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Phospholipids in Whey Protein Ingredients: ^{1}H, ^{13}C and ^{31}P NMR Analysis, and a Review of NMR Studies of Whey Proteins

A methodological review and proposed analytical protocol

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Abstract

The phospholipid fraction of whey protein ingredients is small in mass but disproportionate in technological and commercial consequence. Whey protein concentrates (WPC), whey protein isolates (WPI) and the microfiltration co-product now marketed as whey protein phospholipid concentrate (WPPC) inherit fragments of the milk fat globule membrane (MFGM) that partition into the serum phase during cheesemaking, and the phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylserine (PS) and phosphatidylinositol (PI) carried on those fragments influence foaming, emulsification, oxidative stability, flavor and the nutritional claims attached to MFGM-enriched ingredients. In this white paper the use of ^{1}H, ^{13}C and ^{31}P NMR for the characterization and quantification of these phospholipids is reviewed and a practical, critically assessed methodology is proposed for a mid-field (7.05 T) analytical laboratory. ^{31}P NMR is shown to be the technique of record for class-level quantification: each phosphodiester headgroup yields a single resonance whose integral is proportional to molar amount, no authentic standard is required for identification, and lysophospholipids and dihydrosphingomyelin (DHSM) are resolved where chromatographic methods merge or lose them. The chemistry governing ^{31}P chemical shifts (solvent system, pH, temperature, divalent cations and acyl unsaturation), relaxation and nuclear Overhauser behavior, internal-standard strategies, and the counter-intuitive finding that fields above 9.4 T offer no advantage for micellar preparations are examined, together with the arithmetic that shows why 24 MHz benchtop ^{31}P can resolve PC from SM but cannot resolve the PE/PS/DHSM/SM cluster. ^{1}H NMR is positioned as the structural and stoichiometric complement (choline $N^{+}(CH_3)_3$, sphingoid olefins, plasmalogen vinyl ethers and acyl chains) and as the tool of choice for the aqueous whey phase, where free choline, glycerophosphocholine, phosphocholine, lactose and organic acids report on phospholipid hydrolysis and process history. ^{13}C NMR is assessed as a lower-sensitivity but uniquely informative probe of the glycerol backbone, sn-1/sn-2 acyl distribution and sphingoid base. Literature compositional data for

milk, cream, buttermilk, cheese-whey MFGM and WPPC are tabulated, and the wider body of NMR work on whey proteins — solution structures of β-lactoglobulin and α-lactalbumin, thermal and pressure denaturation followed by ¹H NMR and hydrogen–deuterium exchange, low-field relaxometry of denaturation and gelation, pulsed-field-gradient diffusion in whey gels, ³¹P NMR of phosphorylated species in milk serum, and ¹H NMR metabolite profiling of whey and WPC — is reviewed. The paper closes with a candid inventory of the assumptions and failure modes that an expert reviewer would challenge.

Keywords: whey protein concentrate; whey protein phospholipid concentrate; milk fat globule membrane; phospholipids; sphingomyelin; ³¹P NMR; ¹H NMR; ¹³C NMR; quantitative NMR; β-lactoglobulin; α-lactalbumin; relaxometry.

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1. Introduction

Whey is generated at roughly nine kilograms for every kilogram of cheese, and the fractionation of that stream into lactose, permeate, whey protein concentrate (WPC), whey protein isolate (WPI) and, more recently, whey protein phospholipid concentrate (WPPC) has converted a disposal problem into one of the more valuable ingredient portfolios in the dairy industry.[1,2] The proteins have received almost all of the analytical attention. The lipid that accompanies them has received comparatively little, even though it is the lipid — and specifically the polar lipid — that determines whether a WPC80 foams, whether a WPI beverage develops a cardboard note on storage, whether an infant-formula base can legitimately be described as MFGM-containing, and whether a WPPC stream is worth more as an extractable lecithin than as animal feed.[1,3–5]

The polar lipid of whey is not a homogeneous emulsifier. It is the residue of a biological membrane, the milk fat globule membrane (MFGM), that is torn from small fat globules during cheesemaking, whey clarification and membrane processing, and it therefore carries the full complement of glycerophospholipids and sphingolipids of that membrane: phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylserine (PS), phosphatidylinositol (PI), the minor dihydrosphingomyelin (DHSM), the lysophospholipids (LPC, LPE) that accumulate whenever phospholipases have been active, small amounts of ethanolamine plasmalogen, and glycosphingolipids that carry no phosphorus at all.[3,6,7] Any analytical method proposed for this fraction must therefore answer two questions at once: how much phospholipid is present, and in what class distribution. Chromatographic methods answer both, but only with authentic standards for every class, with detector responses that differ between classes, and with lysophospholipids that are frequently lost in the solvent front or under-recovered by extraction.[8,9]

Phosphorus-31 NMR spectroscopy answers the same two questions by a different route. Every phospholipid class carries exactly one phosphorus atom in a phosphodiester environment whose chemical shift is determined by the headgroup, so the ^{31}P spectrum of a lipid extract is a set of well-separated singlets whose integrals are directly proportional to molar amount, independent of acyl chain length, unsaturation or extinction coefficient.[10,11] The method has been applied to milk, cream, buttermilk, beta serum, cheese, infant formula, MFGM ingredients and — sparingly — to whey-derived materials, and it is the only NMR technique that has been used specifically to quantify phospholipids in whey.[4,12–16] ^{1}H and ^{13}C NMR contribute what ^{31}P cannot: structural confirmation of the sphingoid base and plasmalogen vinyl ether, the acyl-chain profile, the sn-1/sn-2 positional distribution, and, in the aqueous phase, the water-soluble choline and phosphocholine metabolites that betray phospholipid hydrolysis.

This white paper, prepared by Process NMR Associates, LLC (PNA; 84 Patrick Lane, Suite 115, Poughkeepsie, NY 12603), has two purposes. The first is to set out, in enough detail to be adopted directly into a method file, how ^{1}H, ^{13}C and ^{31}P NMR should be applied to the phospholipids of whey protein ingredients, and to be honest about where the literature is thin, contradictory or silent. The second is to review what NMR in all its forms has contributed to the understanding of whey proteins themselves, because a laboratory that acquires whey ^{31}P spectra will, in the same tubes, see the phosphoserine of residual casein and caseinomacropeptide, the inorganic phosphate that dominates acid whey, and the sugar phosphates and glycerophosphodiesters of the serum, and will be asked by clients about protein denaturation, gelation and water binding that have their own NMR literature. A companion PNA white paper devoted to sphingomyelin treats the SM/DHSM chemistry and the field-dependent resolution arithmetic in greater depth; the present paper takes the whey ingredient as its organizing principle.

The structure is as follows. Section 2 describes the whey streams and the origin, classes and reported amounts of their phospholipids. Section 3 summarizes the nuclear properties that make ^{31}P the quantitative nucleus and ^{1}H and ^{13}C the structural nuclei. Sections 4 and 5 treat sample preparation and quantitative ^{31}P methodology, including the chemical-shift chemistry, the field-strength question and the benchtop question. Sections 6 and 7 treat ^{1}H and ^{13}C NMR. Section 8 reviews the dairy and whey ^{31}P literature in chronological order, Section 9 reviews NMR studies of whey proteins, Section 10 sets out a proposed PNA protocol, and Section 11 lists the pitfalls and counter-arguments that a reviewer, an auditor or an opposing expert would raise. Conclusions, references and a closing summary follow.

2. Whey streams, whey protein ingredients and the origin of their phospholipids

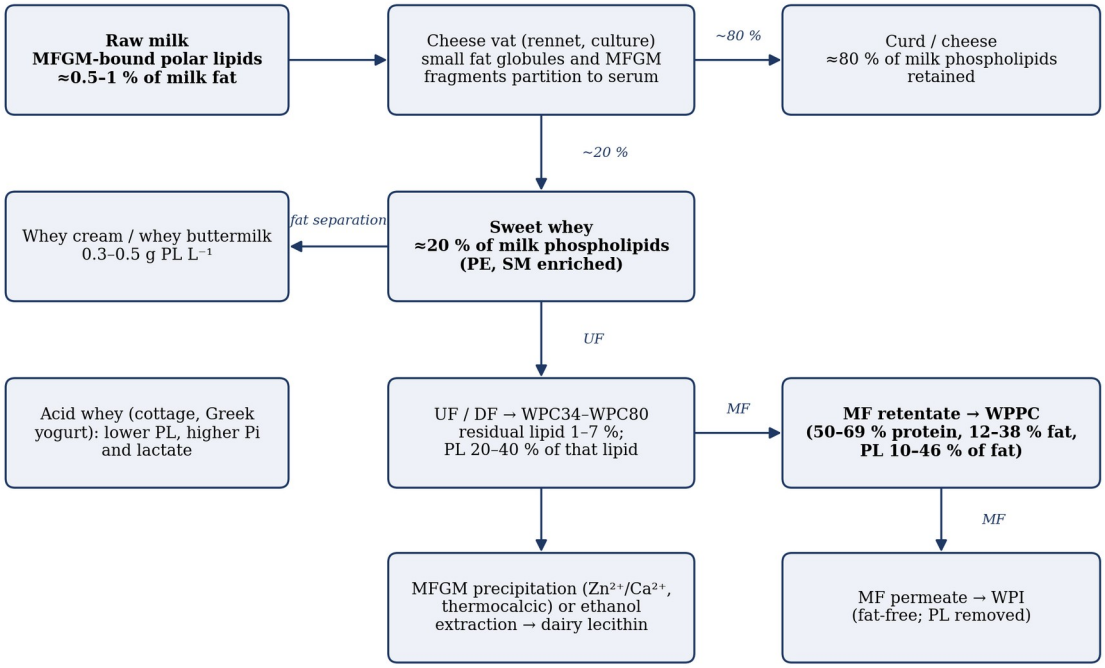
2.1 From milk to whey: the milk fat globule membrane and its partition

Bovine milk fat is secreted as globules of 0.1–15 µm diameter (volume-weighted mean near 4 µm) enclosed by a trilayer membrane that is derived from the apical plasma membrane of the mammary epithelial cell. The MFGM is composed of roughly equal masses of protein and lipid, and its lipid is dominated by the phospholipids and sphingolipids that are otherwise almost absent from the triacylglycerol core.[3,6] Total polar lipid amounts to about 0.5–1 % of milk fat, which corresponds to some 10–40 mg per 100 g of whole milk depending on breed, lactation stage, season and the analytical method used, and the class distribution determined by ^{31}P NMR in bovine milk is remarkably balanced: PC, PE and SM each contribute roughly 20–30 % of the phospholipid phosphorus, PS and PI contribute 2–14 % each, and DHSM contributes a further 4–5 %.[6,12,13,17] Table 2 and Figure 2 collect the published ^{31}P NMR distributions.

Because the polar lipid is membrane-bound rather than dissolved in the fat, it follows the membrane rather than the fat during processing. The systematic mass balance of Rombaut, Van Camp and Dewettinck across the processing of raw milk into skimmed milk, cream, butter, buttermilk, fresh cheese, acid whey, anhydrous milk fat and butterserum established that polar lipids are preferentially enriched in the aqueous phases — skimmed milk, buttermilk and, above all, butterserum, which contained 11.5 % polar lipid on a dry-matter basis and carried 28.4 % of the milk polar lipids in only 0.9 % of the milk mass — and that the polar lipids of acid whey could be recovered by tangential filtration and thermocalcic aggregation.[18] In rennet coagulation the picture is similar. Ferreiro and Rodríguez-Otero, following phospholipid contents through the manufacture of fresh cheese from cows' milk, found that approximately 20 % of the phospholipids drained into the whey, that the whey fraction was relatively enriched in PE and SM, and, unexpectedly, that the sum of phospholipid in whey and cheese exceeded the phospholipid in the starting milk, a surplus attributed to bacterial membrane synthesis during fermentation.[19] The microbial contribution deserves emphasis because it means that the phospholipid profile of a cultured whey is not simply a diluted MFGM profile: starter-culture membranes contribute PG, cardiolipin and PE that carry a bacterial, not a mammary, acyl signature.

Two further process variables modulate the phospholipid load of whey. Fat separation of whey produces whey cream and, on churning, whey buttermilk; Konrad, Kleinschmidt and Lorenz reported 0.30 ± 0.02, 0.42 ± 0.03 and 0.53 ± 0.03 g L^{-1} of phospholipid in whey buttermilks derived from whey creams of 22.0, 30.5 and 38.5 % fat, a linear relationship confirmed by ^{31}P NMR as a control method, and noted that whey buttermilk, unlike sweet buttermilk, is casein-free and is therefore an attractive feedstock for phospholipid

concentrates.[20] Enzyme treatment of cheese milk moves the phospholipid in the opposite direction. When mozzarella milk was hydrolyzed with a fungal phospholipase A_1 before renneting, the lysophospholipids produced were retained in the curd, fat and moisture retention improved, and the amount of phospholipid lost to whey and cooking water fell; the water-binding consequence was followed by ¹H NMR transverse relaxation of the curd.[21] Whey from phospholipase-treated processes therefore carries a lyso-enriched, PE-depleted residue, and any ³¹P method applied to it must resolve LPC and LPE reliably.



PL = phospholipid; MFGM = milk fat globule membrane; UF/DF = ultrafiltration/diafiltration; MF = microfiltration; WPC = whey protein concentrate; WPI = whey protein isolate; WPPC = whey protein phospholipid concentrate; Pi = inorganic phosphate. Partition figures are literature ranges (see text) and vary with cheese type, fat separation efficiency and membrane process.

Figure 1. Partition of MFGM-derived phospholipids (PL) from milk through cheese whey into whey protein ingredients. Percentages and ranges are literature values (Sections 2.1–2.2) and vary with cheese type, fat-separation efficiency and membrane process.[2,18–20,22,23]

2.2 Whey protein concentrates, isolates and whey protein phospholipid concentrate

Commercial WPC is manufactured by ultrafiltration and diafiltration of clarified, pasteurized whey, and residual lipid concentrates with the protein because the MFGM fragments and small fat globules that survive clarification are retained by ultrafiltration membranes. Morr and Ha, reviewing WPC and WPI processing, identified residual lipid as one of the principal causes of variable and often poor functionality in commercial WPC, and the observation has been repeated in every subsequent decade.[1] Vaghela and Kilara compared freeze-dried WPC of 35 and 75 % protein manufactured in the laboratory, with and without a calcium chloride–heat pretreatment and centrifugal clarification, against commercial WPC. Triacylglycerols were the major lipid class, followed by phospholipids, diacylglycerols, free fatty acids, cholesterol esters, cholesterol and monoacylglycerols; the three major phospholipids were SM, PC and PE, followed by PI, PS and cerebrosides; commercial WPC had significantly higher lipid-to-protein ratios than any laboratory product; and the calcium–heat pretreatment lowered total lipid and specifically reduced the

proportion of PE, at the cost of protein losses that increased with the amount of calcium added.[22] Boyd, Drye and Hansen subsequently isolated and characterized whey phospholipids as a fraction in their own right, quantifying PS and PI together, and thereby established the whey phospholipid fraction as a potential ingredient rather than a contaminant.[24]

The most concentrated whey-derived phospholipid stream is produced when high-protein WPC is microfiltered to make WPI. The membrane rejects the fat, the phospholipid-rich MFGM fragments, the lactose that is associated with them and the denatured whey protein, and the retentate, marketed as WPPC (or pro-cream), is compositionally closer to buttermilk powder than to WPC. Levin, Burrington and Hartel characterized WPPC from several suppliers and lots and found protein of 50–69 %, lipid of 11–38 % on a dry basis and phospholipid at 10–45.8 % of the lipid, with an average across eight suppliers near 61 % protein, 19.5 % fat, 4.8 % lactose, 2.9 % ash and 3.6 % moisture; the American Dairy Products Institute standard requires a minimum of 50 % protein and 12 % fat with maxima of 8 % ash and 6 % moisture.[2] Price and co-workers, applying an ethanol extraction developed for egg yolk to WPPC, recovered 1.6 g of phospholipid per 100 g of WPPC by Folch extraction (29.1 % of the total lipid) and showed that a five-stage extraction with 70 % ethanol at 70 °C produced a lipid fraction of 45.8 % phospholipid, with SM- or PS-enriched fractions obtainable by selection of the extraction stage.[5] Ozturk and colleagues, combining glycoproteomics and lipidomics on industrial liquid WPPC, measured a mean fat-globule diameter of 2.64 µm against 4.78 µm in the parent milk and found that phospholipid fatty acids accounted for 20 % of the total fatty acids, both consistent with the preferential enrichment of small, membrane-rich globules in the retentate.[25]

A parallel route to the same material starts from cheese whey rather than from WPC. Zhu and Damodaran isolated MFGM from cheese whey by a divalent-cation precipitation process, obtaining an isolate of 17–19 % lipid and 65–70 % protein in which about half of the lipid was phospholipid and which was rich in PS, SM and the MFGM proteins CD36, butyrophilin, xanthine oxidase and mucin 1; spray-drying and freeze-drying produced morphologically distinct powders with different thermotropic and oxidative behavior.[23] The same group then produced a dairy lecithin from the cheese-whey MFGM by two-stage ethanol extraction at pH 6.5 and 4.5, yielding 17.2 % of the MFGM mass as an extract of about 90 % lipid, 31 % of which was phospholipid, and the phospholipid, quantified by ^{31}P NMR, was 34 % SM, 31 % PC, 27 % PE, 4.6 % PS and 3.1 % PI.[4] That SM-dominant profile is the most directly relevant published ^{31}P NMR composition for a whey-derived phospholipid and it differs materially from the PE-dominant profiles circulated in industry technical literature for WPPC, a discrepancy that is itself an argument for direct measurement rather than reliance on typical values.

2.3 Phospholipid classes, structures and acyl chains

The glycerophospholipids share a sn-glycero-3-phosphate backbone esterified at sn-1 and sn-2 with fatty acids and at the phosphate with a headgroup alcohol: choline in PC, ethanolamine in PE, serine in PS, myo-inositol in PI, glycerol in PG and nothing in phosphatidic acid (PA). Removal of one acyl chain, almost always the sn-2 chain by phospholipase A_2 or the sn-1 chain by phospholipase A_1, gives the corresponding lysophospholipid. Sphingomyelin differs in the backbone: the phosphocholine headgroup is esterified to the primary hydroxyl of a ceramide, in which a long-chain base (predominantly C18 sphingosine with a trans-4 double bond, or sphinganine lacking it) carries an amide-linked fatty acid; SM built on sphinganine is DHSM, which in bovine milk is present at about 4–5 % of the phospholipid phosphorus and can be resolved from SM by ^{31}P NMR under appropriate conditions.[6,7,12] Ethanolamine plasmalogens, in which the sn-1

chain is attached through a vinyl ether, occur at low levels in bovine milk and at higher levels in human and camel milk.[13] Glucosylceramide, lactosylceramide and the gangliosides are members of the same membrane but carry no phosphorus; they are invisible to ^{31}P NMR and their presence in whey — Vaghela and Kilara reported cerebrosides in WPC — must be remembered whenever a ^{31}P result is described as the polar lipid content.[22]

The acyl composition of milk phospholipids differs from that of the triacylglycerol core. The phospholipids contain no butyric or caproic acid, are richer in C18 and longer chains, and carry a higher proportion of polyunsaturated fatty acids; SM is dominated by long saturated amide-linked chains (C16:0, C22:0, C23:0, C24:0) and PS and PI carry a high proportion of C18:0 at sn-1 and unsaturated chains at sn-2.[3,6] Two analytical consequences follow. First, the acyl unsaturation of the glycerophospholipids produces small, consistent upfield ^{31}P shifts of the corresponding molecular species relative to their disaturated analogues, so that a class resonance observed at high field is an envelope of species rather than a single line.[14,26] Second, the ^{1}H and ^{13}C spectra of a whey lipid extract are dominated by the triacylglycerol acyl chains, and the phospholipid acyl signature can be recovered only after fractionation or through the headgroup- and backbone-specific resonances described in Sections 6 and 7.

Table 1. Nuclear properties of the three nuclei used for phospholipid analysis, with resonance frequencies at the two field strengths relevant to a mid-field laboratory (7.05 T) and to benchtop instruments (1.41 T).

Property	^{1}H	^{13}C	^{31}P
Spin	½	½	½
Natural abundance (%)	99.98	1.07	100
Gyromagnetic ratio γ/2π (MHz T^{-1})	42.577	10.708	17.235
Receptivity relative to ^{1}H (natural abundance)	1	1.76×10^{-4}	6.65×10^{-2}
Frequency at 7.05 T (MHz)	300.0	75.4	121.4
Frequency at 1.41 T (MHz)	60.0	15.1	24.3
Useful chemical-shift range	≈ 12 ppm (lipids 0.8–6.0)	≈ 220 ppm (lipids 10–175)	≈ 700 ppm overall; phosphodiesters −1 to +1, phosphomonoesters +3 to +5, Pi +2 to +3 (pH dependent)
Hz per 0.1 ppm at 7.05 T / 1.41 T	30 / 6	7.5 / 1.5	12.1 / 2.4
Dominant relaxation mechanism in lipid solutions	^{1}H–^{1}H dipolar	^{1}H–^{13}C dipolar; CSA for C=O	^{1}H–^{31}P dipolar at low field; CSA increasing as B_0^2 at high field
What it reports for phospholipids	Headgroup stoichiometry, sphingoid olefins, plasmalogen vinyl ether, acyl chains, water-soluble choline metabolites	Backbone and headgroup carbons with $^{2}J/^{3}J(C,P)$ doublets, sn-1/sn-2 carbonyls, sphingoid base C3–C5, acyl profile	Class identity and molar amount from one singlet per class; lysophospholipids and DHSM resolved

2.4 Why the phospholipid fraction of whey ingredients has to be measured

Functionality is the first reason. Residual lipid, and phospholipid in particular, has long been associated with impaired foam formation and stability in WPC, and the removal of MFGM fragments by calcium–heat clarification, by microfiltration or by divalent-cation precipitation is precisely what converts a WPC into a

WPI with reproducible foaming and clarity.[1,22,23] Oxidative stability is the second. Milk phospholipids are the most polyunsaturated lipid fraction in the ingredient and they sit at the interface where oxygen, transition metals and light act; the oxidative stability of cheese-whey MFGM was found to depend on the drying method used to make it, and lipid oxidation in whey powders is a recurrent source of off-flavor complaints.[23] Nutrition and labeling are the third. MFGM phospholipids, and SM in particular, are now formulated into infant formula and adult nutrition products on the strength of neurodevelopmental, gut and cardiometabolic evidence reviewed elsewhere, and ³¹P NMR has been used to fingerprint the phospholipid content of powdered infant formula brands, distinguishing dairy-derived from soy-derived phospholipid by the presence of SM and by the PS/PI balance.[3,15] Valorization is the fourth: the difference between selling WPPC as feed and selling an SM-enriched dairy lecithin is a phospholipid analysis.[4,5] Process control is the fifth, because phospholipase treatments, thermal history and microbial activity each leave a signature in the lysophospholipid and glycerophosphodiester content that only a class-resolved, hydrolysis-product-resolved method can read.[9,21,27]

Table 2. Reported phospholipid class distributions (mol % of phospholipid phosphorus, ³¹P NMR unless stated) and phospholipid contents of milk, dairy streams and whey-derived materials. Values are as published; matrices, extraction and NMR conditions differ between studies and the table is intended to show ranges, not to support direct comparison.

Matrix	PC	PE	PI	PS	SM	Other / notes	Ref.
Bovine milk (three ³¹P NMR studies compiled)	24–29	23–31	3.6–14	1.5–11	20–27	DHSM 4.5–4.6; EPLAS ≈ 0.5	6,13,17
Bovine cream (cholate ³¹P)	26.5	26.7	7.5	11.7	20.8	DHSM 3.9; LPC 1.1; LPE 1.8	6,12
Buttermilk, lipid extract vs direct cholate analysis	27.0 / 26.4	25.7 / 25.8	5.8 / 7.6	9.7 / 11.5	20.4 / 16.9	DHSM 4.6; LPC 0.7; LPE 1.0; agreement of extract and direct routes	6,12
Cheese whey, whole (fresh-cheese make)	—	enriched	—	—	enriched	≈ 20 % of milk PL drained into whey	19
Whey buttermilk (from whey cream of 22–38.5 % fat)	—	—	—	—	—	0.30–0.53 g PL L^{-1}, linear in whey-cream fat	20
Commercial and laboratory WPC35/WPC75 (HPLC-ELSD)	major	major	minor	minor	major	Rank order SM, PC, PE > PI, PS, cerebrosides; TAG the major lipid class	22
Cheese-whey MFGM isolate	—	—	—	—	rich	17–19 % lipid, 65–70 % protein; ≈ 50 % of lipid is PL; PS-rich	23
Cheese-whey dairy lecithin (ethanol extract)	31	27	3.1	4.6	34	31 % PL in extract; ³¹P NMR	4
WPPC (several suppliers)	—	—	—	—	—	protein 50–69 %; lipid 11–38 %; PL 10–45.8 % of lipid; 1.6 g PL/100 g by Folch (29.1 % of lipid)	2,5
Liquid industrial WPPC (lipidomics)	—	—	—	—	—	PL fatty acids 20 % of total FA; MFG diameter 2.64 µm vs 4.78 µm in milk	25

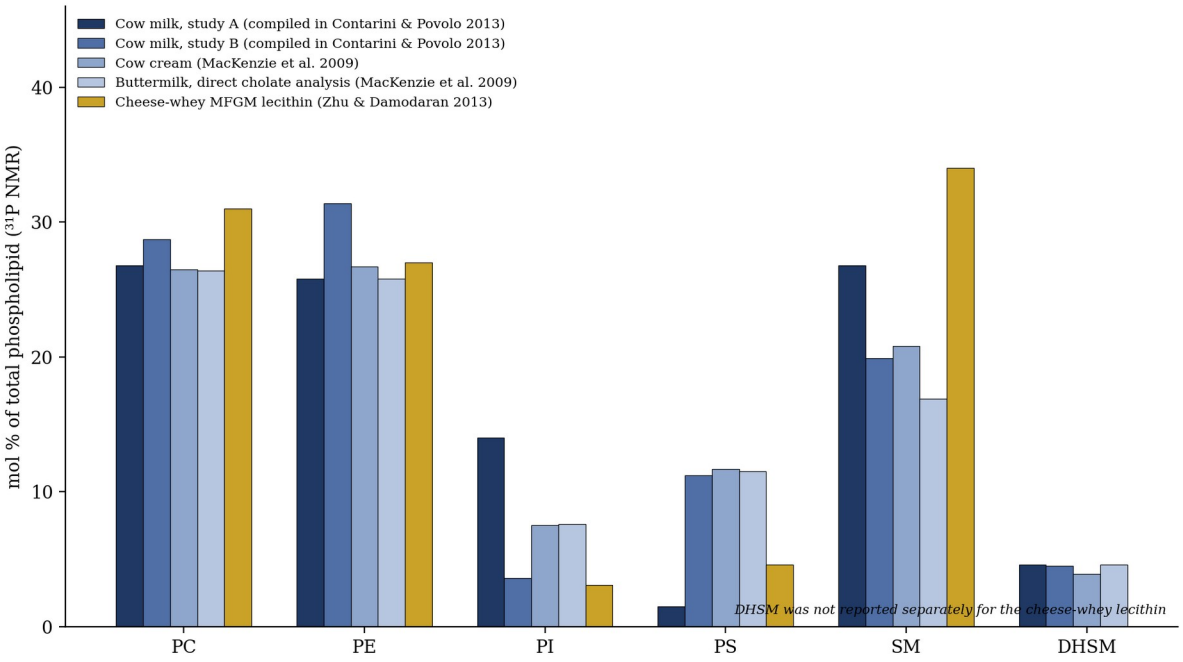


Figure 2. Phospholipid class distributions determined by ³¹P NMR for bovine milk, cream, buttermilk (direct cholate analysis) and cheese-whey MFGM lecithin. Data as compiled by Contarini and Povolo and as reported by MacKenzie et al. and Zhu and Damodaran; DHSM was not reported separately for the cheese-whey lecithin.[4,6,12]

3. Multinuclear NMR of phospholipids: fundamentals

3.1 What each nucleus reports

The properties assembled in Table 1 explain the division of labor. ³¹P is a spin-½ nucleus of 100 % natural abundance with a receptivity about one-fifteenth that of the proton, which places it among the most sensitive heteronuclei, and phosphorus occurs in phospholipids exactly once per molecule and nowhere in the neutral lipids, sugars or proteins that surround them.[6,11] A ³¹P spectrum of a lipid extract is therefore a spectrum of phospholipids only, background-free, in which the number of resonances equals the number of phospholipid classes and the integral of each is proportional to the molar amount of that class. ¹H offers three orders of magnitude more sensitivity but suffers from the opposite problem: every lipid in the extract contributes, the acyl chains of the triacylglycerols dominate, and class-specific information is confined to a few headgroup and backbone resonances in the 3.1–4.4 ppm window and to the sphingoid and vinyl-ether olefins.[28,29] ¹³C, with 1.1 % natural abundance and low γ, is the least sensitive, but it provides the largest chemical-shift dispersion, resolves the sn-1 and sn-2 carbonyls, shows the phosphorus-coupled backbone and headgroup carbons as doublets, and identifies the sphingoid base carbons that have no ¹H counterpart free of overlap.[30,31]

3.2 The chemistry of the ³¹P chemical shift in phospholipids

The phosphodiester phosphorus of a phospholipid is a tetrahedral phosphate with two ester oxygens and two non-esterified oxygens carrying a net negative charge. Its isotropic chemical shift lies within about 2 ppm of the 85 % H_3PO_4 reference, and the differences between classes — the whole of the analytical information —

amount to only 0.05–1.3 ppm. Those differences arise from the headgroup substituent on the second ester oxygen (a quaternary ammonium in PC and SM, a primary ammonium in PE, an ammonium plus a carboxylate in PS, a polyol in PI and PG, a free phosphate in PA), from hydrogen bonding of the headgroup back onto the phosphate, from the protonation state of the headgroup and of the phosphate itself, from ion pairing with counter-cations and from the solvation environment.[10,32] Everything that perturbs these interactions moves the resonances. Divalent cations, especially Ca^{2+} and Mg^{2+}, associate with the anionic phospholipids — cardiolipin showed the highest and PA the next highest interaction potential for Mg^{2+} in the titrations of Merchant and Glonek, with PS and PI following — and the association is signaled by chemical-shift changes, broadening or outright quenching of the affected resonance, which is why every practical solvent system contains EDTA or another chelator.[33] pH moves PS, PE and PA by amounts that can exceed the separation between classes, whereas PC, SM, DHSM and PG are essentially invariant with pH and temperature; PI has a temperature coefficient of about 4×10^{-3} ppm $°C^{-1}$ regardless of pH.[32] Acyl-chain unsaturation produces small, consistent upfield shifts of the unsaturated species of PC, PE, PG and PA relative to the saturated species, roughly twice as large in cholate as in deoxycholate, and these shifts are the origin of the species fine structure seen within a class envelope at high field.[14,26] The practical corollaries are that chemical shifts must be measured, not assumed; that the temperature must be controlled to ±0.1 °C; that the pH of an aqueous or micellar preparation must be fixed and reported; and that assignments must be confirmed by spiking with authentic standards or by ^{1}H–^{31}P correlation rather than by matching to a table.[14,34]

4. Sample preparation from whey protein matrices

4.1 Lipid extraction from protein-rich powders

Whey protein ingredients present the least favorable matrix in dairy lipid analysis: a dry, glassy, lactose- and mineral-containing protein powder in which the lipid is partly enclosed in MFGM fragments, partly adsorbed on protein and partly present as small fat globules. Théodet and Gandemer compared five methods for extracting lipids from whey and its derivatives and found that recovery of the polar fraction depended strongly on the method, a conclusion that has been re-discovered by every group that has since worked on WPC or WPPC.[35] The chloroform–methanol procedures of Folch and of Bligh and Dyer remain the reference methods for total lipid and are the extraction of record for most ^{31}P NMR studies of dairy phospholipids; the Röse–Gottlieb ammonia–ethanol–ether procedure, which is the official method for milk fat, under-recovers phospholipids and should not be used for this purpose.[6,8] For a powder, rehydration is essential before solvent addition because the lipid–protein association must be disrupted; a common approach is to disperse 1–3 g of WPC in water, adjust the pH to near neutrality, and then add chloroform–methanol (2:1 v/v) at 20 volumes per gram of solids, with mild sonication, followed by phase separation against 0.2 volumes of 0.1 M KCl and collection of the lower phase.[5,10] Ethanol and ethanol–water extraction, which precipitates the protein and dissolves the polar lipid, has been shown to be effective for WPPC and cheese-whey MFGM and avoids chlorinated solvents; recoveries and class selectivity depend on the ethanol concentration, temperature and number of stages, and the ethanol route therefore requires its own recovery validation before it is used quantitatively.[4,5,36]

The lysophospholipids are the fraction most likely to be lost. Mayar and co-workers found that the conventional Folch extraction did not fully recover the polar lysophospholipids from emulsions containing enzymatically modified egg yolk and that repeated methanol extractions were required; after solubilization

of the combined extract in cholate micelles, ³¹P NMR recoveries of 96–108 % were achieved.[9] The finding transfers directly to whey from phospholipase-treated cheese processes and to aged or UHT-treated dairy streams, in which LPC and LPE are the analytes of interest rather than a nuisance.[21,27] Any protocol that reports lysophospholipid must therefore include a methanol re-extraction step and a spiked-recovery experiment for LPC and LPE specifically.

4.2 Solvent and detergent systems for ³¹P NMR

Three families of preparation dominate the literature and the choice between them determines resolution, quantitative reliability and the need for extraction. The first is the hydrated chloroform–methanol reagent of Meneses and Glonek, in which the crude lipid extract is dissolved in chloroform and mixed with an aqueous methanol reagent containing 0.2 M EDTA titrated to pH 6 with CsOH (one part aqueous EDTA to four parts methanol, two volumes of chloroform to one of the aqueous methanol reagent); the phospholipids partition into the lower organic phase as inverted micelles, the cesium EDTA scrubs divalent cations, and at 11.75 T the phospholipid resonances are 1.8–3.2 Hz wide with T_1 values of about 3.3 s and NOE enhancements of 50–60 %.[10] Variants of this reagent, with EDTA neutralized by NaOH or KOH and with triphenyl phosphate as the internal standard, have been used for milk, MFGM ingredients, infant formula and cheese.[14–16] The system gives the narrowest lines and the best-documented chemical shifts but it is biphasic, the water content of the organic phase affects the shifts, and it demands an extraction.

The second family is monophasic. Murgia, Mele and Monduzzi introduced a dimethylformamide–triethylamine–guanidinium chloride mixture for milk-fat phospholipids that removed the partition problems of the biphasic reagent and slightly improved resolution, but the mixture formed adducts that led to under-estimation of PE, a limitation acknowledged in the subsequent buffalo-milk study by Andreotti and colleagues that used the same medium.[6,17,37] Chloroform–methanol without added water, optimized by Bosco and colleagues for two-dimensional ¹H-detected heteronuclear multiple-quantum experiments on lecithin phospholipids, is the natural medium when ¹H–³¹P correlation is the objective.[30]

The third family, and the one that has come to dominate dairy applications, is the aqueous bile-salt micelle. Sodium cholate at concentrations of 100–250 mM with 5–10 mM EDTA in D_2O solubilizes phospholipids as small mixed micelles that tumble fast enough to give narrow ³¹P lines, dissolves phospholipid-rich dairy materials directly without extraction, and accepts lipid extracts after solvent evaporation.[9,12,34,38] MacKenzie, Vyssotski and Nekrasov validated the cholate ³¹P method against quantitative two-dimensional TLC for cream, fresh beta serum and buttermilk protein concentrate; the TLC system was more sensitive and detected minor components not seen by NMR, but the NMR analysis took 36 min per sample, quantified PC, PE, PS, PI, SM and DHSM correctly once the detergent pH was raised from 7.1 to 9.5, and gave comparable results for the extracted lipid and for the whole powder dissolved directly in the detergent.[6,12] The pH and temperature dependence of the shifts in cholate was mapped by Puppato and colleagues, whose temperature coefficients are the tool for resolving accidental overlaps.[32] Two cautions apply. The micellar environment raises the rotational correlation time of the phosphorus, so that chemical-shift-anisotropy relaxation, which scales with the square of the field, broadens the lines at high field; Mayar and colleagues showed that 16.4 T offered no advantage over 9.4 T for cholate preparations and that within-laboratory reproducibilities were 7–22 % at 9.4 T and 12–25 % at 16.4 T.[9] And the cholate concentration must be high enough relative to the phospholipid (a cholate-to-phospholipid ratio above about 100 was required for maximal resolution of PC species in 2 % cholate) to keep the micelles small and uniform.[26]

4.3 Direct, extraction-free analysis of phospholipid-rich materials

Where the phospholipid content is high enough — buttermilk protein concentrate, beta serum, MFGM isolates and, by extension, WPPC — the powder can be dispersed directly in the cholate–EDTA medium and analyzed without extraction, as MacKenzie and colleagues demonstrated with agreement between extracted and direct results.[12] The route is attractive for WPPC because it removes the extraction as a source of bias and because the protein, lactose and minerals contribute no ^{31}P signal in the phosphodiester window; the phosphoserine of residual casein and caseinomacropeptide, the inorganic phosphate and the sugar phosphates appear well downfield of the phospholipids and do not interfere. Its limitations are the sensitivity penalty of dissolving whole powder rather than concentrated lipid (a WPPC of 1.6 g phospholipid per 100 g gives, at 300 mg of powder in 0.7 mL, a total phospholipid concentration of about 9 mM, of which the minor classes are 0.3–0.5 mM), the turbidity and residual protein–lipid association that can broaden lines, and the phosphoserine background, which is broad and must be excluded from the baseline correction of the phosphodiester region. For WPC80, whose phospholipid content is typically an order of magnitude lower, direct analysis is not realistic and extraction with concentration is required.

4.4 Internal standards, referencing and calibration

Absolute quantification requires a phosphorus-containing standard of known purity, chemically inert toward the sample, resonating clear of the phosphodiester and phosphomonoester windows, and with a relaxation time no longer than that of the analytes. Triphenyl phosphate (near −17 ppm in the chloroform reagent) is the most widely used in organic media and has been adopted in recent dairy work with the EDTA reagent; trimethyl phosphate, hexamethylphosphoramide (referenced at 29.90 ppm and used at 2 mM by Bruschetta and colleagues in cholate–D_2O milk serum) and methylenediphosphonic acid are used in aqueous and micellar media.[16,38] A coaxial capillary containing the standard removes any possibility of interaction with the sample but introduces a separate susceptibility environment and requires its own calibration; an in-sample standard is simpler and is preferred when its inertness has been demonstrated. Chemical shifts in the chloroform reagent are conventionally referenced to the PC resonance at −0.84 ppm, following Meneses and Glonek, and in cholate to the internal standard.[10,38] Where an internal standard is impractical, the electronic reference approaches (ERETIC and PULCON) that PNA already uses for ^{1}H work can be applied to ^{31}P provided the probe, pulse width and receiver gain are calibrated at the ^{31}P frequency, but their accuracy is limited by radiofrequency inhomogeneity at the higher power required for ^{31}P and they should be cross-checked against a gravimetric standard at least quarterly.

5. ^{31}P NMR: quantitative methodology

5.1 Relaxation, nuclear Overhauser enhancement and acquisition parameters

Two relaxation phenomena govern the accuracy of ^{31}P integrals. The first is longitudinal relaxation. Phospholipid T_1 values of about 3.3 s were reported at 11.75 T in the chloroform–methanol–Cs-EDTA reagent, and the values in cholate micelles at 9.4 and 16.4 T were tabulated by Mayar and colleagues; the internal standards, being small molecules, relax more slowly than the micellar phospholipids.[9,10] A relaxation delay of at least five times the longest T_1 in the sample — which is usually that of the standard — is required for integrals within 1 % of their equilibrium values after a 90° pulse, and in practice delays of 15–30 s are used. The alternative of a short delay with a small flip angle is acceptable for relative class

distributions but not for absolute quantification against a standard whose T_1 differs from the analytes. It should be noted that the claim, made in at least one milk-serum study, that similar integrals were obtained at 1, 10 and 60 s delay because all phosphorus nuclei had similar relaxation times, does not remove the requirement to measure T_1 in each new matrix; it reports a fortunate equality, not a general property.[38]

The second phenomenon is the heteronuclear nuclear Overhauser enhancement. Continuous ^{1}H decoupling during the relaxation delay enhances the ^{31}P signal by up to 50–60 % through ^{1}H–^{31}P dipolar cross-relaxation, and the enhancement differs between classes, between lysophospholipids and diacyl species, and between the standard and the analytes.[10] Inverse-gated decoupling, in which the decoupler is switched on only during acquisition, suppresses the NOE while retaining collapse of the ^{31}P–^{1}H couplings, and it is the mandatory decoupling scheme for quantitative work. Where the NOE is deliberately exploited to gain sensitivity, as in the cross-polarization experiments used at low field, the enhancement must be calibrated per class and the result is no longer a first-principles quantification.[39]

The remaining parameters follow from the chemistry. The spectral window need cover only about 40 ppm centered near 0 ppm if the standard is triphenyl phosphate, or about 60 ppm if hexamethylphosphoramide is used, with the transmitter placed to avoid off-resonance effects on the standard; a 90° pulse calibrated on the sample; an acquisition time of 1.5–2.5 s to define lines of 2–3 Hz; and 256–1024 transients depending on the phospholipid concentration. Processing should use no more than 0.5–1 Hz of exponential line broadening, zero-filling to at least four times the acquired points, careful zero-order phasing across the narrow phosphodiester window, and a baseline correction that is fitted to signal-free regions on both sides of the phosphodiester cluster and explicitly excludes the broad phosphoserine hump present in whole-powder preparations. The automated phase- and baseline-correction module developed at PNA for the AI-60 ^{1}H work is directly applicable here, with the caveat that the phosphodiester cluster spans only 1.5 ppm and a polynomial baseline fitted through it will absorb real intensity from the minor classes unless the fit is constrained to exclude the cluster. Table 6 (Section 10) assembles a parameter set for the PNA 300 MHz instrument.

5.2 Assignment strategy and chemical-shift guidance

Table 3 gives the chemical-shift regions in which each class is expected in the hydrated chloroform–methanol–Cs-EDTA reagent, with PC referenced at −0.84 ppm. The values are approximate literature positions, not constants, and Section 3.2 has already explained why. The assignment sequence recommended for a new matrix is as follows. The unknown extract is first run under the standard conditions and the PC, SM, PE and PI resonances, which are the four most robust, are identified by position and by their expected relative intensities. Authentic standards of PS, LPC, LPE, PA and, if available, DHSM are then added in turn to aliquots and the spectra re-acquired; the class whose signal grows is confirmed and the shift of that class under the actual conditions of the sample is recorded. Where two resonances remain closer than three linewidths, the sample temperature is changed by 10 °C and the resonances are tracked using the published temperature coefficients, which differ between classes and separate PS from SM and PE from LPE in most cases.[32,36] Finally, for any resonance that resists identification, a two-dimensional ^{1}H–^{31}P correlation is acquired: the HMQC/HMBC-type experiment of Bosco and colleagues, the ^{31}P,^{1}H-COSY of Kaffarnik and colleagues that identified ten phospholipids in cheese, or the ^{1}H–^{31}P TOCSY on which the automated autoP assignment and quantification method of Balsgart and colleagues is built, which uses the far more diagnostic ^{1}H shifts of the headgroup protons for identification and the ^{31}P integrals for quantification.[14,30,34] The PS resonance deserves specific attention: it is the anionic class most sensitive to

pH, counter-ion and water content, and a recent pharmaceutical-analysis study has set out the considerations required to quantify PS by ^{31}P NMR reliably, which apply unchanged to dairy PS.[40] SM and PE are the pair most often reported as insufficiently resolved in milk-derived polar lipids; Kato and colleagues resolved the problem by spectral deconvolution rather than integration, and reported that deconvolution gave spiked recoveries closer to 100 % than simple integration.[36]

Table 3. Approximate ^{31}P chemical-shift positions of phospholipid classes in the hydrated $CDCl_3/CH_3OH$/aqueous Cs-EDTA reagent (PC referenced to −0.84 ppm) and the principal factors that move each resonance. Positions are literature guidance only; each class must be confirmed by spiking or by ^{1}H–^{31}P correlation under the actual conditions of the sample.[10,12,14,26,32,33]

Class	Approx. δ (ppm)	Sensitivity to conditions	Comment for whey samples
Phosphatidic acid (PA)	+0.3 to +0.5	Strongly pH dependent (second pK_a); binds Ca^{2+}/Mg^{2+} avidly	Low in fresh whey; rises with phospholipase D activity; broadened without EDTA
Lysophosphatidylethanolamine (LPE)	≈ +0.1 to +0.2 (downfield of PE)	pH dependent	Marker of phospholipase A_1/A_2 action and of storage; under-recovered by Folch
Phosphatidylethanolamine (PE)	0.00 (reference within cluster)	pH dependent; unsaturated species shifted slightly upfield	One of the two dominant classes in whey; envelope of species at high field
Ethanolamine plasmalogen	within ≈ 0.1 ppm of PE	as PE	Minor in bovine milk; confirm by ^{1}H vinyl-ether signal near 5.9 ppm
Dihydrosphingomyelin (DHSM)	slightly downfield of SM (≈ 0.05 ppm)	Invariant with pH and temperature	4–5 % of milk PL phosphorus; resolvable at ≥ 7 T with 2–3 Hz lines
Phosphatidylserine (PS)	within ≈ 0.1 ppm of PE/SM, typically between them	Strongly pH dependent; the classic overlap trap	5–12 % in dairy PL; resolve by pH adjustment (cholate pH 9.5), temperature coefficient or spiking
Sphingomyelin (SM)	≈ −0.09	Invariant with pH and temperature	17–34 % in whey-derived PL; species envelope reflects amide-chain length
Lysophosphatidylcholine (LPC)	≈ −0.28	Small	Hydrolysis marker; requires methanol re-extraction for full recovery
Phosphatidylinositol (PI)	≈ −0.38	Temperature coefficient ≈ 4×10^{-3} ppm $°C^{-1}$; pH insensitive	3–14 % in dairy PL; elevated PI relative to SM indicates plant lecithin
Phosphatidylcholine (PC)	−0.84 (reference)	Invariant with pH and temperature	Reference resonance; species fine structure at ≥ 11.7 T
Phosphocholine, glycerophosphocholine, glycerophosphoethanolamine	phosphomonoesters ≈ +3.5 to +4.3; glycerophosphodiesters ≈ +0.4 to +1.0 (aqueous)	pH dependent	Water-soluble hydrolysis products; appear in the aqueous whey phase, not in the lipid extract
Casein/CMP phosphoserine; inorganic phosphate	SerP broad, ≈ +2.5 to +4; Pi ≈ +2 to +3 (pH dependent)	pH and Ca^{2+} dependent	Present in whole-powder cholate preparations of sweet whey products; exclude from baseline fit

5.3 Resolution, field strength and the benchtop question

The separations that matter for whey phospholipids are of two very different magnitudes. PC lies 0.75 ppm upfield of SM and 0.84 ppm upfield of PE, and PI lies 0.3–0.4 ppm upfield of SM. Within the

PE/PS/DHSM/SM cluster, however, the separations are 0.03–0.1 ppm, and LPE and the plasmalogens sit within 0.1–0.2 ppm of PE. At 121.4 MHz (7.05 T) 0.1 ppm is 12 Hz, so with the 2–3 Hz linewidths attainable in either the chloroform reagent or a well-prepared cholate micelle the whole cluster is resolvable and PS, DHSM and LPE can be integrated as separate lines, as MacKenzie and colleagues did at comparable resolution.[12] At 24.3 MHz (1.41 T, the ³¹P frequency of a 60 MHz benchtop instrument) the same 0.1 ppm is 2.4 Hz. Even with the 1–1.5 Hz linewidths that the best benchtop magnets deliver, PE, PS, DHSM and SM merge into one envelope, LPE is a shoulder, and only PC, PI and the summed PE-cluster can be quantified with confidence. Figure 3 makes the point with simulated spectra of a WPPC-like composition. The benchtop instrument is therefore a legitimate tool for total phospholipid, for PC and for the PC/SM balance in concentrated extracts, but not for the class profile that the applications in Section 2.4 require. Gouilleux and colleagues, in the only systematic evaluation of benchtop ³¹P for lipids, reached the same conclusion from the other direction: by relying on ¹H–³¹P heteronuclear cross-polarization they detected phospholipids in 2 h with limits of detection of 0.5 mM at 1 T and 0.2 mM at 2 T, and they assigned PC, PE, PI, PS and PG headgroups unambiguously only by two-dimensional ¹H–³¹P TOCSY, not by the one-dimensional ³¹P shift.[39] Those detection limits should be compared with the 0.3–0.5 mM at which the minor classes are present in a direct WPPC preparation and with the tens of micromolar at which they are present in a WPC80 extract of realistic mass.

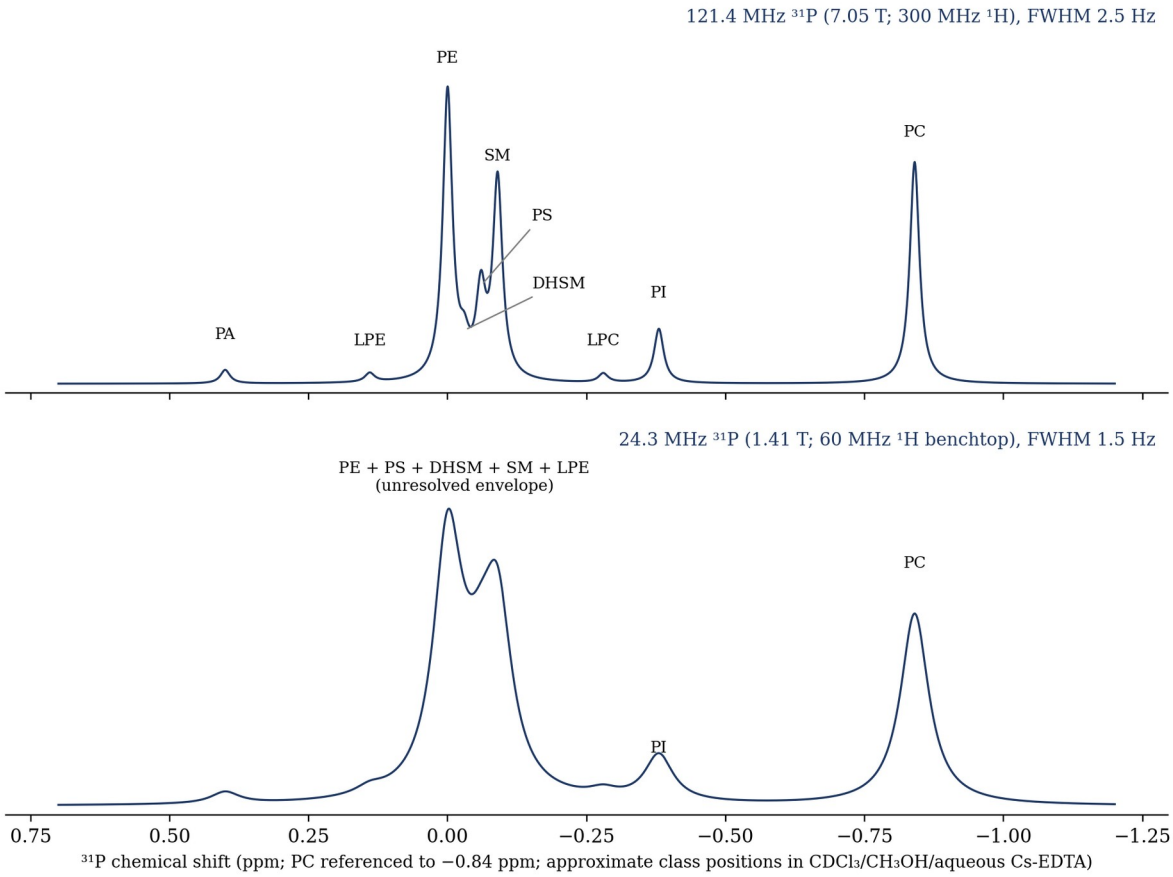


Figure 3. Simulated ³¹P spectra of a WPPC-like phospholipid composition (PE 32, PC 24.5, SM 22, PS 9, PI 6, DHSM 3, PA 1.5, LPE 1, LPC 1 mol %) at 121.4 MHz with 2.5 Hz lines and at 24.3 MHz with 1.5 Hz lines. Class positions are the approximate literature values of Table 3. The simulation is illustrative only; it is not measured data and the relative intensities were chosen for clarity.[12,39]

The upper end of the field range is not the answer either. Because chemical-shift-anisotropy relaxation scales with B_0^2, the micellar phospholipid lines broaden in hertz at high field faster than the chemical-shift dispersion grows, and Mayar and colleagues found no significant advantage of 16.4 T over 9.4 T for cholate preparations, with slightly worse reproducibility at the higher field.[9] Species fine structure within each class also becomes more prominent at high field, which is informative but complicates integration.[14,26] For a 7.05 T laboratory the implication is favorable: the field is high enough to resolve the cluster and low enough that CSA broadening and species splitting are modest, at a sensitivity cost relative to 11.7 T of roughly (7.05/11.7)^1.5, or a factor of about two in signal-to-noise for equal acquisition time.

5.4 Quantification, calculation and reporting bases

The mole fraction of each class is the integral of its resonance divided by the sum of all phospholipid integrals, and it is the most robust quantity the method delivers because it is independent of the standard, of recovery and of weighing. Absolute amounts follow from the ratio of the class integral to the standard integral, the molar amount of standard added, and the mass of sample taken. Conversion of molar amounts to mass requires a molecular weight for each class, and the value chosen must reflect the acyl composition of the dairy phospholipid rather than a synthetic standard: bovine PC averages near 780 g mol^{-1}, PE near 740, PS near 810, PI near 860 and SM near 780–810 depending on the amide chain distribution, and the assumptions used should be stated in the report because they introduce 2–5 % uncertainty in mass-based results. Results are reported on three bases that must not be confused: mol % of phospholipid phosphorus (the native NMR result), mg of each class per 100 g of powder (the labeling basis), and g of phospholipid per 100 g of fat (the basis used in the dairy literature for comparing streams). The moisture content and the fat content of the powder are needed for the last two, and the total phospholipid should be reported both as the sum of the resolved classes and, where the Bartlett or other phosphorus assay is available, as lipid phosphorus × 25 for comparison.[2,12] Lysophospholipids should be reported separately and not folded into their parent classes, and DHSM should be reported separately from SM wherever it is resolved, because the SM/DHSM ratio is a species and processing marker.[7]

5.5 Validation and comparison with chromatographic methods

The validation record for dairy ³¹P NMR is reasonable but not deep. MacKenzie and colleagues validated the cholate method against quantitative two-dimensional TLC and against direct and extracted preparations of the same material; Giuffrida and colleagues, developing an HPLC–ELSD method for human milk, analyzed the same samples by ³¹P NMR and found no statistically significant difference between the two techniques for any class, with HPLC–ELSD repeatability CV(r) of 0.8–13.4 % and intermediate reproducibility CV(iR) of 2.4–25.7 %; Mayar and colleagues reported within-laboratory reproducibility of 7–22 % for phospholipid and lysophospholipid classes in cholate at 9.4 T; and Kato and colleagues reported spiked recoveries for infant-formula polar lipids that depended on whether integration or deconvolution was used.[9,12,36,41] Against these, HPLC–ELSD and HPLC–CAD methods for dairy polar lipids are well established, quantify PE, PC, PI, PS, SM and, with the right column and gradient, glucosyl- and lactosylceramide, but require authentic standards, non-linear detector calibration for each class and careful handling of lysophospholipids.[6,8] LC–MS lipidomics resolves molecular species within each class and is the method of choice for acyl-level questions, as in the WPPC study of Ozturk and colleagues, but its class totals depend on the completeness of the species library and on ionization efficiencies that vary with chain length and unsaturation.[25] Byrdwell and Perry combined liquid chromatography with dual parallel mass

spectrometry and ^{31}P NMR for bovine milk sphingolipids precisely because the NMR provided the class-level quantitative anchor that the MS lacked.[7] Table 4 summarizes the comparison. For a PNA method the minimum validation set is: linearity of each class response against gravimetric standards from 0.1 to 20 mM in the tube; spiked recovery of PC, PE, PS, PI, SM, LPC and LPE at two levels into a WPC80 extract and into a direct WPPC preparation; intermediate precision from six independent preparations of one WPPC lot over three weeks; and a cross-validation of at least ten samples against an HPLC–ELSD or HPLC–CAD laboratory.

Table 4. Comparison of analytical approaches for the phospholipid fraction of whey protein ingredients.

Attribute	^{31}P NMR (7.05 T, EDTA reagent or cholate)	^{31}P NMR (1.41 T benchtop)	HPLC–ELSD / HPLC–CAD	LC–MS lipidomics
Identification	By chemical shift; standards used for confirmation only; 2D ^{1}H–^{31}P for ambiguity	PC, PI and PE-cluster only; 2D TOCSY needed for headgroups	Retention time vs authentic standards	Accurate mass and MS/MS; library dependent
Quantification basis	Molar, one signal per class, response independent of acyl chains	As 7.05 T for resolved classes; NOE/CP calibration if used	Non-linear detector response calibrated per class	Species-level; class totals from summed species
Lysophospholipids	Resolved (LPC, LPE) if extraction recovers them	LPC resolved; LPE merged with PE cluster	Often lost or poorly resolved	Resolved
SM / DHSM	Resolved with 2–3 Hz lines	Not resolved	Not resolved	Resolved by mass
Glycosphingolipids, gangliosides	Not detected (no phosphorus)	Not detected	Detected with suitable gradient	Detected
Typical limit of detection	Tens of µM in the tube after extraction and concentration	0.2–0.5 mM in 2 h with cross-polarization	Low µg on column	ng–pg on column
Precision (literature)	CV 7–22 % within-laboratory (cholate, 9.4 T)	Not established for dairy	CV(r) 0.8–13 %; CV(iR) 2–26 %	Method dependent
Sample preparation	Folch/ethanol extract, or direct cholate for WPPC/MFGM	As 7.05 T; concentrated extract required	Extraction and often SPE clean-up	Extraction, dilution, internal standards per class
Time per sample	30–90 min acquisition at 7.05 T for extracts; longer for direct powders	2 h or more	20–40 min run plus calibration	10–30 min run plus data processing
Principal weakness for whey	Sensitivity for WPC80-level phospholipid; invisible glycolipids	Cluster unresolved; sensitivity	Standards, detector calibration, lyso recovery	Ionization bias; library completeness; cost

6. ^{1}H NMR of whey lipid extracts and of the aqueous whey phase

6.1 Headgroup, backbone and sphingoid resonances

In chloroform–methanol solution the phospholipid headgroups contribute a small number of resonances that lie clear of the acyl-chain envelope and that were assigned for membrane lipid extracts at 500 MHz by Sparling and colleagues.[28] The choline $N^{+}(CH_3)_3$ singlet near 3.2 ppm (nine protons) is common to PC, LPC and SM and is the most useful single ^{1}H marker of choline-containing phospholipid; its integral, divided by nine, gives the molar amount of PC + LPC + SM and can be compared directly with the sum of the corresponding ^{31}P integrals as an internal consistency check. The choline N-CH_2 near 3.6 ppm and the O-CH_2 near 4.25 ppm, the latter split by three-bond coupling to phosphorus, complete the choline pattern. PE

gives an N-CH_2 near 3.1 ppm and a P-O-CH_2 near 4.0 ppm; PS gives the α-CH of serine near 4.0–4.3 ppm; PI gives a cluster of inositol ring protons between 3.3 and 4.2 ppm that overlaps with lactose if any carbohydrate has been carried through the extraction. The glycerol backbone of the diacyl glycerophospholipids gives the sn-2 methine near 5.2 ppm and the sn-1 and sn-3 methylenes between 3.9 and 4.4 ppm, but these overlap with the corresponding triacylglycerol resonances and are not class-specific.[29,31]

Sphingomyelin is recognized by resonances that no glycerophospholipid possesses: the trans-olefinic H-4 (a doublet of doublets near 5.45 ppm) and H-5 (a doublet of triplets near 5.70 ppm) of the sphingosine base, resolved from the cis-olefinic acyl resonances near 5.35 ppm, together with the amide N–H near 7.5–7.9 ppm in chloroform and the H-2 and H-3 methines of the base between 3.9 and 4.1 ppm. Because DHSM lacks the 4,5-double bond, the ratio of the H-4/H-5 integral to the choline-derived SM + DHSM amount from ^{31}P gives an independent estimate of the DHSM fraction. Ethanolamine plasmalogens are identified by the vinyl-ether proton near 5.9 ppm, a doublet with a small coupling, which is the only unambiguous ^{1}H marker of the plasmalogen class and is the appropriate confirmation of a ^{31}P shoulder tentatively assigned to it.[13,29] The acyl-chain region is the same for all lipids: terminal methyl at 0.88 ppm, the bulk methylene at 1.25 ppm, the C-3 methylene at 1.58 ppm, the allylic methylenes at 2.0 ppm, the α-methylene at 2.3 ppm, the bis-allylic methylenes at 2.75–2.8 ppm and the olefins at 5.3–5.4 ppm; in milk lipid the butyrate methyl triplet at 0.95 ppm and the conjugated-diene protons at 5.9–6.3 ppm are additional signatures, though they belong to the triacylglycerol fraction and not to the phospholipids.[29,42]

6.2 Interference from neutral lipid and the role of ^{1}H–^{31}P correlation

A whey lipid extract is 60–90 % triacylglycerol, and a WPC80 extract more so. The ^{1}H spectrum of such an extract is therefore a triacylglycerol spectrum in which the phospholipid headgroup resonances are minor features of 1–10 % of the intensity of the glycerol backbone signals, and the practical roles of ^{1}H NMR are correspondingly limited to three: quantification of total choline-phospholipid from the $N^{+}(CH_3)_3$ singlet, structural confirmation of SM and plasmalogen from their olefins, and cross-checking of the ^{31}P total against the choline total. For class-level quantification of the non-choline classes, ^{1}H is not competitive with ^{31}P. It becomes decisively useful when the two nuclei are correlated. In the two-dimensional ^{1}H–^{31}P experiments, each ^{31}P resonance is connected to the ^{1}H shifts of its own headgroup protons, and because the ^{1}H shifts of choline, ethanolamine, serine, inositol and glycerol headgroups differ by tenths of a ppm rather than hundredths, the identification is unambiguous even when the ^{31}P shifts have moved.[14,30,34] Kaffarnik and colleagues used this approach after liquid–liquid enrichment to identify ten phospholipids in cheese and nine in fish, and noted that the ^{31}P shift variations within a class reflected the fatty acids attached; Balsgart and colleagues automated the assignment for about twenty lipids from the same data.[14,34] At 7.05 T the experiment is accessible with a standard broadband probe at ^{1}H detection, and it should be part of the method file for any new whey matrix, run once per matrix type rather than once per sample.

6.3 ^{1}H NMR of the aqueous whey phase: choline metabolites and the whey metabolome

The water-soluble products of phospholipid hydrolysis do not extract into the organic phase and are invisible in a Folch extract, but they are prominent in the ^{1}H spectrum of whey or of a reconstituted whey powder after protein removal. Glycerophosphocholine, phosphocholine and free choline give $N^{+}(CH_3)_3$ singlets at slightly different shifts near 3.2 ppm, resolved at 300 MHz with adequate shimming, and their

relative amounts record successive stages of PC and SM degradation: phospholipase-mediated deacylation to lysophosphatidylcholine and then to glycerophosphocholine, phosphodiesterase action to phosphocholine, and phosphatase action to free choline.[27,43] Hu and colleagues assigned these and many other species in whole bovine milk without pretreatment, using one- and two-dimensional experiments including ^{1}H–^{31}P correlation, and identified creatine and N-acetyl carbohydrates for the first time in milk by NMR.[43] Sousa and colleagues applied ^{1}H NMR metabolite profiling to whey and WPC-80 from a single Parmigiano Reggiano plant and found that whey was higher in dimethyl sulfone, succinate, orotate, fumarate and lactose, that WPC-80 retained significantly more choline, histidine, formate, glucose plus glucose-6-phosphate and acetate, and that hippurate, valine, lactate and uracil were found in the whey but not in the concentrate, the retention of the polar, multiply charged metabolites in WPC-80 being attributed to their electrostatic and hydrogen-bonding association with the whey proteins during ultrafiltration.[44] The finding that choline is enriched in WPC-80 is directly relevant to the present subject, because choline in the aqueous phase of a whey concentrate is the end product of phospholipid hydrolysis and is a candidate index of the phospholipase history of the stream.

The rest of the aqueous ^{1}H spectrum is dominated by lactose (the anomeric protons of α- and β-lactose near 5.2 and 4.45–4.65 ppm and the ring-proton envelope between 3.3 and 4.0 ppm), citrate (2.5–2.7 ppm), lactate (1.33 and 4.1 ppm), acetate (1.92 ppm), creatine and creatinine (3.0–3.1 ppm), orotate (6.2 ppm), hippurate (7.5–7.9 ppm) and formate (8.45 ppm), and the general milk-NMR literature reviewed by Belloque and Ramos and, more recently, in the metabolomics literature provides the assignments.[43–45] For whey protein ingredients the practical value of this spectrum lies in lactose and lactate quantification (WPC34 versus WPC80, sweet versus acid whey), in the organic-acid and orotate profile as a marker of the cheese process, and in the choline metabolites as the hydrolysis index described above; none of it substitutes for the ^{31}P analysis of the lipid.

7. ^{13}C NMR

7.1 Backbone and headgroup carbons and the carbon–phosphorus couplings

The ^{13}C spectrum of a phospholipid contains a set of carbons that are diagnostic because they are coupled to phosphorus. In the glycerophospholipids the sn-3 carbon (CH_2–O–P) near 63.5 ppm appears as a doublet with $^{2}J(C,P)$ of about 5 Hz, the sn-2 methine near 70.5 ppm as a doublet with $^{3}J(C,P)$ of about 8 Hz, and the sn-1 carbon near 62.9 ppm as a singlet; the headgroup carbons show the same pattern, with the choline O–CH_2 near 59.5 ppm (^{2}J) and N–CH_2 near 66.5 ppm (^{3}J) and the $N(CH_3)_3$ near 54.4 ppm as a sharp singlet, and with the PE O–CH_2 near 62 ppm and N–CH_2 near 40.8 ppm.[30,31] These doublets identify a carbon as belonging to a phospholipid rather than to a triacylglycerol, whose backbone carbons (sn-1,3 at 62.1 ppm and sn-2 at 68.9 ppm) are singlets, and they survive in a whey lipid extract even when the phospholipid is a minor component, provided that the signal-to-noise ratio is adequate. The carbonyl region is the second diagnostic window: the sn-1 and sn-2 ester carbonyls of the glycerophospholipids resonate near 173.6 and 173.3 ppm, separated by about 0.3–0.4 ppm, and are themselves distinct from the triacylglycerol carbonyls at 173.2 (sn-1,3) and 172.8 ppm (sn-2), so that the positional distribution of acyl chains between sn-1 and sn-2 can be read from the carbonyl fine structure of a purified phospholipid fraction.[29,31]

Sphingomyelin and DHSM are distinguished most cleanly in the ^{13}C spectrum. The sphingosine base of SM gives the trans-olefinic C-4 and C-5 near 130.5 and 134.2 ppm, an allylic C-3 methine near 71.5 ppm, C-2

near 54 ppm and the amide carbonyl near 174–175 ppm, whereas DHSM lacks the olefinic pair and its C-3 is a saturated methine shifted upfield of the SM C-3; the C-3 resonance is therefore the SM/DHSM discriminator in ^{13}C, and the companion PNA white paper on sphingomyelin treats this assignment and its use in more detail. The phosphorus couplings of the C-1 carbon of the base (near 65 ppm, doublet) and of the choline carbons are the same as in PC and confirm the phosphocholine headgroup.

7.2 Acyl-chain profiling and milk-fat signatures

The acyl-chain carbons of all lipid classes overlap, so that the ^{13}C spectrum of a whey lipid extract reports the acyl profile of the whole extract, dominated by triacylglycerol. Even so, the ^{13}C spectrum of milk lipid is distinctive. The butyrate C-4 methyl near 13.7 ppm and the butyrate C-2 near 36.2 ppm, the caproate and caprylate signals, the olefinic carbons of oleate at 129.7 and 130.0 ppm and of the trans and conjugated isomers between 128 and 135 ppm, the bis-allylic carbon of linoleate at 25.6 ppm and the C-2 carbons at 34.0–34.2 ppm, which differ by 0.2–0.3 ppm between sn-1,3 and sn-2 positions, together characterize the species of origin and the positional distribution; Andreotti and colleagues used exactly this information to distinguish cow, buffalo and other milks from the ^{13}C spectra of their triacylglycerols.[42] For the phospholipid fraction specifically, the acyl profile is accessible by ^{13}C only after the phospholipids have been separated from the neutral lipid, for example by silica solid-phase extraction, at which point the absence of butyrate, the higher proportion of C18:0 and C18:1, the long saturated amide chains of SM and the polyunsaturated sn-2 chains of PS and PI become visible, and the quantitative ^{13}C methods reviewed by Mannina and colleagues and by Alexandri and colleagues can be applied.[6,29,31]

7.3 Practical limits and two-dimensional alternatives

The limitation of ^{13}C is sensitivity. With a receptivity four orders of magnitude below ^{1}H and phospholipid present at a few percent of the extract, a quantitative one-dimensional ^{13}C spectrum of a whey phospholipid fraction at 75.4 MHz requires tens of milligrams of isolated phospholipid, inverse-gated decoupling, relaxation delays of 10–20 s for the carbonyl carbons and an overnight acquisition, and the result is a profile of the bulk phospholipid rather than of any class. The efficient alternative is the ^{1}H-detected two-dimensional correlation. A ^{1}H–^{13}C HSQC of the extract acquired in a few hours at 7.05 T places each headgroup and backbone methylene at its ^{13}C shift, resolves the choline, ethanolamine and sphingoid carbons from the acyl envelope and from lactose, and, combined with the ^{1}H–^{31}P correlation of Section 6.2, ties every ^{31}P resonance to a specific headgroup and backbone. In the multinuclear fingerprinting of krill oil, which is the closest published analogue to a whey lipid extract in its mixture of triacylglycerol and phospholipid, the combination of ^{31}P for class quantification, ^{1}H for total choline and acyl profile, and ^{13}C for structural confirmation was found to be complementary rather than redundant, and the same conclusion is reached here for whey.[46,47] ^{13}C is recommended for method development and for the structural characterization of a new ingredient, not for routine batch analysis.

8. Dairy and whey applications of ^{31}P NMR: a chronological account

The application of ^{31}P NMR to milk began not with phospholipids but with the phosphorus of the serum and the casein micelle. Belton, Lyster and Richards reported the first ^{31}P spectrum of cows' milk in 1985, identifying inorganic phosphate, the broad phosphoserine resonance of micellar casein, and the sharp resonances of low-molecular-weight phosphoesters and glycerophosphodiesters, and thereby defined the

three regions of the milk ^{31}P spectrum that every subsequent study has used.[48] Meneses and Glonek published the chloroform–methanol–Cs-EDTA reagent for extracted phospholipids in 1988, with the linewidths, T_1 and NOE values quoted above, and it became the reference preparation for tissue and, later, food phospholipids.[10] Merchant and Glonek then documented the competition of Mg^{2+}, Ca^{2+}, Na^{+} and K^{+} for the anionic phospholipids and the chemical-shift, broadening and quenching effects that follow, establishing the chelator requirement.[33]

Murgia, Mele and Monduzzi were the first to apply ^{31}P NMR quantitatively to milk-fat phospholipids, in 2003, comparing the biphasic reagent with a monophasic dimethylformamide–triethylamine–guanidinium chloride medium and reporting PC, PE, PI, PS and SM in cow and ewe cream, with LPC, monomethyl-PE and LPE detected qualitatively.[17] Andreotti, Trivellone and Motta extended the monophasic approach to buffalo milk in 2006, analyzing the Folch lipid, the whole milk and a ten-fold ultrafiltration concentrate of defatted milk, and identified in the concentrate N-acetylglucosamine-1-phosphate, galactose-1-phosphate, phosphocholine, phosphoethanolamine, glycerol-1-phosphate and glucose-6-phosphate in addition to inorganic phosphate, glycerophosphoethanolamine and glycerophosphocholine.[37] Byrdwell and Perry, in 2007, combined liquid chromatography with dual parallel mass spectrometry and ^{31}P NMR for bovine milk sphingolipids and quantified SM and DHSM, the NMR providing the class-level anchor for the species-level mass spectrometry.[7]

The decisive methodological paper for dairy is that of MacKenzie, Vyssotski and Nekrasov in 2009, which introduced the sodium cholate detergent preparation, validated it against two-dimensional TLC for cream, beta serum and buttermilk protein concentrate, demonstrated direct analysis of high-phospholipid powders without extraction, showed the need to raise the detergent pH from 7.1 to 9.5 to separate PS, and reported a 36 min analysis time.[12] Zhu and Damodaran used ^{31}P NMR to characterize the phospholipids of cheese-whey MFGM and of the dairy lecithin extracted from it in 2011–2013, providing the SM-rich profile of Table 2 and the only ^{31}P NMR quantification of a whey-derived phospholipid concentrate in the peer-reviewed literature.[4,23] Garcia and colleagues optimized a ^{31}P procedure for the phospholipid fingerprints of human, cow, camel and mare milk in 2012, quantifying twelve phospholipid species including lysophospholipids and plasmalogens and reporting total phospholipid of 0.265 mM in cow milk against 0.324 mM in human milk, with the observation that the optimal temperature for resolution depended on the species.[6,13] Kaffarnik and colleagues introduced the two-dimensional ^{31}P,^{1}H approach for cheese and fish in 2013 and Konrad and colleagues used ^{31}P NMR as the control method for phospholipid enrichment of whey buttermilk by ultrafiltration in the same year.[14,20]

The second decade of the century broadened the applications. Balsgart and colleagues published the automated ^{1}H–^{31}P TOCSY assignment method in 2016; Kato and colleagues addressed the SM/PE overlap in infant-formula polar lipids in 2018 with an ethanol–water extraction and a surfactant dispersion that avoided chloroform; Zhu, Frew and colleagues fingerprinted the phospholipids of four commercial infant-formula brands in 2019, quantifying nine phospholipids and using multivariate analysis to separate brands on PE, SM and PA content; Gouilleux and colleagues evaluated benchtop ^{31}P for lipids in the same year; Mayar and colleagues established the recovery, field-strength and precision characteristics of the cholate method for food emulsions in 2020; and Bruschetta and colleagues applied ^{31}P NMR of milk serum in cholate, with principal component analysis and class-modeling, to the authentication of milk from six species in 2021, quantifying phosphocreatine, glycerophosphocholine, glycerophosphoethanolamine, N-acetylglucosamine-1-phosphate, lactose-1-phosphate, galactose-1-phosphate, phosphocholine and glucose-6-phosphate.[9,15,34,36,38,39] Most recently, Zhao and colleagues used the EDTA reagent with triphenyl

phosphate to follow phospholipid classes through the preparation of MFGM ingredients enriched in polar lipids by microfiltration and diafiltration, finding 5.8–6.4 % phospholipid in the ingredients with the order PC > PE > SM > PS > PI, and Chen and colleagues reviewed the use of NMR for the identification of phospholipids in foods.[16,47] Ferreiro and Rodríguez-Otero, whose group has used ³¹P NMR for milk and quark phospholipids, provided the whey partition data cited in Section 2.1.[19]

Set against the dozens of chromatographic studies of dairy phospholipids reviewed by Rombaut and Dewettinck and by Contarini and Povolo, the ³¹P NMR literature on whey-derived materials is thin: one cheese-whey MFGM lecithin, one whey-buttermilk ultrafiltration study, one fresh-cheese partition study, and the buttermilk protein concentrate of the MacKenzie validation.[6,8] No published ³¹P NMR class profile of a commercial WPC80 or of a commercial WPPC lot was located in the present search, and the compositional statements circulating for WPPC rest on HPLC, TLC or lipidomic determinations of variable provenance. That gap is an opportunity for a laboratory equipped and inclined to fill it.

9. NMR studies of whey proteins: a review

The NMR literature on whey proteins is larger than the phospholipid literature by an order of magnitude and falls into six groups: high-resolution solution NMR of the two principal proteins, β-lactoglobulin and α-lactalbumin; ¹H NMR and hydrogen–deuterium exchange studies of processing-induced denaturation; low-field relaxometry of denaturation, gelation and water binding; pulsed-field-gradient diffusion in whey protein solutions and gels; ³¹P NMR of the phosphorylated species of milk serum; and ¹H NMR profiling of whey and its concentrates. Table 5 summarizes representative studies in each group.

9.1 Solution NMR of β-lactoglobulin

β-Lactoglobulin (β-Lg; 162 residues, 18.3 kDa) is a lipocalin: eight antiparallel β-strands (A–H) form a calyx, a ninth strand (I) forms the dimer interface, and a three-turn α-helix packs against the outer face of the barrel. The protein is a dimer at neutral pH and a monomer below about pH 3, and the monomeric acid state, which retains the native fold, is the state in which its solution structure was determined by NMR. Kuwata and colleagues reported the solution structure and backbone dynamics of the bovine A variant at pH 2, and Uhrínová and colleagues determined the structure of the monomer and described the structural changes that accompany the pH-induced dissociation of the dimer, showing that the strand I interface and the AB loop are the regions that respond.[49,50] Sakurai and Goto later followed the ¹H–¹⁵N HSQC spectrum of β-Lg across the full pH range and applied principal component analysis to the chemical-shift trajectories, separating the dimer dissociation at low pH from the Tanford transition near pH 7, in which the EF loop swings open to expose the calyx, and demonstrating that the two transitions involve different regions of the protein.[51] The Tanford transition is the structural basis of the ligand-binding behavior of β-Lg that is directly relevant to whey lipids: the calyx binds fatty acids, retinol and other hydrophobic ligands, and Ragona and colleagues mapped the binding of palmitic acid to the calyx by NMR chemical-shift perturbation and exchange measurements, while Kontopidis, Holt and Sawyer reviewed the binding properties, structure and putative function of the protein.[52,53] For the present subject the implication is that a fraction of the free fatty acid and possibly of the lysophospholipid of a whey stream is carried on β-Lg rather than on MFGM fragments, and that it is released, or not, according to the pH of the extraction.

9.2 α-Lactalbumin and the molten globule

α-Lactalbumin (α-La; 123 residues, 14.2 kDa) binds Ca^{2+} and adopts, on acidification or calcium removal, the partially folded state that gave the molten globule its name. The acid-denatured state was characterized by two-dimensional ^{1}H NMR by Alexandrescu and colleagues, who found persistent native-like helical structure in the α-domain alongside a disordered β-domain, and Schulman and colleagues obtained a residue-specific view of the non-cooperative unfolding of the molten globule, in which different regions lose their residual structure at different denaturant concentrations.[54,55] The molten globule is not an academic curiosity for dairy technology: it is the state through which α-La passes during acid whey processing, during the heat treatments used to fractionate α-La from β-Lg, and at the low pH of the ^{31}P NMR extraction media, and its exposed hydrophobic surface binds lipid.

9.3 Processing-induced changes followed by ^{1}H NMR and hydrogen–deuterium exchange

The bridge between structural NMR and dairy processing was built by Belloque and Smith, who followed the thermal denaturation of β-Lg at pH 2 and 7.4 by ^{1}H NMR and deuterium exchange. At pH 2 much of the structure was preserved and two-dimensional spectra could be assigned; assigned amide resonances from different parts of the protein were then followed as they disappeared during heating at 45, 55 and 75 °C in $^{2}H_2O$, and the denaturation was found to proceed in stages, with strand E and the A–B loop unfolding at 55 °C ahead of the rest of the barrel.[56] The same authors applied ^{1}H NMR to the effect of high hydrostatic pressure, again using exchange to locate the regions destabilized by pressure treatment.[57] Edwards, Jameson, Palmano and Creamer used NMR-monitored H/D exchange to identify the heat-resistant structural features of β-Lg A, a set of amide protons in the core of the barrel that remained protected after heating and that define the nucleus from which refolding, or irreversible aggregation, proceeds.[58] These studies are the reference points for any NMR-based claim about the denaturation state of a whey protein ingredient, and they should be cited whenever a relaxometric or diffusion result is interpreted in structural terms.

9.4 Low-field relaxometry: denaturation, gelation and the state of water

Low-resolution, low-field ^{1}H NMR entered whey protein science through the work of Lambelet and colleagues at the Nestlé Research Centre. In 1989 they described a method in which the transverse relaxation of water protons, measured at 20 °C after a thermal treatment, was used to follow whey protein denaturation, showed that the parameter responded to denaturation and not to gelation, and applied it qualitatively to WPC, β-Lg, α-La, bovine serum albumin and immunoglobulins and quantitatively to the degree of denaturation in WPC solutions.[59] In 1992 they extended the approach to milk proteins, showing that the increase in the water proton transverse relaxation rate above the denaturation onset temperature paralleled the classical Rowland fractionation, and that ionic strength, caseinate and casein micelles modified the response.[60] The method is the direct ancestor of every time-domain NMR assay of whey protein denaturation offered today and it retains its principal virtue, that it requires no extraction and no optical clarity, and its principal limitation, that the relaxation rate integrates denaturation, aggregation, ionic strength and protein concentration into one number that must be calibrated against a reference method for each matrix.

The state of water in whey protein systems has been studied in more detail by the Mariette group. Le Dean, Mariette and Marin measured ^{1}H relaxation of water in caseinate–whey protein mixtures and interpreted the multiexponential behavior in terms of exchange between water populations associated with the two protein types.[61] Colsenet, Mariette and Cambert combined transverse relaxation with pulsed-field-gradient water self-diffusion in whey protein solutions and gels, found that water diffused freely over 15–47 µm with no restriction that could explain the reduction in mobility, attributed the relaxation changes on aggregation to diffusion and chemical exchange between water and protein protons, and modeled the concentration dependence of diffusion with an obstruction model and a two-compartment cell model.[62] Hinrichs, Götz and Weisser used the same relaxation methods to characterize water-holding capacity and structure in hydrocolloid gels, WPC gels and yogurts.[63] Mariette reviewed the application of NMR and MRI to food colloids, including whey protein gels, and set out the interpretive framework — exchange, obstruction, compartmentation — within which relaxometric results in whey systems should be read.[64]

9.5 Diffusion NMR in whey protein solutions and gels

Pulsed-field-gradient NMR has been used to map the mesh size and connectivity of whey protein gels from the diffusion of probe molecules and of the proteins themselves. The Mariette group measured the self-diffusion of poly(ethylene glycol) probes of 1080 to 82,250 g mol^{-1} in whey protein solutions and heat-set gels over a concentration range of 6.5–40 % protein, finding a strong dependence on probe size that increased with protein concentration, and, counter-intuitively, that heat-induced aggregation increased the diffusion coefficient of the larger probes because the aggregated network left larger pores than the unaggregated solution; ionic strength and denaturation time were subsequently shown to modulate the effect, and the diffusion of the residual non-aggregated whey protein could be measured in the same experiment.[64,65] For a phospholipid analysis these experiments are relevant in one respect: the same pulsed-field-gradient capability distinguishes a phospholipid signal arising from small mixed micelles from one arising from large MFGM fragments or vesicles, and a diffusion-edited ^{1}H or ^{31}P experiment is the cleanest way to demonstrate that a direct cholate preparation of a whey powder has actually solubilized the phospholipid rather than merely dispersed it.

9.6 ^{31}P NMR of phosphorylated species in milk serum and whey

The serum-phase ^{31}P literature is the natural companion of the phospholipid literature because the two are acquired in the same spectra. Belton and colleagues defined the three spectral regions; Belloque, de la Fuente and Ramos then carried out a qualitative and quantitative analysis of the phosphorylated compounds of milk by ^{31}P NMR, assigning inorganic phosphate, the phosphoserine residues of casein, the glycerophosphodiesters, phosphocholine and phosphoethanolamine, and the sugar phosphates, and establishing the pH and chelator conditions under which the small molecules could be quantified against the broad casein background.[48,66] Belloque and Ramos exploited the phosphoserine resonance directly to determine the casein content of bovine milk, an approach that transfers to the measurement of residual casein and caseinomacropeptide in sweet whey products, in which the single phosphoserine of caseinomacropeptide (SerP149 of κ-casein) and the phosphopeptides of the proteose-peptone fraction contribute a small phosphomonoester signal.[67] In UHT milk, Belloque, Carrascosa and López-Fandiño followed the phosphoglyceride composition during storage by ^{31}P NMR and attributed the changes to thermoresistant microbial phospholipases surviving the heat treatment, which is the clearest demonstration in the dairy literature that ^{31}P NMR can read enzymatic history from a phospholipid profile.[27] Andreotti and

colleagues added the buffalo-milk serum assignments, and Bruschetta and colleagues quantified eight monophosphorylated metabolites in the milk serum of six species in cholate–EDTA–D_2O with hexamethylphosphoramide as standard, finding in cow milk serum glycerophosphocholine at 1.0–1.3 mM, glycerophosphoethanolamine at 0.17–0.32 mM, N-acetylglucosamine-1-phosphate at 0.6–2.5 mM and galactose-1-phosphate at 0.15–0.47 mM, with the caveat that lactose-1-phosphate overlapped the inorganic phosphate resonance in cow and goat samples and that their classification models were built on ten samples per species.[37,38] A ^{31}P spectrum of a reconstituted whey powder in cholate therefore contains, downfield of the phospholipids, a serum profile that reports on the process (sweet versus acid, degree of demineralization), on the residual casein and on the phospholipid hydrolysis products, all from the same acquisition.

9.7 ^{1}H NMR profiling of whey and whey protein concentrates

The ^{1}H NMR profiling of milk was reviewed by Belloque and Ramos in 1999 and has since become a mature metabolomics discipline, but its application to whey and to whey protein ingredients is recent and sparse; the comparison of whey and WPC-80 by Sousa and colleagues in 2025 appears to be the first systematic ^{1}H NMR metabolite profile of a whey protein concentrate, and its finding that polar and multiply charged metabolites are retained by ultrafiltration through association with the proteins is both new and analytically consequential.[44,45] The whole-milk assignments of Hu and colleagues remain the most complete reference for the small molecules of the serum phase.[43]

9.8 Gaps in the whey protein NMR literature

Three gaps are evident. There is no published ^{31}P NMR study of the phospholipid class profile of commercial WPC80 or WPPC lots, and no study relating that profile to foaming, emulsification or oxidative stability of the same lots. There is no study that reports the phospholipid, lysophospholipid and glycerophosphodiester profiles of the same whey stream together, which is the combination that would identify phospholipase activity unambiguously. And there is no study that combines the relaxometric denaturation assay of Section 9.4 with a phospholipid analysis, although the two are the dominant quality attributes of a WPC and both are accessible on the same instrument. Each gap is a defined project.

Table 5. Representative NMR studies of whey proteins and whey-related phosphorylated species, grouped by technique.

Group / technique	System	Principal finding	Ref.
Solution NMR structure	Bovine β-Lg A, pH 2 monomer	Solution structure and backbone dynamics of the lipocalin fold	49
Solution NMR structure	Bovine β-Lg, pH titration	Structural changes accompanying dimer dissociation; AB loop and strand I respond	50
^{1}H–^{15}N HSQC with PCA	Bovine β-Lg, pH 2–9	Dimer dissociation and Tanford (EF-loop) transition separated as distinct principal components	51
Chemical-shift perturbation	β-Lg with palmitic acid	Fatty acid bound in the calyx; residues affected mapped	52,53
2D ^{1}H NMR, acid state	α-Lactalbumin molten globule	Native-like α-domain helices persist; β-domain disordered; non-cooperative unfolding	54,55
^{1}H NMR + H/D exchange	β-Lg heated at pH 2 and 7.4	Staged denaturation: strand E and A–B loop unfold at 55 °C before the barrel	56
^{1}H NMR + H/D exchange	β-Lg after high pressure	Pressure-destabilized regions located by exchange	57

Group / technique	System	Principal finding	Ref.
H/D exchange	β-Lg A heated	Heat-resistant core of protected amides identified	58
Low-field ^{1}H T_2	WPC, β-Lg, α-La, BSA, Ig solutions	Water T_2 after heating reports denaturation, not gelation; quantitative for WPC	59,60
^{1}H relaxometry	Caseinate–whey protein mixtures	Water populations in exchange between protein types	61
T_2 and PFG diffusion	Whey protein solutions and heat-set gels	Free water diffusion over 15–47 µm; relaxation explained by exchange; obstruction and cell models	62
PFG probe diffusion	PEG probes in whey protein solutions and gels	Probe-size and concentration dependence; heat-set network leaves larger pores than the solution	65
Low-field NMR	WPC gels, hydrocolloid gels, yogurt	Water-holding capacity and structure from relaxation distributions	63
Review	Food colloids incl. whey gels	Interpretive framework for NMR/MRI of colloids	64
^{31}P NMR of milk	Cows' milk	Pi, casein SerP and phosphoesters defined the three spectral regions	48
^{31}P NMR of milk	Bovine milk, pH/EDTA series	Qualitative and quantitative assignment of phosphorylated compounds	66
^{31}P NMR	Bovine milk	Casein content from the phosphoserine integral	67
^{31}P NMR	UHT milk in storage	Phosphoglyceride changes attributed to thermoresistant microbial phospholipases	27
^{31}P NMR in cholate, PCA/SIMCA/k-NN	Milk serum of six species	Eight monophosphorylated metabolites quantified; species classification	38
1D/2D ^{1}H, ^{13}C, ^{1}H–^{31}P NMR	Whole bovine milk, no pretreatment	Assignment of serum metabolites; creatine and N-acetyl carbohydrates detected	43
^{1}H NMR metabolomics	Whey vs WPC-80	Choline, histidine, formate and acetate enriched in WPC-80; hippurate, lactate, valine, uracil only in whey	44

10. A proposed PNA protocol for the ^{1}H, ^{13}C and ^{31}P analysis of whey protein ingredients

The protocol below is written for the PNA 300 MHz spectrometer with a broadband probe and for the benchtop instrument where its use is defensible. It is presented as a draft for validation, not as a validated method, and the validation set of Section 5.5 must be completed before results are reported to clients under the ISO/IEC 17025 scope.

10.1 Sample intake and preparation

Powders are equilibrated to room temperature in sealed containers before weighing to avoid moisture uptake, and the moisture content is determined on a separate portion. For WPC34, WPC80 and WPI, 2.0 g of powder is dispersed in 10 mL of water at pH 6.5–7.0, held for 30 min with gentle agitation, and extracted with 40 mL of chloroform–methanol (2:1, v/v) with 5 min of bath sonication; the mixture is centrifuged, the lower phase is collected, the residue is re-extracted twice with 10 mL of methanol to recover lysophospholipids, and the combined extracts are washed with 0.2 volumes of 0.1 M KCl, dried under nitrogen and weighed. For WPPC, MFGM isolates and buttermilk powders, two preparations are made in parallel: the Folch extract as above from 1.0 g of powder, and a direct preparation in which 300 mg of

powder is dispersed in 0.7 mL of 250 mM sodium cholate with 10 mM EDTA in D_2O, adjusted to pH 7.1 (and, in a second tube, to pH 9.5 for PS resolution), sonicated for 5 min and centrifuged; agreement between the two preparations is the acceptance criterion for the direct route.[5,9,12] For the aqueous ^{1}H and serum ^{31}P profile, 1.0 g of powder is reconstituted in 10 mL of D_2O-containing phosphate buffer at pH 7.0, the proteins are removed by ultrafiltration through a 3 kDa membrane, and the permeate is analyzed with an internal standard.

10.2 ^{31}P acquisition on the 300 MHz instrument

The dried lipid extract (20–60 mg) is dissolved in 2.0 mL of $CDCl_3$ containing triphenyl phosphate at a known concentration (typically 1–2 µmol per tube), 1.0 mL of the aqueous methanol–Cs-EDTA reagent is added, the mixture is shaken and centrifuged, and 0.7 mL of the lower phase is transferred to a 5 mm tube; a 10 mm tube with the full lower phase is used for WPC80 extracts of low phospholipid content. The parameters of Table 6 are applied. T_1 of every resonance, including the standard, is measured by inversion recovery on the first sample of each new matrix and the relaxation delay is set to five times the longest value. A ^{1}H–^{31}P correlation (HMBC-type, ^{1}H detected) is acquired once per matrix type for assignment. The pH 9.5 cholate preparation of WPPC is used to confirm the PS integral obtained from the pH 7.1 preparation and from the chloroform reagent, and the three PS values are required to agree within their combined uncertainty before the PS result is reported.

Table 6. Proposed acquisition and processing parameters for quantitative ^{31}P NMR of whey phospholipid extracts and direct WPPC preparations on the PNA 7.05 T spectrometer, with the corresponding limits for a 1.41 T benchtop instrument.

Parameter	7.05 T (121.4 MHz ^{31}P)	1.41 T benchtop (24.3 MHz ^{31}P)
Sample	Folch/ethanol extract in $CDCl_3$–CH_3OH–Cs-EDTA reagent, or WPPC directly in 250 mM cholate/10 mM EDTA/D_2O	Concentrated extract only; direct powder preparations not recommended
Internal standard	Triphenyl phosphate (organic) or hexamethylphosphoramide / methylenediphosphonate (cholate), purity-corrected; T_1 measured	As 7.05 T; larger amount to offset sensitivity
Temperature	25.0 ± 0.1 °C; shifts recorded at 25 and 35 °C for assignment	As stable as the magnet permits; record actual value
Pulse	Calibrated 90° on sample	Calibrated 90°; or ^{1}H–^{31}P cross-polarization with per-class calibration
Decoupling	Inverse-gated WALTZ-16 (NOE suppressed)	Inverse-gated if available; CP schemes require calibration
Spectral width / offset	≈ 40 ppm centered at −8 ppm (to include TPP) or ≈ 60 ppm if HMPA	As 7.05 T
Acquisition time	1.6–2.5 s (2–3 Hz lines)	2–4 s
Relaxation delay	≥ 5 × longest T_1; expect 15–30 s	≥ 5 × T_1; T_1 may be longer at low field (measure)
Transients	256–1024 (1.5–8 h) depending on phospholipid mass	≥ 2 h; LOD 0.2–0.5 mM per Gouilleux et al.
Processing	0.5–1 Hz exponential; ≥ 4× zero-fill; manual zero-order phase; baseline fitted outside the phosphodiester cluster and excluding SerP hump	As 7.05 T

Parameter	7.05 T (121.4 MHz ^{31}P)	1.41 T benchtop (24.3 MHz ^{31}P)
Resolved classes	PC, LPC, PI, SM, DHSM, PS, PE, LPE, PA, (EPLAS by ^{1}H)	PC, PI, PE-cluster (PE + PS + SM + DHSM + LPE) only
Reporting	mol % of PL phosphorus; mg/100 g powder (state MW assumptions); g/100 g fat; LPL and DHSM reported separately	Total PL, PC and PC/PE-cluster ratio only, with the resolution limitation stated

10.3 ^{1}H and ^{13}C acquisition

A quantitative ^{1}H spectrum of the same lower-phase solution is acquired at 300 MHz with a 30° pulse, a 10 s relaxation delay and 64 transients, and the choline $N^{+}(CH_3)_3$ integral, the SM H-4/H-5 integral and the plasmalogen vinyl-ether integral are extracted; the total choline-phospholipid from ^{1}H is compared with PC + LPC + SM + DHSM from ^{31}P and a discrepancy above 10 % triggers a review of the assignments and of the relaxation delay. A ^{1}H–^{13}C HSQC (4–6 h) is acquired for each new matrix type and a one-dimensional quantitative ^{13}C spectrum with inverse-gated decoupling (overnight) is acquired only when the acyl profile or the SM/DHSM C-3 discrimination is required for a characterization report. The aqueous permeate is analyzed by ^{1}H NMR with water presaturation and a 30 s delay for lactose, lactate, citrate, orotate, choline, phosphocholine and glycerophosphocholine, and by ^{31}P NMR in the same tube after addition of EDTA for inorganic phosphate, phosphomonoesters, glycerophosphodiesters and residual phosphoserine.

10.4 Quality control and reporting

Each batch carries a reagent blank, a gravimetric phospholipid standard mixture (PC, PE, PS, PI, SM) prepared to mimic a whey profile, and one previously analyzed WPPC control. Acceptance requires the control to fall within the established intermediate-precision limits for every class, the standard mixture to be recovered within 5 % for the major and 10 % for the minor classes, and the ^{1}H–^{31}P choline cross-check to agree within 10 %. The report states, for each class, the mol %, the mass per 100 g of powder with the molecular-weight assumptions, the extraction route, the solvent system and pH, the temperature, the relaxation delay and the standard used, and it identifies explicitly which resonances were confirmed by spiking or by two-dimensional correlation. Results from the benchtop instrument are reported only as total phospholipid, PC and the PE-cluster, with a statement of the resolution limitation, and are never reported as a class profile.

11. Critical assessment: pitfalls, confounders and what a reviewer would challenge

The method described above is sound in principle, but the following points are where it fails in practice and where an auditor, a journal referee or an opposing expert would concentrate. They are listed in order of the damage they can do.

Extraction bias is the largest and least visible error. The phospholipid of a whey powder is bound in MFGM fragments and adsorbed on protein, and every published extraction — chloroform–methanol, ethanol, pressurized fluid, supercritical CO_2 — recovers a different fraction of it with a different class selectivity; Théodet and Gandemer showed this for whey thirty-five years ago and Price and colleagues showed it again for WPPC, where the selection of extraction stage changed the SM and PS content of the recovered lipid.[5,35]

A ^{31}P class profile is therefore a profile of what was extracted, and it is not a profile of the powder unless recovery has been demonstrated by a mass balance or by agreement with a direct preparation, which is only possible for phospholipid-rich materials. The lysophospholipids are the extreme case, being lost by the Folch procedure and recovered only by repeated methanol extraction.[9] Any WPC80 result reported without a recovery study is a relative class distribution, and it should be labeled as such.

Misassignment is the second. The chemical shifts of PS, PE, LPE, DHSM and the plasmalogens lie within 0.1 ppm of one another, move with pH, cation content, water content and temperature, and are routinely reported in the literature from tables rather than from spiking; the pH shift of PS alone can exceed its separation from SM.[32,33,40] The consequence is that the minor anionic classes, which are precisely the ones with nutritional and labeling significance, are the ones most often misreported. A profile in which PS, DHSM or LPE has been quantified without a spiking experiment or a ^{1}H–^{31}P correlation should be treated as provisional.

Relaxation and NOE errors are the third. A relaxation delay chosen for the phospholipids and not for the standard under-estimates the standard and inflates every absolute result; continuous decoupling inflates the phospholipids relative to the standard by a class-dependent 20–60 %; cross-polarization schemes used to gain sensitivity at low field transfer polarization with class-dependent efficiency.[10,39] These errors do not affect mol % distributions much, because all classes are affected similarly, but they affect absolute contents and they are invisible in the spectrum.

The benchtop temptation is the fourth. A 60 MHz instrument with a ^{31}P channel will produce a clean-looking spectrum of a WPPC extract showing PC, a large envelope and PI. The envelope is PE + PS + SM + DHSM + LPE, and no processing — deconvolution included — can extract five classes from one Lorentzian of 2.4 Hz per 0.1 ppm with any reliability. Reporting a class profile from such a spectrum is not defensible; reporting total phospholipid, PC and the envelope is.[39]

The glycolipid blind spot is the fifth. Glucosylceramide, lactosylceramide and the gangliosides, which are real components of whey MFGM and of the health claims attached to it, contain no phosphorus. A ^{31}P result described as the polar lipid content of an ingredient is an under-statement by the glycolipid fraction, which can be several percent of the polar lipid, and the description must be corrected to phospholipid content.[3,22]

Compositional variability of the material is the sixth. WPPC lots from a single supplier six months apart, and lots from different suppliers, differ in fat content by a factor of three and in phospholipid content of the fat by a factor of four.[2] A single measurement characterizes a lot, not a product, and typical values quoted for WPPC have no better standing than the sampling behind them. The bacterial contribution to the whey phospholipid of cultured processes, which adds PG and cardiolipin absent from MFGM, is a related confounder that has been reported but not, so far, quantified by NMR.[19]

Chemometric over-reach is the seventh, and it applies to the literature rather than to the method. Classification of milk species from ^{31}P serum profiles on ten samples per class, and separation of infant-formula brands on four brands, are demonstrations of feasibility, not validated models, and they should not be cited as evidence that ^{31}P NMR authenticates dairy ingredients until they have been reproduced on independent sample sets of adequate size.[15,38] The same restraint applies to any PNA fingerprinting claim built on the whey phospholipid profile.

Finally, the mass basis is the eighth. Conversion of ^{31}P mole fractions to mass per 100 g requires a molecular weight per class that depends on the acyl composition of the dairy phospholipid, which is itself not measured

by ^{31}P; the assumption introduces 2–5 % uncertainty in the mass of each class and up to 3 % in the total, and it is rarely stated. The molar result is the primary result and should be reported first.

12. Conclusions

^{31}P NMR is the appropriate technique of record for the class-level quantification of phospholipids in whey protein ingredients. It requires no authentic standard for identification, gives one resonance per class with a molar response independent of acyl composition, resolves the lysophospholipids and dihydrosphingomyelin that chromatography merges or loses, and can be applied directly, without extraction, to phospholipid-rich materials such as WPPC and MFGM isolates. The chemistry that determines its reliability — solvent system, chelation of divalent cations, pH, temperature, relaxation and NOE control — is well understood and is summarized in Sections 3–5, and a 7.05 T instrument is a good field for the purpose: high enough to resolve the PE/PS/DHSM/SM cluster, low enough that chemical-shift-anisotropy broadening and species fine structure remain modest. A 24 MHz benchtop instrument can quantify total phospholipid, PC and the PE-cluster but not the class profile.

^{1}H NMR supplies the choline stoichiometry that cross-checks the ^{31}P total, the sphingoid and vinyl-ether olefins that confirm SM, DHSM and plasmalogen assignments, and, in the aqueous phase, the choline-metabolite and lactose–organic acid profile that reports on hydrolysis and process history. ^{13}C NMR supplies the carbon–phosphorus-coupled backbone and headgroup carbons, the sn-1/sn-2 carbonyl distribution and the sphingoid-base carbons that discriminate SM from DHSM, at a sensitivity cost that confines it to characterization work. The three nuclei are complementary, and a ^{1}H–^{31}P correlation acquired once per matrix type is the single most valuable addition to a routine ^{31}P method.

The literature on whey-derived phospholipids by NMR is thin — one cheese-whey lecithin, one whey buttermilk study, one fresh-cheese partition study and the buttermilk protein concentrate of a validation paper — and no published ^{31}P class profile of commercial WPC80 or WPPC exists. The wider NMR literature on whey proteins, from the solution structures of β-lactoglobulin and α-lactalbumin through processing-induced denaturation, low-field relaxometry, diffusion in gels, serum-phase ^{31}P and ^{1}H metabolite profiling, is mature and provides the interpretive context within which whey phospholipid results should be read. The pitfalls of Section 11 — extraction bias, misassignment of the anionic classes, relaxation and NOE errors, the benchtop resolution limit, the glycolipid blind spot, lot-to-lot variability, chemometric over-reach and the molecular-weight assumption — are each manageable, but only if they are managed explicitly and stated in the report.

References

All references were verified against the CrossRef metadata record (title, author list, journal, year, volume, pagination and DOI) on August 27, 2026. Titles are reproduced as published, including original spelling.

1. Morr CV, Ha EYW. Whey protein concentrates and isolates: processing and functional properties. Crit Rev Food Sci Nutr. 1993;33:431–476. doi:10.1080/10408399309527643
2. Levin MA, Burrington KJ, Hartel RW. Composition and functionality of whey protein phospholipid concentrate and delactosed permeate. J Dairy Sci. 2016;99:6937–6947. doi:10.3168/jds.2016-10974
3. Dewettinck K, Rombaut R, Thienpont N, Le TT, Messens K, Van Camp J. Nutritional and technological aspects of milk fat globule membrane material. Int Dairy J. 2008;18:436–457. doi:10.1016/j.idairyj.2007.10.014

4. Zhu D, Damodaran S. Dairy lecithin from cheese whey fat globule membrane: its extraction, composition, oxidative stability, and emulsifying properties. J Am Oil Chem Soc. 2013;90:217–224. doi:10.1007/s11746-012-2152-5
5. Price N, Fei T, Clark S, Wang T. Extraction of phospholipids from a dairy by-product (whey protein phospholipid concentrate) using ethanol. J Dairy Sci. 2018;101:8778–8787. doi:10.3168/jds.2018-14950
6. Contarini G, Povolo M. Phospholipids in milk fat: composition, biological and technological significance, and analytical strategies. Int J Mol Sci. 2013;14:2808–2831. doi:10.3390/ijms14022808
7. Byrdwell WC, Perry RH. Liquid chromatography with dual parallel mass spectrometry and ^{31}P nuclear magnetic resonance spectroscopy for analysis of sphingomyelin and dihydrosphingomyelin. II. Bovine milk sphingolipids. J Chromatogr A. 2007;1146:164–185. doi:10.1016/j.chroma.2007.01.108
8. Rombaut R, Dewettinck K. Properties, analysis and purification of milk polar lipids. Int Dairy J. 2006;16:1362–1373. doi:10.1016/j.idairyj.2006.06.011
9. Mayar M, de Roo N, Hoos P, van Duynhoven J. ^{31}P NMR quantification of phospholipids and lysophospholipids in food emulsions. J Agric Food Chem. 2020;68:5009–5017. doi:10.1021/acs.jafc.0c00404
10. Meneses P, Glonek T. High resolution ^{31}P NMR of extracted phospholipids. J Lipid Res. 1988;29:679–689. doi:10.1016/S0022-2275(20)38513-8
11. Spyros A, Dais P. ^{31}P NMR spectroscopy in food analysis. Prog Nucl Magn Reson Spectrosc. 2009;54:195–207. doi:10.1016/j.pnmrs.2008.09.002
12. MacKenzie A, Vyssotski M, Nekrasov E. Quantitative analysis of dairy phospholipids by ^{31}P NMR. J Am Oil Chem Soc. 2009;86:757–763. doi:10.1007/s11746-009-1403-6
13. Garcia C, Lutz NW, Confort-Gouny S, Cozzone PJ, Armand M, Bernard M. Phospholipid fingerprints of milk from different mammalians determined by ^{31}P NMR: towards specific interest in human health. Food Chem. 2012;135:1777–1783. doi:10.1016/j.foodchem.2012.05.111
14. Kaffarnik S, Ehlers I, Gröbner G, Schleucher J, Vetter W. Two-dimensional ^{31}P,^{1}H NMR spectroscopic profiling of phospholipids in cheese and fish. J Agric Food Chem. 2013;61:7061–7069. doi:10.1021/jf4021812
15. Zhu D, Hayman A, Kebede B, Stewart I, Chen G, Frew R. ^{31}P NMR-based phospholipid fingerprinting of powdered infant formula. J Agric Food Chem. 2019;67:10265–10272. doi:10.1021/acs.jafc.9b03902
16. Zhao P, Ji G, Lin R, Zhang L, Li F, Zhang S, Chen Y, Wei W, Wang X. Preparation of milk fat globule membrane ingredients enriched in polar lipids: composition characterization and digestive properties. J Dairy Sci. 2024;107:4320–4332. doi:10.3168/jds.2023-24462
17. Murgia S, Mele S, Monduzzi M. Quantitative characterization of phospholipids in milk fat via ^{31}P NMR using a monophasic solvent mixture. Lipids. 2003;38:585–591. doi:10.1007/s11745-003-1500-3
18. Rombaut R, Van Camp J, Dewettinck K. Phospho- and sphingolipid distribution during processing of milk, butter and whey. Int J Food Sci Technol. 2006;41:435–443. doi:10.1111/j.1365-2621.2005.01091.x
19. Ferreiro T, Rodríguez-Otero JL. Evolution and distribution of phospholipids in cheese and whey during the manufacturing of fresh cheese from cows' milk. Int J Dairy Technol. 2018;71:820–823. doi:10.1111/1471-0307.12499
20. Konrad G, Kleinschmidt T, Lorenz C. Ultrafiltration of whey buttermilk to obtain a phospholipid concentrate. Int Dairy J. 2013;30:39–44. doi:10.1016/j.idairyj.2012.11.007
21. Lilbæk HM, Broe ML, Høier E, Fatum TM, Ipsen R, Sørensen NK. Improving the yield of mozzarella cheese by phospholipase treatment of milk. J Dairy Sci. 2006;89:4114–4125. doi:10.3168/jds.S0022-0302(06)72457-2
22. Vaghela M, Kilara A. Lipid composition of whey protein concentrates manufactured commercially and in the laboratory. J Dairy Sci. 1996;79:1172–1183. doi:10.3168/jds.S0022-0302(96)76471-8

23. Zhu D, Damodaran S. Composition, thermotropic properties, and oxidative stability of freeze-dried and spray-dried milk fat globule membrane isolated from cheese whey. J Agric Food Chem. 2011;59:8931–8938. doi:10.1021/jf201688w
24. Boyd LC, Drye NC, Hansen AP. Isolation and characterization of whey phospholipids. J Dairy Sci. 1999;82:2550–2557. doi:10.3168/jds.S0022-0302(99)75509-8
25. Ozturk G, Liang N, Bhattacharya M, Robinson RC, Shankar S, Huang Y-P, Paviani B, Taha AY, Barile D. Glycoproteomic and lipidomic characterization of industrially produced whey protein phospholipid concentrate with emphasis on antimicrobial xanthine oxidase, oxylipins and small milk fat globules. Dairy. 2022;3:277–302. doi:10.3390/dairy3020022
26. Pearce JM, Komoroski RA. Resolution of phospholipid molecular species by ^{31}P NMR. Magn Reson Med. 1993;29:724–731. doi:10.1002/mrm.1910290603
27. Belloque J, Carrascosa AV, López-Fandiño R. Changes in phosphoglyceride composition during storage of ultrahigh-temperature milk, as assessed by ^{31}P-nuclear magnetic resonance: possible involvement of thermoresistant microbial enzymes. J Food Prot. 2001;64:850–855. doi:10.4315/0362-028X-64.6.850
28. Sparling ML, Zidovetzki R, Muller L, Chan SI. Analysis of membrane lipids by 500 MHz ^{1}H NMR. Anal Biochem. 1989;178:67–76. doi:10.1016/0003-2697(89)90358-8
29. Alexandri E, Ahmed R, Siddiqui H, Choudhary MI, Tsiafoulis CG, Gerothanassis IP. High resolution NMR spectroscopy as a structural and analytical tool for unsaturated lipids in solution. Molecules. 2017;22:1663. doi:10.3390/molecules22101663
30. Bosco M, Culeddu N, Toffanin R, Pollesello P. Organic solvent systems for ^{31}P nuclear magnetic resonance analysis of lecithin phospholipids: applications to two-dimensional gradient-enhanced ^{1}H-detected heteronuclear multiple quantum coherence experiments. Anal Biochem. 1997;245:38–47. doi:10.1006/abio.1996.9907
31. Mannina L, Sobolev AP, Viel S. Liquid state ^{1}H high field NMR in food analysis. Prog Nucl Magn Reson Spectrosc. 2012;66:1–39. doi:10.1016/j.pnmrs.2012.02.001
32. Puppato A, DuPré DB, Stolowich N, Yappert MC. Effect of temperature and pH on ^{31}P nuclear magnetic resonances of phospholipids in cholate micelles. Chem Phys Lipids. 2007;150:176–185. doi:10.1016/j.chemphyslip.2007.07.002
33. Merchant TE, Glonek T. ^{31}P NMR of tissue phospholipids: competition for Mg^{2+}, Ca^{2+}, Na^{+} and K^{+} cations. Lipids. 1992;27:551–559. doi:10.1007/BF02536139
34. Balsgart NM, Mulbjerg M, Guo Z, Bertelsen K, Vosegaard T. High throughput identification and quantification of phospholipids in complex mixtures. Anal Chem. 2016;88:2170–2176. doi:10.1021/acs.analchem.5b03798
35. Théodet C, Gandemer G. Comparaison de cinq méthodes pour extraire les lipides du lactosérum et de ses dérivés. Lait. 1991;71:41–54. doi:10.1051/lait:199113
36. Kato T, Nishimiya M, Kawata A, Kishida K, Suzuri K, Saito M, Fujita K, Igarashi T, Inagaki M. Quantitative ^{31}P NMR method for individual and concomitant determination of phospholipid classes in polar lipid samples. J Oleo Sci. 2018;67:1279–1289. doi:10.5650/jos.ess18062
37. Andreotti G, Trivellone E, Motta A. Characterization of buffalo milk by ^{31}P-nuclear magnetic resonance spectroscopy. J Food Compos Anal. 2006;19:843–849. doi:10.1016/j.jfca.2006.03.014
38. Bruschetta G, Notti A, Lando G, Ferlazzo A. A promising ^{31}P NMR-multivariate analysis approach for the identification of milk phosphorylated metabolites and for rapid authentication of milk samples. Biochem Biophys Rep. 2021;27:101087. doi:10.1016/j.bbrep.2021.101087
39. Gouilleux B, Christensen NV, Malmos KG, Vosegaard T. Analytical evaluation of low-field ^{31}P NMR spectroscopy for lipid analysis. Anal Chem. 2019;91:3035–3042. doi:10.1021/acs.analchem.8b05416
40. Rebollar-Ramos D, Chen S-N, Lankin DC, Kleps RA, Pauli GF. Key considerations for phosphatidylserine quantification by ^{31}P NMR in pharmaceutical analysis. J Pharm Biomed Anal. 2026;270:117248. doi:10.1016/j.jpba.2025.117248

41. Giuffrida F, Cruz-Hernandez C, Flück B, Tavazzi I, Thakkar SK, Destaillats F, Braun M. Quantification of phospholipids classes in human milk. Lipids. 2013;48:1051–1058. doi:10.1007/s11745-013-3825-z

42. Andreotti G, Lamanna R, Trivellone E, Motta A. ¹³C NMR spectra of TAG: an easy way to distinguish milks from different animal species. J Am Oil Chem Soc. 2002;79:123–127. doi:10.1007/s11746-002-0445-2

43. Hu F, Furihata K, Ito-Ishida M, Kaminogawa S, Tanokura M. Nondestructive observation of bovine milk by NMR spectroscopy: analysis of existing states of compounds and detection of new compounds. J Agric Food Chem. 2004;52:4969–4974. doi:10.1021/jf049616o

44. Sousa I, Meoni G, Tenori L, Pozza M, De Marchi M, Niero G. ¹H NMR for comparative metabolic analysis of whey and WPC-80. Metabolites. 2025;15:770. doi:10.3390/metabo15120770

45. Belloque J, Ramos M. Application of NMR spectroscopy to milk and dairy products. Trends Food Sci Technol. 1999;10:313–320. doi:10.1016/S0924-2244(00)00012-1

46. Burri L, Hoem N, Monakhova YB, Diehl BWK. Fingerprinting krill oil by ³¹P, ¹H and ¹³C NMR spectroscopies. J Am Oil Chem Soc. 2016;93:1037–1049. doi:10.1007/s11746-016-2836-3

47. Chen D-W, Mo X, Xiao J, Zhao Z, Wan P. Use of nuclear magnetic resonance spectroscopy to identify phospholipids in food: a brief review. J Food Compos Anal. 2025;140:107268. doi:10.1016/j.jfca.2025.107268

48. Belton PS, Lyster RLJ, Richards CP. The ³¹P nuclear magnetic resonance spectrum of cows' milk. J Dairy Res. 1985;52:47–54. doi:10.1017/S0022029900023888

49. Kuwata K, Era S, Hoshino M, Forge V, Goto Y, Batt CA. Solution structure and dynamics of bovine β-lactoglobulin A. Protein Sci. 1999;8:2541–2545. doi:10.1110/ps.8.11.2541

50. Uhrínová S, Smith MH, Jameson GB, Uhrín D, Sawyer L, Barlow PN. Structural changes accompanying pH-induced dissociation of the β-lactoglobulin dimer. Biochemistry. 2000;39:3565–3574. doi:10.1021/bi992629o

51. Sakurai K, Goto Y. Principal component analysis of the pH-dependent conformational transitions of bovine β-lactoglobulin monitored by heteronuclear NMR. Proc Natl Acad Sci USA. 2007;104:15346–15351. doi:10.1073/pnas.0702112104

52. Ragona L, Zetta L, Fogolari F, Molinari H, Pérez DM, Puyol P, De Kruif K, Löhr F, Rüterjans H. Bovine β-lactoglobulin: interaction studies with palmitic acid. Protein Sci. 2000;9:1347–1356. doi:10.1110/ps.9.7.1347

53. Kontopidis G, Holt C, Sawyer L. Invited review: β-lactoglobulin: binding properties, structure, and function. J Dairy Sci. 2004;87:785–796. doi:10.3168/jds.S0022-0302(04)73222-1

54. Alexandrescu AT, Evans PA, Pitkeathly M, Baum J, Dobson CM. Structure and dynamics of the acid-denatured molten globule state of α-lactalbumin: a two-dimensional NMR study. Biochemistry. 1993;32:1707–1718. doi:10.1021/bi00058a003

55. Schulman BA, Kim PS, Dobson CM, Redfield C. A residue-specific NMR view of the non-cooperative unfolding of a molten globule. Nat Struct Biol. 1997;4:630–634. doi:10.1038/nsb0897-630

56. Belloque J, Smith GM. Thermal denaturation of β-lactoglobulin. A ¹H NMR study. J Agric Food Chem. 1998;46:1805–1813. doi:10.1021/jf9709313

57. Belloque J, López-Fandiño R, Smith GM. A ¹H-NMR study on the effect of high pressures on β-lactoglobulin. J Agric Food Chem. 2000;48:3906–3912. doi:10.1021/jf000241q

58. Edwards PJB, Jameson GB, Palmano KP, Creamer LK. Heat-resistant structural features of bovine β-lactoglobulin A revealed by NMR H/D exchange observations. Int Dairy J. 2002;12:331–344. doi:10.1016/S0958-6946(02)00029-8

59. Lambelet P, Berrocal R, Ducret F. Low resolution NMR spectroscopy: a tool to study protein denaturation. I. Application to diamagnetic whey proteins. J Dairy Res. 1989;56:211–222. doi:10.1017/S0022029900026431

60. Lambelet P, Berrocal R, Renevey F. Low-field nuclear magnetic resonance relaxation study of thermal effects on milk proteins. J Dairy Res. 1992;59:517–526. doi:10.1017/S0022029900027187

61. Le Dean A, Mariette F, Marin M. ¹H nuclear magnetic resonance relaxometry study of water state in milk protein mixtures. J Agric Food Chem. 2004;52:5449–5455. doi:10.1021/jf030777m

62. Colsenet R, Mariette F, Cambert M. NMR relaxation and water self-diffusion studies in whey protein solutions and gels. J Agric Food Chem. 2005;53:6784–6790. doi:10.1021/jf050162k

63. Hinrichs R, Götz J, Weisser H. Water-holding capacity and structure of hydrocolloid-gels, WPC-gels and yogurts characterised by means of NMR. Food Chem. 2003;82:155–160. doi:10.1016/S0308-8146(02)00539-3

64. Mariette F. Investigations of food colloids by NMR and MRI. Curr Opin Colloid Interface Sci. 2009;14:203–211. doi:10.1016/j.cocis.2008.10.006

65. Colsenet R, Söderman O, Mariette F. Pulsed field gradient NMR study of poly(ethylene glycol) diffusion in whey protein solutions and gels. Macromolecules. 2006;39:1053–1059. doi:10.1021/ma0519922

66. Belloque J, de la Fuente MA, Ramos M. Qualitative and quantitative analysis of phosphorylated compounds in milk by means of ^{31}P-NMR. J Dairy Res. 2000;67:529–539. doi:10.1017/S0022029900004477

67. Belloque J, Ramos M. Determination of the casein content in bovine milk by ^{31}P-NMR. J Dairy Res. 2002;69:411–418. doi:10.1017/S0022029902005630

Summary

This white paper has examined how ^{1}H, ^{13}C and ^{31}P NMR can be used to characterize and quantify the phospholipids that whey protein ingredients inherit from the milk fat globule membrane, and has reviewed the broader NMR literature on whey proteins within which such measurements are interpreted.

The phospholipid fraction of whey is the residue of a membrane. Roughly a fifth of the phospholipid of cheese milk drains into sweet whey, enriched in phosphatidylethanolamine and sphingomyelin, and it is then concentrated by ultrafiltration into WPC, removed by microfiltration to make WPI, and collected as the retentate co-product WPPC, whose lipid is 10–46 % phospholipid. The only peer-reviewed ^{31}P NMR composition of a whey-derived phospholipid concentrate — a cheese-whey MFGM lecithin — is sphingomyelin-dominant (34 % SM, 31 % PC, 27 % PE), and no ^{31}P class profile of a commercial WPC80 or WPPC lot has been published. Industry typical values are therefore unsupported by NMR measurement, and the gap is one that a laboratory with a broadband probe can fill.

^{31}P NMR is the technique of record for class quantification because each phospholipid class gives one resonance whose integral is molar and independent of acyl composition, because no standard is required for identification, and because the lysophospholipids and dihydrosphingomyelin that chromatography merges are resolved. The reliability of the method rests on chemistry that is well characterized in the literature: chelation of divalent cations by EDTA, control of pH and temperature because the anionic classes (PS, PE, PA) move with both, inverse-gated decoupling to suppress a class-dependent NOE of up to 60 %, relaxation delays set by the standard rather than by the analytes, and confirmation of assignments by spiking or by ^{1}H–^{31}P correlation rather than by table look-up. Two preparation routes are recommended: the chloroform–methanol–Cs-EDTA reagent for extracts, and the sodium cholate micelle for direct, extraction-free analysis of phospholipid-rich powders such as WPPC, with the pH raised to 9.5 in a second tube to resolve PS.

Field strength is a bounded choice. Chemical-shift-anisotropy relaxation broadens micellar phospholipid lines as the square of the field, so that 16.4 T offers no advantage over 9.4 T; and at 24.3 MHz (a 60 MHz benchtop instrument) the 0.03–0.1 ppm separations within the PE/PS/DHSM/SM cluster collapse to 1–2 Hz and cannot be resolved by any processing, leaving only PC, PI and the cluster total. A 7.05 T instrument resolves the full profile at a modest sensitivity cost relative to 11.7 T.

^{1}H NMR contributes the choline $N^{+}(CH_3)_3$ stoichiometry that cross-checks the ^{31}P total, the sphingoid H-4/H-5 olefins and plasmalogen vinyl ether that confirm the sphingolipid and plasmalogen assignments, and, in the aqueous whey phase, the free choline, phosphocholine and glycerophosphocholine that record phospholipid hydrolysis, together with the lactose, lactate, citrate and orotate profile of the process. ^{13}C NMR contributes the carbon–phosphorus-coupled backbone and headgroup doublets, the sn-1/sn-2 carbonyl distribution and the sphingoid-base carbons that discriminate SM from DHSM, at a sensitivity that confines it to characterization rather than routine work; the ^{1}H-detected ^{1}H–^{13}C and ^{1}H–^{31}P correlations are the efficient route to the same information.

The NMR literature on whey proteins is mature. Solution structures of β-lactoglobulin and α-lactalbumin, the pH-dependent dimer dissociation and Tanford transition, ligand binding in the β-lactoglobulin calyx, staged thermal denaturation followed by ^{1}H NMR and hydrogen–deuterium exchange, low-field relaxometry of denaturation and gelation, pulsed-field-gradient diffusion in whey gels, ^{31}P NMR of the phosphorylated species of milk serum and ^{1}H NMR metabolite profiling of whey and WPC-80 together define the interpretive context of any whey phospholipid measurement, and the phosphoserine of residual

casein and caseinomacropeptide, the inorganic phosphate and the sugar phosphates of the serum will be present in the same ^{31}P spectra as the phospholipids.

A draft PNA protocol has been set out for the 300 MHz instrument, with a validation set (linearity, spiked recovery including LPC and LPE, intermediate precision, and cross-validation against HPLC–ELSD or HPLC–CAD) that must be completed before results are reported under the accredited scope. The eight failure modes that an auditor or opposing expert would raise — extraction bias, misassignment of the anionic classes, relaxation and NOE errors, the benchtop resolution limit, the glycolipid blind spot, lot-to-lot variability, chemometric over-reach and the molecular-weight assumption in mass-basis results — are each manageable, provided that they are managed explicitly and stated in the report.

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