovery experiments (1.7 %). And the molar mass of PS, for which the conventional value of 797 g mol^{-1} lies within the range spanned by the free acids and the sodium salts of the PS species derived from soybean and sunflower lecithins (roughly 775–810 g mol^{-1}), is represented by a rectangular distribution of half-width 2.2 % (1.3 %). Sampling of a capsule blend, the TPP weighing, the fill weight, incomplete relaxation, the purity of the standard and the signal-to-noise ratio contribute less. The combined relative standard

uncertainty for the current procedure is 3.76 %, and the expanded uncertainty with a coverage factor of 2 is 7.5 %. For the softgel of Section 8.3 (105.3 mg per softgel) this corresponds to ± 7.9 mg, and for the capsule blend of Section 8.4 (103.7 mg per serving) to ± 7.8 mg. After the proposed refinements (validated integration, documented recovery, duplicate preparations, a declared molar-mass basis, a larger TPP mass and a measured T_1), the combined relative standard uncertainty falls to 1.77 % and the expanded uncertainty to 3.5 %, or about ± 3.7 mg for both products. The budget assumes that the T_1 of TPP in the reagent does not exceed about 4 s. If it were 7 s, every result would be biased high by 4.6 % with a 90° pulse and by 8.3 % with a 144° pulse (Section 5.4), a bias that no uncertainty budget can legitimately absorb and that must be excluded by measurement.

Two further points should be made. First, the budget treats the integration model as a random component, but the analysis of Section 8.6 shows that it is largely systematic: narrow windows bias the PS result low in a predictable way. A laboratory that has established the bias with gravimetric mixtures should correct for it (and carry the uncertainty of the correction), not merely widen its uncertainty. Second, the molar-mass term disappears entirely if the result is reported in moles of PS per serving, or on a phosphorus-molar basis, and it can be reduced to a few tenths of a percent if the mean molar mass is calculated from a fatty-acid profile and the salt form is declared. The choice of reporting basis is thus not only a matter of convention but a lever on the uncertainty.

10.3 Decision rules near specification limits

ISO/IEC 17025 requires that, when a statement of conformity to a specification is made, the decision rule, including the way measurement uncertainty is taken into account, be agreed with the customer and documented, and that the statement identify the rule applied[18]. Under simple acceptance, the result is compared directly with the limit and the risk of a false acceptance can approach 50 % when the true value lies at the limit. Under guarded acceptance, the acceptance limit is moved inside the specification by a guard band, commonly equal to the expanded uncertainty, so that a result is declared conforming only when the probability that the true value lies outside the specification is small.

The two PS products of Section 8 illustrate the consequences. With the current expanded uncertainty of about ± 7.8–7.9 mg, neither the softgel (105.3 mg against a lower limit of 100 mg) nor the capsule blend (103.7 mg against 100 mg) can be declared conforming under a guarded-acceptance rule, although both conform under simple acceptance. With the refined uncertainty of about ± 3.7 mg, the softgel would conform under guarded acceptance, while the capsule blend, whose result minus its expanded uncertainty is almost exactly 100 mg, would lie at the boundary. If the integration bias identified in Section 8.6 is confirmed by gravimetric validation and corrected, both results would be expected to rise by several milligrams, and the conformity decisions would change accordingly. The change is not uniformly favorable: the line-fitted capsule-blend result of 111.3 mg would clear the lower limit under guarded acceptance, but with the current expanded uncertainty its upper bound (about 119.7 mg) would lie within 0.3 mg of the 120 mg upper limit, so that a two-sided specification of this width leaves almost no room for either a bias or an uncertainty of the present size. These cases show that for PS products formulated close to their label claims the

analytical method, and not only the product, determines whether a lot passes, and that the decision rule must be settled with the client before, not after, a borderline result is obtained. They also show that the most valuable single improvement to the PNA method is the resolution of the integration question, since it affects both the bias and the uncertainty.

10.4 Reference materials, proficiency testing and control charts

No certified reference material for phospholipid class composition is in general circulation, which is one reason the trueness of ³¹P methods has usually been established indirectly. The options available to a routine laboratory are nonetheless adequate. Certified lecithin standards of the kind analyzed by MacKenzie and co-workers[3], pure synthetic phospholipids whose purity has been established by ¹H qNMR against an SI-traceable reference material, as Kato and co-workers did for DOPC[4], and gravimetric mixtures of such phospholipids with the internal standard, provide trueness checks for the major classes. The krill-oil proficiency test described by Zailer and co-workers is the interlaboratory scheme for ³¹P phospholipid analysis most directly relevant to PNA's work[14], and participation in it, or in bilateral comparisons with laboratories using the automated lecithin method of Monakhova and Diehl[13], would provide external evidence of comparability. Internally, an in-house reference lecithin and an in-house PS material, analyzed with every batch and plotted on control charts of the principal class contents and of the total phospholipid, would detect drift in the reagent, the spectrometer or the integration practice long before it affected a client result.

11. Critical Assessment

11.1 What the method does well

The strengths of ^{31}P NMR for phospholipid analysis are not in dispute and have been confirmed repeatedly in the literature reviewed here. The method counts phosphorus atoms, so a single internal standard quantifies every class, including classes for which no reference material exists; it needs no response factors and is indifferent to fatty-acid composition, which chromatographic detectors are not; it resolves classes that co-elute in common chromatographic systems, such as SM and DHSM or the two lyso-PC isomers; and it tolerates complex finished-product matrices because only phosphorus is observed[3–5,8]. The PNA implementation adds a large sample capacity, which suits heterogeneous products, and an alkaline reagent that removes PA from the crowded phosphodiester region. The four datasets of Section 8 show narrow lines, high signal-to-noise ratios and principal-class results that are internally consistent and, for the one dataset that was re-processed from raw data, reproducible. What follows concerns the points on which the method is vulnerable, in the order of their likely effect on a reported result, and it is written from the standpoint of a reviewer or an opposing expert looking for the weakest link.

11.2 Integration is the largest single source of error

The analysis of Section 8.6 shows that, for a PS product, the choice between narrow analyst-placed windows and line fitting changes the PS result by about 7 % and redistributes a tenth of the PS signal into the PI, PE, lyso-PC and unassigned windows. This is larger than every other identified component of the uncertainty budget, it is systematic rather than random, and it is not documented in the current method. Its consequences are two-fold. The principal class is biased low, which for PS products formulated near their label claims can decide conformity; and the minor classes adjacent to a large line are biased high, so that lyso-PC, PI and "other phospholipid" results for PS and high-PC products are overstated and could be mistaken for evidence of degradation or impurity. The variability of the TPP window, from 0.20 to 0.72 ppm in four analyses, introduces a between-analysis bias of up to about 2.6 % in every absolute result. An opposing expert would not need to argue that the method is wrong, only that its integration rule is undefined and that its results therefore depend on the analyst. A second systematic effect, potentially larger still, has not been examined at all: the T_1 of the internal standard in the PNA reagent is unknown, and because the repetition time is fixed, a T_1 of 7 s rather than 3–4 s would bias every absolute result high by about 5 % with a 90° pulse and by about 8 % with the 144° pulses recorded for two of the four analyses (Section 5.4). Unlike the integration bias, this one would inflate the PS content of a finished product, and it is the first question an expert reviewer should be expected to ask.

11.3 Assignment rests on a table that does not describe the reagent

The shift table printed in the routine report template lists positions (PA 1.50 ppm, LPA 2.78 ppm, PS −0.18 ppm, PE −0.07 ppm) that correspond to a near-neutral reagent, not to the alkaline PNA reagent in which the spectra are recorded, and the assignments made in practice rely on the

analyst's experience rather than on that table. Several assignments have not been confirmed by standard addition in the PNA reagent: the two phosphomonoester signals near 4.5 and 5.0 ppm, which are labeled differently in lecithin and PS-product analyses; the lyso-PC isomers, whose labels follow a convention opposite to the chemically unambiguous one and whose window integrals, in ratios between 1:1 and 2.3:1 in the four analyses, are incompatible with the roughly 90:10 equilibrium expected at alkaline pH; and the minor signals between PE and PG in PS products, which are currently integrated as LPE, cardiolipin and N-acyl-PE but which, on the evidence of Section 8.5, may include lyso-PS. Unassigned signals are reported at a nominal molar mass of 770 g mol^{-1}, which is defensible as a convention but should be flagged in the report as an estimate. None of these points is likely to change a principal-class result by more than a few tenths of a percent; all of them affect the minor-class results that clients increasingly specify, such as lyso-PC maxima and PA limits.

11.4 Partition into the measured phase has not been demonstrated

The PNA procedure measures only the lower phase of a chloroform–methanol–water partition, and it assumes that every phospholipid class is recovered in that phase. The literature contains direct evidence that this assumption can fail: lower recoveries of PS and LPC in a chloroform-based reagent than in cholate[25], and partial transfer of lysophospholipids to the upper phase, strongly dependent on the oil load of the lower phase[16]. The PNA reagent differs from both of those systems in its counter-ion, its pH and its volume ratio, and it may well recover all classes quantitatively; but until recovery has been demonstrated for each product type, and in particular for oil-based softgel fills and lyso-rich materials, an opposing expert could argue that the PS and lyso results are lower bounds rather than measurements. The same argument applies to mineral-containing formulations, in which the 0.2 mmol of EDTA in the reagent could be insufficient to release PS and PA from calcium or magnesium.

11.5 Reporting conventions change the numbers

The distinction between the phosphorus mole percentage of a class and its weight percentage in the sample is fundamental, and a report that states one where the other is meant can mislead the client by a large factor; in the PS-enriched powder of Section 8.2 the two are 32.2 % and 23.7 %. The weight percentage depends in addition on the molar mass assigned to each class, and for PS the conventional value of 797 g mol^{-1} lies between the free acids and the sodium salts of the common species, so that the result for a product supplied as the calcium or sodium salt, or made from a substrate with an unusual fatty-acid composition, carries a bias of up to about 3 %. Reporting the molar content, stating the molar-mass basis, and reserving weight percentages for results expressed explicitly on that basis would remove the ambiguity.

11.6 Documentation must match what was done

The acquisition parameters stated in routine reports should be those recorded in the spectrometer files. In the analyses examined for this paper, the spectral width, acquisition time, relaxation delay

and pulse width actually used (6.998 kHz, 6.86 s, 15 s and 11–20 µs) differed from the nominal values printed in the report template, the internal standard was labeled with the symbol of a different compound, and the spectrum was referenced about 0.07 ppm away from the stated position of the standard. None of these discrepancies need invalidate a result, and the as-run relaxation delay and acquisition time are, if anything, longer than the nominal ones, although the pulse widths varied from about 79° to 144°; but an assessor would treat each as a nonconformity, and an opposing expert would use them to question the reliability of everything else in the report. The remedy is procedural and inexpensive: report templates populated from the parameter files, a single named standard, and a documented referencing step.

11.7 Single determinations near specification limits

The PS results of Section 8 for the two finished products lie 3.7 % and 5.3 % above their lower specification limits, and the provisional expanded uncertainty of a single determination is 7.5 %. Both products conform under simple acceptance and neither under guarded acceptance. Neither report stated a decision rule or an uncertainty, and neither was based on replicate preparations. This is the most consequential exposure in the current practice, because it concerns the conformity statement itself, and it is resolved not by changing the chemistry but by validation, by duplicate preparations for release testing, and by agreeing the decision rule with each client[18].

11.8 The strongest counterarguments

Three counterarguments deserve to be addressed directly. The first is that a validated chromatographic method with class-specific calibration would be preferable for regulatory testing of PS products. The response is that ³¹P NMR is closer to a primary ratio method than any chromatographic technique, because its response factor is fixed by the number of phosphorus atoms; but that claim is only as strong as the integration and recovery that underlie it, and both must be demonstrated before the method can be defended as superior. The second is that the aqueous cholate system, which gave better recoveries of PS and LPC in one direct comparison, should replace the biphasic reagent[25]. The response is that the cholate system has its own weaknesses for these products (PI close to PC, limited capacity for oil-rich samples, CSA broadening at high field, and the need to exclude water-soluble phosphorus), and that the correct course is a direct comparison of the two reagents on the product types that PNA tests, which would either justify the current choice or indicate where the cholate system should be used instead. The third is that benchtop instruments could make the analysis cheaper and more widely available. The published benchtop data and the simulation of Section 3.8 show that this is realistic for total phospholipid and for the major classes of simple lecithins, but not for the class distribution of PS products or for lyso-isomer determination, which require the resolution of a high-field instrument[17].

12. Conclusions and Recommendations

Phosphorus-31 NMR is the most direct method available for the quantitative class analysis of lecithins and phospholipid products. It resolves the phospholipid classes of soybean, sunflower, rapeseed and niche-oilseed lecithins, egg yolk, milk-fat-globule-membrane ingredients, krill oil and marine tissues, together with the lyso, N-acyl, ether-linked and phosphono species that characterize individual sources and processing histories, and it quantifies all of them against one internal standard. Across the processing routes reviewed in Section 7 it answers specific questions at twenty-seven checkpoints, from the non-hydratable phosphatides of crude oil to the stability of PS in finished capsules, and the PI-anchored balance of Section 8.5 shows how two class profiles can be turned into a quantitative account of a phospholipase D conversion. Its principal limitations are chemical-shift variability with the sample matrix, which makes assignment a matter of evidence rather than of tables, the dependence of the result on the integration model when lines overlap, and, in biphasic reagents, the assumption that every class partitions into the measured phase.

The current PNA method is sound in its chemistry and produces narrow, well-resolved spectra, but its integration rule, its assignment table, its recovery and its documentation have not yet been brought to the standard that ISO/IEC 17025 accreditation, and the scrutiny of a contested result, will require. The following actions are recommended, in order of priority. The integration procedure should be validated with gravimetric mixtures of PS, PC and PE standards with triphenyl phosphate in the PNA reagent, and either line fitting with a documented model or rule-based windows of fixed width relative to the line width should be adopted for the standard and the analytes alike. Recovery into the lower phase should be demonstrated for each product type by analysis of the upper phase and by standard additions, with particular attention to oil-based softgel fills, lyso-rich materials and mineral-containing blends. The assignment table should be re-measured in the PNA reagent at pH 9.7 with authentic standards, including lyso-PS, lyso-PI and both LPA isomers, and the unambiguous lyso-isomer nomenclature should be adopted. The T_1 of triphenyl phosphate and of the principal classes should be measured in the reagent and the pulse width calibrated and fixed. Results should be reported as molar contents and phosphorus mole percentages with the molar-mass basis of any weight percentage stated, and report templates and calculation workbooks should be generated from, and verified against, the recorded parameters and the equations of Section 3.7. Finally, an uncertainty budget based on PNA validation data should replace the provisional budget of Section 10.2, duplicate preparations should be used for release testing, in-house reference materials should be charted, and decision rules should be agreed with clients before results near specification limits are reported.

For the industry more broadly, three conclusions follow. Class compositions should always be quoted with their basis (phosphorus mole percent, mole percent of molecules or weight percent of sample, and in the last case the molar masses used), since literature values on different bases are routinely compared as if they were equivalent. The preparation system should be chosen for the question: biphasic cesium-EDTA reagents for lecithins, concentrates and finished products,

cholate for dairy materials and whenever water-soluble phosphorus must be observed, and buffered deoxycholate for degumming studies. And the most informative use of ^{31}P NMR in process control is often not the absolute content of the principal class but the complete class balance before and after a process step, which no other single technique provides.

Appendix A. Methods Used for Synthesized Spectra, Re-processing and Calculations

A.1 Synthesized spectra

All synthesized spectra in this paper were calculated as sums of pseudo-Voigt lines (85 % Lorentzian, 15 % Gaussian) whose areas are proportional to the phosphorus-molar amounts of the classes in the stated composition, with Gaussian noise added at a fixed fraction of the largest signal (random-number seed 20260923, so that every figure can be regenerated exactly). Unless otherwise stated the line width was 3.0 Hz at 121.44 MHz, with 5.0 Hz for PA, 6.0 Hz for LPA and 6.0 Hz for unassigned signals. Spectra on the PNA scale use the nominal positions listed in Table 16, which were taken as typical values from the four PNA datasets; spectra on literature scales use the shift tables of Mayar et al.[16], MacKenzie et al.[3], Bettjeman et al.[24] and van Rijn et al.[15] as stated in the captions. The positions given for alkylacyl-PC, lyso-alkylacyl-PC and lyso-PS on the PNA scale are estimates and are marked with an asterisk in the figures. For the field-strength simulation (Figure 2) the line width of each phosphodiester class was modeled as $\sqrt{[(0.012\ \text{ppm}\cdot\nu_0)^2 + (1.8\ \text{Hz})^2]}$, that of PA and LPA as $\sqrt{[(0.025\ \text{ppm}\cdot\nu_0)^2 + (2.5\ \text{Hz})^2]}$, and the noise was scaled so that the signal-to-noise ratio varied as $B_0^{3/2}$ for equal acquisition time. The compositions used are listed in Table 16; except where a literature source is named, they are representative or illustrative and are not measurements.

Table 16. Compositions and scales used for the synthesized spectra (phosphorus-molar units unless stated).

Figure and panel	Shift scale	Composition
Nominal PNA-scale positions (ppm)	PNA	LPA 4.50; PA 3.92; unassigned 0.62; PG 0.48; LPE 0.40; LPS* 0.33; DPG 0.28; APE 0.13; PE 0.02; PS −0.10; 1-acyl-LPC −0.25; PI −0.34; 2-acyl-LPC −0.50; PC −0.77 (AAPC* −0.72; LAAPC* −0.20)
Soybean products, Figure 4(a) fluid lecithin	PNA	PC 35; PE 31; PI 18; PA 8; 1-acyl-LPC 1.7; 2-acyl-LPC 0.3; APE 1.5; PG 1; DPG 0.8; LPE 1.2; PS 0.5; LPA 0.4; unassigned 0.6
(b) PC-enriched fraction	PNA	PC 62; PE 20; PI 3; PA 2; 1-acyl-LPC 6.2; 2-acyl-LPC 0.8; APE 1; PG 1; LPE 2; DPG 0.5; PS 0.3; LPA 0.2; unassigned 1
(c) PI/PE-enriched fraction	PNA	PC 12; PE 30; PI 32; PA 16; 1-acyl-LPC 0.9; 2-acyl-LPC 0.1; APE 2; PG 1; DPG 1.5; LPE 1; PS 1; LPA 1; unassigned 1.5
(d) High-purity PC	PNA	PC 94; 1-acyl-LPC 2.6; 2-acyl-LPC 0.4; PE 1; PA 0.5; unassigned 1.5
(e) PLA_2-hydrolyzed lecithin	PNA	PC 23; 1-acyl-LPC 13.5; 2-acyl-LPC 1.5; PE 20; LPE 11; PI 18; PA 6; LPA 2.5; APE 1.5; PG 1; DPG 0.8; PS 0.4; unassigned 0.8
PLD time course, Figure 12, t = 0 h	PNA	PC 62; PE 20; PI 3; PA 2; 1-acyl-LPC 6.2; 2-acyl-LPC 0.8; APE 1; PG 1; LPE 2; DPG 0.5; PS 0.3; LPA 0.2; unassigned 1
t = 1 h	PNA	PC 48; PE 18.5; PI 3; PA 4.1; 1-acyl-LPC 5.5; 2-acyl-LPC 0.7; APE 1; PG 1; LPE 2; DPG 0.5; PS 13.6; LPA 0.3; LPS* 0.7; unassigned 1
t = 3 h	PNA	PC 28.9; PE 15.9; PI 3; PA 7.1; 1-acyl-LPC 4.2; 2-acyl-LPC 0.5; APE 1; PG 1; LPE 2; DPG 0.5; PS 32.4; LPA 0.5; LPS* 1.9; unassigned 1
t = 8 h	PNA	PC 8.1; PE 10.8; PI 3; PA 10.7; 1-acyl-LPC 2.2; 2-acyl-LPC 0.3; APE 1; PG 1; LPE 2; DPG 0.5; PS 54.7; LPA 0.8; LPS* 3.9; unassigned 1
t = 24 h	PNA	PC 0.1; PE 3.2; PI 3; PA 12.8; 1-acyl-LPC 0.3; APE 1; PG 1; LPE 2; DPG 0.5; PS 68.2; LPA 1.1; LPS* 5.8; unassigned 1

Figure and panel	Shift scale	Composition
Egg yolk, Figure 7(a)	Mayar et al.	PC 74; PE 17; SM 2.5; LPC (major) 2.0; LPC (minor) 0.3; PI 1.5; LPE (major) 0.7; LPE (minor) 0.2; minor 1.8
Enzyme-modified yolk, (b)	Mayar et al.	LPC (major) 55; LPC (minor) 6; LPE (major) 12; LPE (minor) 1.5; PC 15; PE 4; SM 2.5; PI 1; minor 3
Cream polar lipids, (c)	MacKenzie et al.	PC 26.5; PI 7.5; PS 11.7; LPC 1.1; PE 26.7; SM 20.8; DHSM 3.9; LPE 1.8
Buttermilk protein concentrate, (d)	MacKenzie et al.	PC 26.4; PI 7.6; PS 11.5; LPC 0.7; PE (incl. lactosyl-PE) 31.2; SM 16.9; DHSM 4.6; LPE 1.0
Krill oil, Figure 8(a)	PNA (estimated)	PC 40.3; AAPC* 5.98; 1-acyl-LPC 17.3; 2-acyl-LPC 1.85; LAAPC* 1.57; PE 2.33; LPE 0.93; LPE (minor) 0.90; APE 1.89; CL 1.79; PG 0.16; PA 0.3
Hoki roe oil, (b) (µmol g^{-1})	Bettjeman et al.	PC 12.33; PCP 0.74; LPC 0.53; LPCP 0.87; PE 0.40; PA 0.09; CL 0.77; LDPG 0.24
Squid mantle oil, (c) (µmol g^{-1})	Bettjeman et al.	PC 3.57; PCP 0.92; PE 1.13; PEP 0.32; SM 0.49; DHSM 0.31; PS 0.48; PI 0.22; LPC 0.20; PG 0.34; PA 0.27; LDAG-AEP 0.52; DAG-AEP 0.04; minor others 0.17
Degumming, Figure 10(a) water-degummed oil (µmol/100 g)	van Rijn et al.	PC 250; PE 200; PI 150; PA 180; 1LPC 8; 1LPE 6; P_i 10
(b) after PLC (PC/PE-specific)	van Rijn et al.	PC 25; PE 60; PI 150; PA 180; CP 225; EP 140; P_i 12; 1LPC 8; 1LPE 6
(c) after phospholipase A	van Rijn et al.	PC 40; PE 30; PI 60; PA 40; 1LPC 190; 2LPC 21; 1LPE 150; 2LPE 17; LPI 90; LPA 140; P_i 10
Field-strength simulation, Figure 2	PNA	PS capsule blend of Section 8.4: PS 56.5; PA 14.7; PE 6.7; PI 5.3; unassigned 3.8; DPG 3.1; APE 3.1; PC 1.8; LPC 1.4 + 1.3; LPA 1.0; LPE 0.7; PG 0.6

* Estimated positions. Compositions without a named source are representative or illustrative.

A.2 Re-processing and line fitting of the capsule-blend spectrum

The raw free-induction decay of the capsule-blend analysis (48 000 complex points, spectral width 6997.9 Hz, ^{31}P frequency 121.435 MHz) was read with the nmrglue library, multiplied by an exponential window corresponding to 0.8 Hz line broadening, with the first point halved, zero-filled to 524 288 points and Fourier transformed. Zero- and first-order phase corrections were optimized automatically by minimizing the entropy of the first derivative of the real spectrum with a penalty on negative intensity. The chemical-shift axis was set so that the TPP resonance lay at −17.80 ppm, and the routine integration windows were applied after a uniform displacement of −0.066 ppm, the value that minimized the root-mean-square difference between the re-computed and the reported window integrals. The region from −0.47 to +0.16 ppm, normalized to the TPP window integral, was fitted by non-linear least squares (trust-region reflective algorithm) with ten pseudo-Voigt components and a linear baseline; each center was constrained to ±0.02 ppm of its starting position, each full width at half-height to 0.004–0.09 ppm and each Lorentzian fraction to 0–1, and component areas were calculated analytically from the fitted heights, widths and Lorentzian fractions. Two very small components converged to widths at or below the applied line broadening (0.5 and 0.8 Hz) and represent noise features; their areas, like that of a third small component near 0.05 ppm, are below 0.1 unit and negligible. The window-by-window distribution of each component in Table 13 was obtained by numerical integration of the fitted functions over each window.

A.3 Recalculation of the PNA results

The integrals recorded in the routine integration tables (TPP = 100) were recalculated with Eq. (2), Eq. (3) and Eq. (4), using a TPP purity of 99.95 %, a TPP molar mass of 326.28 g mol^{-1}, the class molar masses of Table 1, two phosphorus atoms per molecule for cardiolipin and one for every other class, and a molar mass of 770 g mol^{-1} for unassigned signals, which were summed. Total phospholipid phosphorus (µmol P g^{-1}) was calculated as the sum of all phospholipid integrals multiplied by the amount of TPP and divided by the sample mass. Milligrams per serving were calculated with Eq. (5) from the stated mean fill weight and the number of units per serving. All calculations, together with the PI-anchored balance, the line-fitting results, the uncertainty budget and the PCA, are reproduced with live formulas in the companion workbook.

A.4 Principal component analysis

The class profiles used in Section 9.4 were taken from the recalculated PNA results and from the published tables of the sources cited, converted where necessary to a phosphorus-molar basis, grouped into ten variables and closed to 100 %. The data were mean-centered and Pareto-scaled (each centered variable divided by the square root of its standard deviation) and decomposed by singular value decomposition. For the log-ratio variant, zeros were replaced by 0.05 %, the profiles re-closed, and the centered log-ratio transform applied before centering and decomposition. Inputs, preprocessing parameters, scores, loadings and explained variances for both variants are given in the companion workbook.

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Summary

In this white paper the use of phosphorus-31 NMR spectroscopy for the quantitative analysis of lecithins and phospholipid products from soybean, sunflower, rapeseed and niche oilseeds, egg yolk, dairy milk-fat-globule-membrane materials, krill oil, fish roe and shellfish has been reviewed, and the method applied at Process NMR Associates has been described and critically assessed. The chemical basis of the technique was set out: the ^{31}P chemical shift is governed by the head group, so that each phospholipid class gives a resolved resonance, and the integral counts phosphorus atoms, so that all classes are quantified against a single internal standard without response factors. The dependence of the shifts on pH, counter-ion, solvent composition, temperature and matrix was shown to be the principal obstacle to assignment, and the phosphomonoesters PA and LPA were shown to move by several parts per million with pH and medium. Biphasic cesium-EDTA reagents, monophasic organic reagents and aqueous cholate and deoxycholate micelles were compared, and their relative merits for lecithins, finished products, dairy materials and degumming studies were identified.

The PNA procedure, a biphasic $CDCl_3$/methanol/cesium-EDTA method at pH 9.7 with triphenyl phosphate as internal standard and measurement at 121.4 MHz with inverse-gated decoupling, was described as actually executed, including the recorded acquisition parameters and the integration windows used in four recent analyses. The manufacturing routes of fluid, deoiled, fractionated, enzyme-modified, high-PC and PS products, and of egg, dairy and marine phospholipids, were mapped against twenty-seven ^{31}P NMR checkpoints. Genericized PNA spectra of a sunflower lecithin, a PS-enriched sunflower powder, a high-purity PS softgel fill and a PS capsule blend were presented with recalculated results; a PI-anchored balance was used to quantify an apparent phospholipase D conversion, and the evidence that lyso-PS may be hidden among signals currently assigned to other minor classes was examined. Re-processing and line fitting of a raw free-induction decay showed that the integration model changes the PS result of a capsule blend by about 7 % and inflates the adjacent minor classes, which makes integration the largest quantified source of error, while the unmeasured relaxation time of the internal standard was identified as a potential bias of similar or greater size. Synthesized spectra illustrated products and process stages not represented by PNA data and the effect of magnetic field strength, including benchtop instruments, and an exploratory principal component analysis showed that class profiles separate vegetable, dairy, egg, marine and PS materials along a few chemically interpretable contrasts. A provisional uncertainty budget gave an expanded uncertainty of about 7.5 % for PS per serving by the current procedure and about 3.5 % after the proposed refinements, and it was shown that for products formulated close to their label claims the decision rule determines conformity. Validation of the integration procedure and of recovery into the measured phase, re-measurement of the assignment table in the PNA reagent, measured relaxation times, explicit reporting bases, and agreed decision rules were identified as the priorities for operation under ISO/IEC 17025.