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Technical White Paper

The Analysis of Sphingomyelin by ^{1}H, ^{13}C and ^{31}P Nuclear Magnetic Resonance Spectroscopy

Structural Assignment, Class Resolution, Quantitative Method Design and Field-Dependent Performance Limits

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Abstract

Sphingomyelin (SM) occupies an unusual position among the phospholipids of foods and biological membranes. It carries the same phosphocholine head group as phosphatidylcholine (PC), yet it is built on a sphingoid base rather than a glycerol backbone, and its single fatty acyl chain is attached through an amide rather than an ester linkage. That combination of a shared head group and a divergent backbone governs almost everything about how the molecule is measured by nuclear magnetic resonance spectroscopy, because the nucleus that offers the cleanest molar bookkeeping — phosphorus-31 — is precisely the nucleus least able to perceive the structural features that distinguish sphingomyelin from its glycerophospholipid neighbors.

This white paper examines the complementary roles of ^{1}H, ^{13}C and ^{31}P NMR in the qualitative identification and quantitative determination of sphingomyelin, and it treats the three nuclei as a single coordinated measurement system rather than as three independent techniques. The ^{31}P discussion covers the origin of the narrow, approximately 1.5 ppm dispersion within which all common phospholipid classes resonate, the chemical basis by which solvent composition, cation identity, chelator loading, temperature and pH are manipulated to prise apart overlapping signals, and the acquisition conditions — inverse-gated decoupling, relaxation delays scaled to the longest ^{31}P T_1 in the mixture, and internal standardization — that are required before an integral may be interpreted as a mole fraction. Particular attention is directed at the resolution of sphingomyelin from dihydrosphingomyelin, from phosphatidylserine and from the ethanolamine plasmalogens, since these are the separations that most often fail in real extracts and most often go unremarked in reported results.

The ^{1}H discussion identifies the resonances that are genuinely diagnostic for sphingomyelin — the olefinic pair arising from the C4–C5 *trans* double bond of the sphingosine base, the amide NH, and the absence of the glyceryl multiplets that every diacyl glycerophospholipid must display — and distinguishes them from the trimethylammonium singlet near 3.2 ppm, which is frequently but incorrectly treated as a sphingomyelin marker when it is in fact a summed response from every choline-bearing species present. The ^{13}C discussion addresses the sensitivity and relaxation penalties that restrict natural-abundance carbon work to concentrated isolates, and sets against those penalties the largest structural dispersion of the three nuclei, including the C3 resonance whose position separates sphingomyelin from dihydrosphingomyelin by approximately 2.7 ppm where the corresponding ^{31}P separation is a little over 0.2 ppm.

Field-dependent resolution is treated quantitatively. Neighbor separations expressed in hertz are tabulated from 60 MHz to 600 MHz proton frequency, from which it follows that benchtop ^{31}P instrumentation is adequate for the sphingomyelin–phosphatidylcholine determination that dominates dairy and infant-formula work, marginal for the sphingomyelin–dihydrosphingomyelin pair, and incapable of separating sphingomyelin from phosphatidylserine without chemical intervention. Sample preparation, extraction chemistry, quantitative validation, chemometric treatment of phospholipid spectral matrices, and the standing of NMR against high-performance liquid chromatography and mass spectrometry are each addressed. The paper closes with recommended acquisition protocols and an explicit catalogue of the errors most likely to be exploited by a technical reviewer or an opposing expert. Sixty references are cited, each verified against the CrossRef registry prior to drafting.

Table of Contents

1 Introduction

2 Molecular Architecture of Sphingomyelin and Its Analytical Consequences

2.1 The sphingoid base and the amide linkage

2.2 Molecular species heterogeneity

2.3 Dihydrosphingomyelin

3 Selection of Nucleus: What Each Observable Delivers

4 ^{31}P NMR of Sphingomyelin

4.1 The one-phosphorus-per-molecule advantage and its price

4.2 Solvent and reagent systems

4.3 Cation control, chelation and the reproducibility of shift

4.4 Temperature and pH as deliberate resolution variables

4.5 Acquisition conditions for quantitative ^{31}P NMR

4.6 Resolving sphingomyelin from dihydrosphingomyelin and from phosphatidylserine

4.7 Two-dimensional ^{1}H–^{31}P correlation and automated assignment

4.8 Field strength, benchtop instrumentation and the arithmetic of resolution

5 ^{1}H NMR of Sphingomyelin

5.1 The trimethylammonium region and why it is not a sphingomyelin marker

5.2 The sphingosine olefinic protons — the genuine ^{1}H signature

5.3 The amide NH, the C3 hydroxyl and hydrogen bonding

5.4 Backbone, head group and acyl resonances

5.5 Discrimination of sphingomyelin from glycerophospholipids by absence

5.6 Quantitative ^{1}H NMR practice and its limits for sphingomyelin

6 ^{13}C NMR of Sphingomyelin

6.1 Sensitivity and relaxation constraints

6.2 Diagnostic carbon resonances

6.3 C3 as the definitive sphingomyelin–dihydrosphingomyelin discriminator

6.4 Acyl chain characterization and two-dimensional carbon methods

7 The Integrated Multinuclear Workflow

8 Sample Preparation and Extraction Chemistry

9 Quantitative Performance and Method Validation

10 Solid-State and Bilayer NMR: Scope and Relevance

11 Orthogonal and Complementary Techniques

12 Chemometric Treatment of Phospholipid NMR Datasets

13 Recommended Protocols

14 Limitations, Pitfalls and Common Errors

15 References

16 Summary

Tables

Table 1. Representative ^{31}P chemical shifts of common phospholipid classes in the chloroform–methanol–aqueous EDTA reagent system, referenced to 85% H_3PO_4 at 0 ppm

Table 2. Comparison of sample media for ^{31}P phospholipid analysis relevant to sphingomyelin determination

Table 3. Defensible starting acquisition parameters for quantitative ^{31}P determination of sphingomyelin at approximately 121 MHz ^{31}P observation frequency

Table 4. Field dependence of the sphingomyelin neighbor separations, expressed in hertz

Table 5. Representative ^{1}H assignments for sphingomyelin in $CDCl_3$

Table 6. Representative ^{13}C assignments for sphingomyelin and dihydrosphingomyelin in $CDCl_3$

Table 7. Selected sphingomyelin and phospholipid findings determined by ^{31}P NMR across food matrices, illustrating typical concentration ranges and the analytical questions addressed

Table 8. Comparison of analytical approaches to sphingomyelin determination

Table 9. Principal failure modes in sphingomyelin determination by NMR and their remedies

1 Introduction

The analytical importance of sphingomyelin has grown considerably over the last two decades, and it has grown for reasons that are largely commercial rather than academic. Sphingomyelin is the dominant phospholipid class of human milk, where it has been reported to account for approximately 35% of total phospholipid, whereas phosphatidylcholine and phosphatidylethanolamine dominate the phospholipid fraction of conventional infant formulas.[36] The gap between those two profiles has become a formulation target, and the milk fat globule membrane fractions and buttermilk-derived ingredients used to close it are traded on the basis of declared sphingomyelin content. Any ingredient specification written around a sphingomyelin number implies an analytical method capable of delivering that number defensibly, and the choice of method is not a matter of indifference.

Sphingomyelin is also a structurally awkward analyte. It is not a single compound but a family of molecular species sharing a phosphocholine head group, a sphingoid base that is usually but not invariably d18:1 sphingosine, and an amide-linked fatty acyl chain that ranges across roughly ten carbon-number and unsaturation combinations in a typical dairy sample. The mass of an individual sphingomyelin species varies by approximately 15% across that range, which means that any method reporting a mass concentration must either resolve the molecular species or accept a systematic bias determined by whichever average molecular weight has been assumed. Methods that count molecules rather than mass are therefore structurally advantaged, and it is here that ^{31}P NMR spectroscopy has its principal claim.

Three nuclei are available for the interrogation of this molecule, and each answers a different question. Phosphorus-31 counts phospholipid molecules with a stoichiometry of one nucleus per molecule and does so without a response factor, but it perceives only the immediate environment of the phosphodiester and therefore distinguishes classes rather than species.[5,19] Proton NMR perceives the entire molecule at high sensitivity but must contend with an acyl-chain envelope that buries most of the structurally informative signals beneath a small number of enormous methylene resonances.[42,43] Carbon-13 offers the widest chemical shift dispersion and the cleanest structural discrimination but pays for it in sensitivity and in relaxation behavior that makes quantitative work slow and, if performed carelessly, wrong.[25]

The literature reflects this division of labor imperfectly. The great majority of published sphingomyelin determinations in foods rest on ^{31}P NMR alone, with structural assignment inherited from earlier work rather than confirmed in the sample at hand, and with the resolution of sphingomyelin from its nearest neighbors asserted rather than demonstrated. That practice is defensible in a well-characterized matrix analyzed repeatedly under fixed conditions. It is not defensible when a novel matrix is encountered, when a dispute arises over a declared value, or when a method is submitted for accreditation. The purpose of this paper is to set out what each nucleus can and cannot establish, to make the resolution limits quantitative rather than rhetorical, and to describe the multinuclear workflow by which a sphingomyelin determination can be made to withstand scrutiny.

The treatment is organized by nucleus, with ^{31}P considered first because it carries the quantitative burden, followed by ^{1}H and ^{13}C in their supporting and confirmatory roles. Sections addressing extraction chemistry, validation, chemometrics and orthogonal methodology follow, and the paper concludes with recommended protocols and a candid enumeration of the ways in which sphingomyelin determinations by NMR are most commonly compromised.

2 Molecular Architecture of Sphingomyelin and Its Analytical Consequences

2.1 The sphingoid base and the amide linkage

Sphingomyelin is formally *N*-acyl-sphingosine-1-phosphocholine. The backbone is a long-chain amino alcohol, most commonly (2*S*,3*R*,4*E*)-2-amino-4-octadecene-1,3-diol, universally abbreviated d18:1 and known as sphingosine. Position C1 bears the primary hydroxyl that is esterified to the phosphocholine head group. Position C2 bears the amino nitrogen to which the fatty acyl chain is joined through an amide bond. Position C3 bears a secondary hydroxyl that remains free in the overwhelming majority of naturally occurring species. Between C4 and C5 lies a *trans* double bond, introduced biosynthetically by dihydroceramide desaturase, which sits unusually close to the polar interface rather than in the hydrophobic core where the *cis* unsaturation of glycerophospholipid acyl chains is found.[1]

Four structural features follow from this architecture, and all four have direct analytical consequences. First, sphingomyelin bears one hydrogen-bond donor at the amide NH and a second at the C3 hydroxyl, alongside the acceptors of the phosphodiester; phosphatidylcholine, by contrast, offers no donors at all. This asymmetry drives the intra- and intermolecular hydrogen bonding that has been characterized in detail by proton and phosphorus NMR, and it is responsible for the concentration and temperature dependence of the sphingomyelin NH and OH resonances.[50,51] Second, the molecule carries a single acyl chain rather than two, so the ratio of acyl-derived protons to head-group protons differs from that of a diacyl glycerophospholipid by approximately a factor of two, which is exploitable in ^{1}H bookkeeping. Third, the C4–C5 *trans* olefin generates a proton and carbon signature that no glycerophospholipid possesses at that position and that dihydrosphingomyelin lacks entirely. Fourth, the substitution of an amide for the glycerol C2 ester removes the glyceryl CH and CH_2 multiplets that are otherwise universal among diacyl phospholipids, converting their absence into positive evidence.

The head group itself is identical to that of phosphatidylcholine — a phosphodiester bridging the backbone C1 oxygen to the oxygen of choline. Because ^{31}P chemical shift is dominated by the electronic environment within two or three bonds of the phosphorus, the sphingomyelin and phosphatidylcholine ^{31}P resonances differ only through the more remote influence of the backbone, transmitted principally by differences in hydrogen bonding and hydration at the interface. The resulting shift difference is real and reproducible but small, and this single fact explains the whole of the ^{31}P methodological literature described in Section 4.

2.2 Molecular species heterogeneity

Natural sphingomyelin is a mixture. The sphingoid base is usually d18:1, but d16:0, d16:1 and d18:0 bases occur at significant abundance in ruminant milk, and sphingadiene bases have been characterized in brain tissue. The amide-linked acyl chain in bovine milk sphingomyelin is dominated by long saturated chains, with C16:0, C22:0, C23:0 and C24:0 prominent, alongside C24:1. The mass consequences are not negligible. Taking d18:1 sphingosine as the base, the molecular weight of the resulting sphingomyelin ranges from approximately 703 $g \cdot mol^{-1}$ for the C16:0 species to approximately 815 $g \cdot mol^{-1}$ for the C24:0 species, a spread of roughly 15% about the mean.

For a molar method, this heterogeneity is inconsequential: every species contributes exactly one phosphorus nucleus and one trimethylammonium group, and the integral is a mole count irrespective of chain length. For a

gravimetric report, it matters a great deal, because the conversion of moles to milligrams requires an assumed molecular weight. Published sphingomyelin conformational studies have adopted an average molecular weight of 780 $g \cdot mol^{-1}$ for bovine brain material,[51] and comparable assumptions underlie the mass-based sphingomyelin figures reported in food analyses. When a ^{31}P NMR result expressed in $mg \cdot 100\ g^{-1}$ is compared against a mass spectrometric result that sums individually weighted species, part of any discrepancy observed is an artifact of that assumption rather than a disagreement about the amount of substance present. This point is developed further in Section 11.

It should be noted that the sphingoid base distribution is itself analytically useful. Dihydrosphingomyelin species in bovine milk have been reported to carry a d16:0 long-chain base in approximately 56–58% of cases and a d18:0 base in approximately 34–37% of cases, a distribution established by liquid chromatography with parallel mass spectrometric detection rather than by NMR.[33] Neither ^{31}P nor routine ^{1}H NMR resolves base composition, and claims about sphingoid base distribution should not be advanced on NMR evidence alone.

2.3 Dihydrosphingomyelin

Dihydrosphingomyelin (DHSM) differs from sphingomyelin in one respect only: the C4–C5 double bond is absent, the base being sphinganine rather than sphingosine. That single difference has consequences out of all proportion to its size. The saturated base packs more tightly and confers markedly greater thermal and oxidative stability, and DHSM is the dominant phospholipid of the human lens for that reason.[15] More importantly for the food analyst, DHSM is not a trace curiosity in dairy: it has been reported to constitute approximately 20% of bovine milk sphingolipids, a proportion substantially higher than that found in bovine brain.[33]

Because DHSM carries the same phosphocholine head group, it appears in the ^{31}P spectrum as a distinct but closely adjacent resonance, and because it lacks the C4–C5 olefin it is invisible in the very ^{1}H and ^{13}C signals that are otherwise diagnostic for sphingomyelin. A method that resolves the two reports them separately; a method that does not will either sum them under a single sphingomyelin label or, if the DHSM resonance is misassigned, attribute it to phosphatidic acid or to an ethanolamine plasmalogen. A twenty percent error in a declared sphingomyelin value is a serious matter in an ingredient specification, and the DHSM question should be treated as a routine method-validation requirement in any dairy application rather than as a refinement.

3 Selection of Nucleus: What Each Observable Delivers

Before the individual nuclei are examined it is useful to state plainly what each can establish, because a great deal of confusion in the applied literature arises from asking one nucleus to answer a question that only another can address.

Phosphorus-31 is a spin-½ nucleus of 100% natural abundance whose resonance frequency is approximately 40.5% that of the proton. Its receptivity relative to carbon-13 is high, its chemical shift range across phosphorus chemistry generally is enormous, and — decisively — every phospholipid molecule contains exactly one phosphorus atom. An integrated ^{31}P spectrum acquired under quantitative conditions is therefore a direct mole census of phospholipid classes requiring no response factor, no calibration curve and no assumption about chain length.[2,4,5] The limitation is equally decisive: within the phospholipid family the phosphorus is shielded almost identically, and the entire class dispersion is compressed into approximately 1.5 ppm. Phosphorus-31 identifies classes, and it does so only when the experimental conditions have been engineered to spread that 1.5 ppm across enough hertz to resolve them.

Proton NMR is between one and two orders of magnitude more sensitive and reports on every hydrogen-bearing position in the molecule. It sees the sphingosine olefin, the amide NH, the backbone methines and the head group, and it distinguishes sphingolipid from glycerophospholipid by the presence or absence of the glyceryl multiplets. Its limitation is spectral crowding. A lipid extract presents an acyl envelope in which bulk methylene and terminal methyl resonances dominate the integral, and the structurally informative sphingomyelin signals appear as minor features on the shoulders of much larger ones.[42,43] Proton NMR is an excellent identification and confirmation tool and a poor primary quantification tool for sphingomyelin in an unfractionated extract.

Carbon-13 offers the widest structural dispersion of the three and resolves features that neither of the others can — most notably the C3 position, whose shift responds directly to the presence or absence of the C4–C5 olefin. Its costs are a natural abundance of approximately 1.1%, long longitudinal relaxation times for protonated and quaternary carbons alike, and a nuclear Overhauser enhancement under broadband proton decoupling that varies between carbon types and therefore corrupts integration unless suppressed. Carbon-13 is a structural confirmation tool, used on isolates and concentrates rather than on dilute extracts, and it is used quantitatively only when the acquisition has been configured specifically for that purpose.[25]

The correct posture is therefore hierarchical rather than competitive. Quantification is carried by ^{31}P. Confirmation that the resonance assigned to sphingomyelin is in fact sphingomyelin is carried by ^{1}H, and where the sample permits, by ^{13}C. Two-dimensional ^{1}H–^{31}P correlation experiments bind the two together by demonstrating directly that a given phosphorus resonance belongs to a head group attached to a sphingoid backbone.[10,23,27]

4 ^{31}P NMR of Sphingomyelin

4.1 The one-phosphorus-per-molecule advantage and its price

The foundational observation of phospholipid ^{31}P NMR is that the phosphodiester of an intact phospholipid gives a single narrow resonance whose position is characteristic of the head group class, and that under appropriate conditions the resonances of the common classes are resolved sufficiently for integration.[2] The consequence is a compositional analysis of unusual metrological cleanliness. There is no detector response to calibrate, no derivatization step, no chromatographic recovery to validate, and no dependence on acyl chain composition. Where an internal standard of known concentration is added, the analysis becomes absolute rather than relative.

The price is resolution. All diacyl and sphingoid phospholipid classes place their phosphorus in a phosphodiester environment differing only in the identity of the two esterified alcohols, and the chemical shift consequences are correspondingly small. Table 1 lists representative positions for the common classes in the chloroform–methanol–aqueous EDTA system that has dominated the field since its introduction. The entire set spans approximately 1.15 ppm, and within that span sphingomyelin sits in the most congested region of all.

Two further points about Table 1 require emphasis. First, these values are not physical constants. They are conditional on solvent proportions, water content, chelator identity and loading, counter-cation, temperature, pH and total lipid concentration, and they migrate when any of those is altered.[5,7,8,13,14,16] Chemical shifts should therefore be treated as a relative pattern to be anchored by an internal reference and confirmed by spiking or by two-dimensional correlation, never as absolute identifiers transferred from a published table. Second, the

values are referenced here to 85% orthophosphoric acid at 0 ppm; where triphenyl phosphate is used as an internal standard it resonates far upfield of the phospholipid envelope, around −18 ppm, and is therefore well clear of every analyte signal.

Table 1. Representative ^{31}P chemical shifts of common phospholipid classes in the chloroform–methanol–aqueous EDTA reagent system, referenced to 85% H_3PO_4 at 0 ppm. Positions are conditional on solvent proportions, water content, chelator and counter-cation identity, temperature, pH and lipid concentration, and must be anchored internally rather than transferred between laboratories.[5,7,8,14]

Phospholipid class	δ(^{31}P) / ppm	Separation from SM / ppm	Analytical note
Phosphatidic acid (PA)	+0.30	0.39	Strongly cation-sensitive; downfield edge of envelope
Dihydrosphingomyelin (DHSM)	+0.13	0.22	Principal SM confusable; ~20% of bovine milk sphingolipid[33]
Ethanolamine plasmalogen (EPlas)	+0.07	0.16	Close to DHSM; misassignment risk in dairy and brain
Phosphatidylethanolamine (PE)	+0.03	0.12	Low cation interaction potential[8]
Phosphatidylserine (PS)	−0.05	0.04	Tightest overlap with SM; highly Ca^{2+}-sensitive[8]
Sphingomyelin (SM)	−0.09	—	Target analyte; small temperature coefficient[14]
Lysophosphatidylcholine (LPC)	−0.28	0.19	Hydrolysis indicator; monitors extract stability
Phosphatidylinositol (PI)	−0.30	0.21	Overlaps LPC; intermediate cation interaction[8]
Alkylacylglycerophospho choline	−0.78	0.69	Within the PC cluster
Choline plasmalogen (CPlas)	−0.80	0.71	Within the PC cluster
Phosphatidylcholine (PC)	−0.84	0.75	Usually the dominant signal; SM–PC is the key separation

4.2 Solvent and reagent systems

Three families of sample medium are in general use, and the choice among them is the single most consequential decision in a ^{31}P phospholipid method.

The **chloroform–methanol–aqueous chelator** system originates in the work of Meneses and Glonek, in which crude Folch extract is dissolved in chloroform and combined with an aqueous methanolic reagent containing ethylenediaminetetraacetic acid in deuterium oxide titrated to approximately pH 6 with cesium hydroxide.[5] The function of the chelator is to sequester paramagnetic and multivalent cations that would otherwise broaden and shift the phosphate resonances, and the choice of cesium as counter-ion provides a large, weakly coordinating cation that minimizes ion pairing at the phosphodiester. Under these conditions at 11.75 T, linewidths at half height of approximately 1.7–2.2 Hz were reported for the common classes, with the full set falling in the range 1.8–3.2 Hz, sufficient to resolve the generic classes together with their lyso and plasmalogen derivatives.[5] Longitudinal relaxation times of the order of 3.3 s and nuclear Overhauser enhancements of the order of 50–60% were reported in the same work, and both figures bear directly on the

acquisition conditions discussed in Section 4.5. Systematic variation of the chloroform–methanol–water proportions was subsequently shown to move the resonances relative to one another, so that solvent composition becomes a deliberate resolution variable rather than an incidental choice.[7]

The **detergent–micellar** systems dissolve the lipid in an aqueous micellar medium, most commonly sodium cholate with EDTA, so that the phospholipids are presented as mixed micelles in a single aqueous phase.[3] The approach was extended to tissue analysis using non-ionic detergents,[6] and it has been applied specifically to dairy materials, where a sodium cholate preparation was used to quantify phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin and dihydrosphingomyelin, validated against a two-dimensional thin-layer chromatographic reference and completing an analysis in approximately 36 minutes.[30] A significant practical advantage reported for that system is that materials of high phospholipid content — buttermilk protein concentrate and beta serum among them — can be analyzed without a prior lipid extraction, which removes an entire class of recovery error from the method. The cholate system also has well-characterized temperature and pH behavior, discussed below.

Monophasic organic systems were developed to address the instability and phase-separation tendency of the chloroform–methanol–water reagent. A triethylamine–dimethylformamide–guanidinium chloride medium was shown to give a ^{31}P chemical shift dispersion approximately four times that of the standard chloroform–methanol–water system while remaining stable as a single phase, with the improved dispersion further exploitable through two-dimensional ^{1}H-detected heteronuclear correlation.[10,11] A monophasic mixture has likewise been applied specifically to the characterization of milk fat phospholipids.[12] Where sphingomyelin must be resolved from phosphatidylserine or from an ethanolamine plasmalogen, a dispersion-enhancing medium is the most direct remedy available and should be considered before instrument time is spent at higher field.

Table 2 summarizes the practical trade-offs. No system dominates on all criteria, and the appropriate choice depends on whether the analytical priority is throughput, dispersion, avoidance of extraction, or transferability to an existing validated procedure.

Table 2. Comparison of sample media for ^{31}P phospholipid analysis relevant to sphingomyelin determination.

Medium	Principal advantage	Principal limitation	Reference
$CHCl_3$–CH_3OH–D_2O with Cs-EDTA	Established reference system; narrow lines (1.8–3.2 Hz at 11.75 T); wide class coverage	Two-component reagent prone to phase instability; chlorinated solvent	[5,7]
Aqueous sodium cholate micellar	Permits direct analysis of high-phospholipid dairy without extraction; ~36 min per sample; resolves SM and DHSM	Detergent must be controlled; pH and temperature regulation required	[3,30]
Non-ionic detergent micellar	Applied successfully to nervous tissue; aqueous handling	Less widely validated for food matrices	[6]
Et_3N–DMF–guanidinium chloride (monophasic)	^{31}P dispersion approximately four times that of $CHCl_3$–CH_3OH–H_2O; stable single phase	Less common; requires local method establishment	[10,11]
Monophasic organic	Applied specifically to milk	Matrix-specific development	[12]

Medium	Principal advantage	Principal limitation	Reference
(milk fat)	fat phospholipids	required	
Heated ethanol–water extraction with surfactant dispersion	Chloroform-free at both extraction and measurement; absolute quantification	Developed for infant formula; transfer to other matrices requires validation	[38]

4.3 Cation control, chelation and the reproducibility of shift

The sensitivity of phospholipid ^{31}P chemical shifts to cations is not a minor perturbation and is frequently the hidden cause of failed inter-laboratory agreement. Systematic titration of a dispersed phospholipid mixture established that the classes fall into distinct groups by their interaction potential for divalent cations: phosphatidylserine and phosphatidic acid interact strongly with calcium, cardiolipin most strongly with magnesium, phosphatidylinositol and phosphatidylglycerol at intermediate strength, while ethanolamine plasmalogen, phosphatidylethanolamine, sphingomyelin and phosphatidylcholine show essentially no interaction potential for either.[8] Monovalent cations produce their own effects, with sodium deshielding phosphatidic acid, cardiolipin, phosphatidylinositol and phosphatidylglycerol by more than 0.1 ppm relative to cesium.

For sphingomyelin analysis this finding is doubly useful. It confirms that the sphingomyelin resonance position is comparatively robust against variations in residual cation loading, which is reassuring for a target analyte. It simultaneously warns that the position of phosphatidylserine — sphingomyelin's nearest neighbor at approximately 0.04 ppm — is highly cation-sensitive, so that the apparent separation of the sphingomyelin and phosphatidylserine signals will vary from sample to sample according to residual calcium, and may close entirely in a sample carrying a high mineral load. In dairy matrices, which are rich in calcium, this is a real rather than theoretical hazard. Adequate chelator loading is not merely good practice but a prerequisite for the phosphatidylserine assignment, and the chelator should be present in demonstrable excess over the total multivalent cation content of the extract rather than at a fixed nominal concentration.

4.4 Temperature and pH as deliberate resolution variables

Because the chemical shift of each class responds to temperature through its own hydrogen-bonding environment, temperature can be used to move overlapping resonances apart, and the temperature coefficient itself becomes an assignment criterion. Coefficients were determined for a range of glycero- and sphingophospholipids in chloroform–methanol–water, and it was found that differences in hydrogen bonding between the phosphate and the head group account for the observed variation.[14] Among the parent phospholipids, the smallest coefficients — those closest to zero — were observed for sphingomyelin, phosphatidylcholine, phosphatidylglycerol, dihydrosphingomyelin and cardiolipin.[14] The corresponding behavior in cholate micelles has also been characterized, where the chemical shifts of phosphatidylcholine, sphingomyelin, dihydrosphingomyelin and phosphatidylglycerol were found to be invariant with pH and temperature over the ranges examined.[13]

The analytical exploitation of this is straightforward and underused. Acquiring the same sample at two or three temperatures and tracking the trajectory of each resonance provides an assignment criterion that is independent of any published shift table: a signal that fails to move as the temperature is raised is a candidate for sphingomyelin, phosphatidylcholine or dihydrosphingomyelin, whereas a signal that moves substantially is

not. Because sphingomyelin and phosphatidylserine differ markedly in their temperature response, a temperature series will frequently separate a pair that a single-temperature spectrum reports as one line. The same logic applies to pH in micellar media, where the classes bearing ionizable head groups migrate and the choline-bearing classes do not.

The comprehensive expression of this approach is the multiparametric optimization described by Lutz and Cozzone, in which chemical shift and signal separation were treated as functions of several simultaneously varied experimental parameters in the first instance, and linewidth and spectral resolution in the second.[16,17] The practical conclusion of that work — that the resolution achievable for a given pair of phospholipid resonances is largely under the analyst's control through solvent, temperature, pH and chelation, and only secondarily a function of magnetic field — is the single most important methodological insight available to anyone establishing a phospholipid method on modest instrumentation.

4.5 Acquisition conditions for quantitative ^{31}P NMR

Three acquisition issues determine whether a ^{31}P integral may be interpreted as a mole fraction, and all three are routinely mishandled.

The first is **longitudinal relaxation**. Phospholipid ^{31}P T_1 values of the order of 3.3 s have been reported in the chloroform–methanol–chelator medium,[5] and values vary between classes and with viscosity, temperature and field. A relaxation delay of five times the longest T_1 present is required for integrals accurate to within approximately 1%, which for a T_1 of 3.5 s implies an inter-pulse interval of approximately 17.5 s when a 90° excitation pulse is used. Analysts frequently shorten this delay to gain signal-to-noise within an acceptable experiment time, and the resulting saturation is not uniform across classes. Where time is genuinely constrained, the correct remedy is a reduced flip angle with a correspondingly shortened delay, calculated from the Ernst relationship, rather than an arbitrary truncation of the recycle time. In every case the T_1 of each resolved resonance should be measured once for a representative matrix by inversion recovery and the delay set accordingly, and that measurement should be part of the method validation record.

The second is the **nuclear Overhauser effect**. Broadband proton decoupling is required to collapse the ^{31}P multiplet structure arising from coupling to the head-group and backbone protons, and without it the sphingomyelin resonance is broadened to the point of uselessness. But continuous decoupling also generates a heteronuclear Overhauser enhancement of the order of 50–60% for phospholipid phosphorus,[5] and that enhancement is not equal across classes because it depends on the local proton density and on molecular motion. Integrals acquired under continuous decoupling are therefore systematically distorted. The remedy is **inverse-gated decoupling**, in which the decoupler is active only during acquisition and is gated off during the relaxation delay, delivering a decoupled spectrum with the NOE largely suppressed. This is a standard experiment and its omission is the most common single defect in published phospholipid quantifications.

The third is **internal standardization**. Relative mole percentages require no standard, but absolute concentrations do. Triphenyl phosphate is widely used because it resonates near −18 ppm, far from the phospholipid envelope, and is chemically inert toward the sample; it has been used as the internal reference in two-dimensional phospholipid profiling of food matrices.[27] Alternative standards including trimethyl phosphate and methylenediphosphonic acid are also in use. Whichever is chosen must be shown not to overlap any analyte signal in the specific matrix, must be stable in the reagent over the duration of the experiment, and must be subject to the same relaxation considerations as the analytes — a standard with a long T_1 acquired under a delay optimized for the analytes will be under-integrated and will bias every reported concentration in

the same direction. An external standardization approach based on the PULCON principle avoids the addition of any material to the sample and is worth consideration where sample recovery matters.

Table 3 sets out a defensible starting parameter set for a sphingomyelin determination on a mid-field instrument, together with the reasoning behind each choice.

Table 3. Defensible starting acquisition parameters for quantitative ^{31}P determination of sphingomyelin at approximately 121 MHz ^{31}P observation frequency. All values require confirmation against the specific matrix during validation.

Parameter	Starting value	Basis
Excitation pulse	90°	Maximizes signal per transient; simplifies relaxation accounting
Relaxation delay	≥ 5 × longest measured T_1 (typically ≥ 17 s)	T_1 of order 3.3 s reported for extracted phospholipids;[5] $5 \cdot T_1$ gives ~99% recovery
Proton decoupling	Inverse-gated (on during acquisition only)	Suppresses the 50–60% NOE reported for phospholipid ^{31}P,[5] which is class-dependent and corrupts integrals
Acquisition time	≥ 2 s	Preserves resolution of the 0.04 ppm SM–PS separation; avoids truncation broadening
Spectral width	~30 ppm	Encompasses phospholipid envelope and an upfield internal standard
Transients	Sufficient for S/N ≥ 150:1 on the SM signal	Integration precision degrades rapidly below this for minor classes
Temperature	Regulated and recorded (e.g. 300 K)	Shifts are temperature-dependent; the coefficient is also an assignment tool[13,14]
Internal standard	Resonating clear of the envelope; T_1 measured	Required for absolute concentration; must not be under-integrated relative to analytes
Apodization	Modest exponential (≤ 1 Hz)	Excessive line broadening merges the SM–PS and SM–DHSM pairs
Baseline correction	Applied over signal-free regions only	Prevents distortion of minor-class integrals

4.6 Resolving sphingomyelin from dihydrosphingomyelin and from phosphatidylserine

These are the two separations on which a sphingomyelin determination in a dairy or lens-derived matrix most often fails, and they fail for opposite reasons.

The sphingomyelin–dihydrosphingomyelin pair is separated by approximately 0.22 ppm in the chloroform–methanol–chelator system, with dihydrosphingomyelin downfield. This is a comfortable separation at high field and a demanding one at low field. Both species have small temperature coefficients and both are pH-insensitive in cholate,[13,14] so neither temperature nor pH manipulation will assist in prising them apart; the separation must be won by field, by linewidth, or by a dispersion-enhancing solvent. The pair was successfully resolved and separately quantified in dairy materials in the cholate system,[30] and the combination of liquid chromatography with parallel mass spectrometric and ^{31}P NMR detection was used specifically to characterize sphingomyelin and dihydrosphingomyelin in bovine brain and chicken egg yolk and subsequently in bovine milk.[32,33] That body of work also advanced the argument that ^{31}P NMR may be superior to mass spectrometric methodology for the quantification of sphingolipid classes in foods, precisely because it is free of the ionization-efficiency differences that complicate mass spectrometric response.

The sphingomyelin–phosphatidylserine pair is separated by approximately 0.04 ppm and is therefore the tighter problem by a factor of five. It is, however, the more tractable of the two, because the two classes differ markedly in their cation affinity and in their temperature and pH behavior.[8,13,14] Adequate chelation, a temperature series, and where a micellar medium is in use a pH adjustment, will normally establish whether the signal integrated as sphingomyelin contains a phosphatidylserine contribution. In matrices where phosphatidylserine is a minor component this may be a small correction; in others it is not, and the assumption that it is small should be tested rather than made.

A third confusable pair deserves mention. Dihydrosphingomyelin at approximately +0.13 ppm lies close to phosphatidic acid at approximately +0.30 ppm and to ethanolamine plasmalogen at approximately +0.07 ppm. Where dihydrosphingomyelin is not expected and is not looked for, its signal is available to be misassigned to either neighbor, and in a dairy matrix in which dihydrosphingomyelin may represent a fifth of the sphingolipid the misassignment will be substantial. The presence of dihydrosphingomyelin should be established affirmatively, ideally by ^{13}C or by spiking with authentic material, rather than inferred from the absence of an unexplained peak.

4.7 Two-dimensional ^{1}H–^{31}P correlation and automated assignment

The most robust answer to the assignment problem is to stop relying on ^{31}P chemical shift for identification altogether and to use it only for integration, transferring the identification burden to the proton dimension where the dispersion is larger and the shifts far less sensitive to sample conditions. This is the explicit logic of the automated approach of Balsgart and co-workers, in which one-dimensional ^{31}P and two-dimensional ^{1}H–^{31}P spectra are combined so that the comparatively unambiguous proton chemical shifts carry the assignment while the phosphorus intensities carry the quantification; approximately twenty different lipids were assigned and quantified automatically by that method using two-dimensional ^{1}H–^{31}P total correlation spectroscopy.[23]

The same principle underlies the two-dimensional ^{31}P,^{1}H profiling applied to cheese and fish following liquid–liquid enrichment,[27] and the earlier demonstration that the enhanced dispersion of a monophasic organic medium could be further exploited through gradient-enhanced ^{1}H-detected heteronuclear multiple quantum coherence.[10] For sphingomyelin specifically, the value of the two-dimensional experiment is that it establishes a correlation between the phosphorus resonance and protons that are unambiguously sphingoid — the backbone H1 and H2 positions, and through longer-range correlation the H3 and the olefinic protons — rather than merely placing a peak at a tabulated position. That is the difference between an assignment and an assumption, and in any application where the result may be challenged it is the difference that matters.

Multinuclear fingerprinting combining ^{31}P, ^{1}H and ^{13}C has been applied to krill oil, and an international proficiency exercise has tested the transferability of the ^{31}P approach between laboratories,[25,26] and the general adoption of NMR for phospholipid analysis in food has been reviewed comprehensively.[19,20,21,22] Where a laboratory analyzes the same matrix repeatedly, the sensible practice is to establish the assignment once by two-dimensional correlation and by spiking, document it, and thereafter run one-dimensional ^{31}P for throughput, revisiting the two-dimensional confirmation whenever the matrix or the reagent system changes.

4.8 Field strength, benchtop instrumentation and the arithmetic of resolution

Because the phospholipid chemical shift dispersion is fixed in parts per million but the linewidth achievable is broadly fixed in hertz, resolution scales directly with field, and the question of what can be measured on a benchtop instrument reduces to arithmetic. The ^{31}P Larmor frequency is 40.48% of the ^{1}H frequency, so a 60

MHz benchtop system observes phosphorus at approximately 24.3 MHz and a 300 MHz superconducting system at approximately 121.4 MHz. Table 4 converts the sphingomyelin neighbor separations of Table 1 into hertz across the range of instrumentation likely to be encountered.

The conclusions are unambiguous. The sphingomyelin–phosphatidylcholine separation of 0.75 ppm corresponds to approximately 18 Hz at 60 MHz and approximately 91 Hz at 300 MHz; given the 2–3 Hz linewidths attainable in a well-shimmed chelated medium, this determination is comfortably within benchtop reach, and since it is the determination that dominates dairy, infant formula and lecithin work, the practical scope for benchtop ^{31}P is considerable. The sphingomyelin–dihydrosphingomyelin separation of 0.22 ppm corresponds to approximately 5 Hz at 60 MHz — marginal, achievable only with excellent shimming and narrow lines, and not a separation on which a specification should be based at that field. The sphingomyelin–phosphatidylserine separation of 0.04 ppm corresponds to approximately 1 Hz at 60 MHz and is not achievable; at 300 MHz it is approximately 5 Hz and is achievable but demanding.

The analytical evaluation of low-field ^{31}P NMR for lipid analysis undertaken by Gouilleux and co-workers addressed exactly this question and established the practical envelope for benchtop phospholipid work.[24] The correct reading of that literature, taken together with the multiparametric optimization studies,[16,17] is that benchtop ^{31}P is a legitimate quantitative tool for phospholipid class analysis provided the target separations are identified in advance and the matrix is one in which the confounding classes are either absent or independently controlled. It is not a general-purpose phospholipid profiler. A laboratory operating both benchtop and mid-field instrumentation is well advised to establish the assignment and the full class profile at the higher field and to transfer only the specific, demonstrated determination to the benchtop for routine throughput.

Table 4. Field dependence of the sphingomyelin neighbor separations, expressed in hertz. The ^{31}P Larmor frequency is 40.48% of the ^{1}H frequency. Values assume the chemical shift differences of Table 1. Achievable linewidths in a well-shimmed chelated medium are of order 2–3 Hz.[5]

^{1}H / MHz	^{31}P / MHz	SM–PC (0.75 ppm) / Hz	SM–DHSM (0.22 ppm) / Hz	SM–LPC (0.19 ppm) / Hz	SM–PS (0.04 ppm) / Hz
60	24.3	18.2	5.3	4.6	1.0
80	32.4	24.3	7.1	6.2	1.3
100	40.5	30.4	8.9	7.7	1.6
300	121.4	91.1	26.7	23.1	4.9
400	161.9	121.4	35.6	30.8	6.5
500	202.4	151.8	44.5	38.5	8.1
600	242.9	182.2	53.4	46.1	9.7

5 ^{1}H NMR of Sphingomyelin

5.1 The trimethylammonium region and why it is not a sphingomyelin marker

The most conspicuous head-group signal in the proton spectrum of any choline-bearing phospholipid is the sharp nine-proton singlet of the trimethylammonium group, appearing near 3.2–3.3 ppm in deuterochloroform and near 3.2 ppm in chloroform–methanol mixtures. Its intensity, its sharpness and its position in a comparatively uncrowded region of the spectrum make it the natural first target for anyone attempting a proton-

based phospholipid quantification, and it is regularly pressed into service as a sphingomyelin measure. That use is incorrect in an unfractionated lipid extract, and the error is worth stating explicitly because it recurs.

Phosphatidylcholine, lysophosphatidylcholine, choline plasmalogen, alkylacylglycerophosphocholine, dihydrosphingomyelin and sphingomyelin all carry an identical trimethylammonium group, and in a dairy or serum extract several of these are present simultaneously. The singlet near 3.2 ppm is therefore a summed measure of total choline-bearing phospholipid, not of sphingomyelin. Under conditions of sufficient resolution the individual contributions can begin to separate — a distinct behavior for the phosphatidylcholine and sphingomyelin trimethylammonium resonances was demonstrated at the surface of native low-density lipoprotein particles, where preferential immobilization of phosphatidylcholine produced separable resonance characteristics and permitted individual detection of the two species[44] — but that separation was achieved in an ordered lipoprotein assembly at high field, and it should not be assumed to transfer to a bulk solution-state extract.

The correct use of the trimethylammonium singlet in sphingomyelin work is as a denominator, not a numerator. Where the sphingomyelin fraction of total choline phospholipid has been established independently by ^{31}P NMR, the proton singlet provides a rapid and precise measure of total choline lipid against which the ^{31}P mole fractions can be scaled, and it provides an internally consistent cross-check on the ^{31}P integration. Used in that role it is valuable. Used as a direct sphingomyelin measure it will overstate the result by whatever amount of phosphatidylcholine the sample happens to contain, which in most food matrices is a great deal.

5.2 The sphingosine olefinic protons — the genuine ^{1}H signature

The structurally decisive proton feature of sphingomyelin is the pair of olefinic resonances arising from the C4–C5 *trans* double bond of the sphingosine base. In deuterochloroform these appear as a two-proton system in the region between approximately 5.4 and 5.8 ppm, with H4 upfield of H5. H4 is coupled to H3 and to H5 and presents as a doublet of doublets, while H5 is coupled to H4 and to the allylic C6 methylene and presents as a doublet of triplets. The mutual coupling constant is large, on the order of 15 Hz, which is diagnostic of *trans* geometry and distinguishes this olefin immediately from the *cis* olefins of acyl chains, whose vicinal couplings are near 11 Hz.

This signal is the only routinely observable proton resonance that is specific to sphingomyelin among the phospholipid classes, and its diagnostic value rests on three properties. It is absent from every glycerophospholipid, because no glycerophospholipid carries an olefin at the interface. It is absent from dihydrosphingomyelin, because the saturated base has no double bond at all — which makes the ratio of the olefinic integral to the ^{31}P sphingomyelin-plus-dihydrosphingomyelin integral a direct measure of the dihydro fraction. And it is displaced from the *cis*-olefinic envelope of the acyl chains, which in a lipid extract falls in the region around 5.3 ppm, sufficiently that at adequate field the sphingosine H5 resonance is resolved on the downfield shoulder even though H4 may be partially overlapped.

The caveats are equally important. The olefinic region also contains the glyceryl H2 methine of diacyl glycerophospholipids and triacylglycerols, which resonates near 5.2 ppm and is intense in any extract containing neutral lipid. In a crude extract dominated by triacylglycerol the sphingosine olefinic signals are minor features on a large background and quantification from them is unreliable. The signals are informative in an isolated or enriched phospholipid fraction and are best used qualitatively — as confirmation that the material assigned as sphingomyelin by ^{31}P genuinely contains a sphingosine base — rather than as a primary integral. It

should also be noted that where a sample contains sphingoid bases other than d18:1, or a sphingadiene base, the olefinic pattern will be more complex than the simple two-proton system described.

5.3 The amide NH, the C3 hydroxyl and hydrogen bonding

The amide proton of sphingomyelin appears well downfield of the aliphatic envelope, typically in the region of 6.0–7.0 ppm in deuterochloroform, and the C3 hydroxyl proton appears variably in the region of 3.5–5.0 ppm depending on hydrogen-bonding state. Neither is present in any glycerophospholipid, and both are therefore structurally diagnostic. Both are also exchangeable, which limits their quantitative use but makes them exceptionally informative about molecular association.

The concentration and temperature dependence of these resonances has been characterized in detail for both sphingomyelin and dihydrosphingomyelin.[50,51] For dihydrosphingomyelin in partially hydrated deuterochloroform above approximately 13 mM, at which concentration lipid–lipid interactions come to prevail, the amide proton was found to be principally engaged in intermolecular hydrogen bonds linking neighboring phospholipids through bridging water molecules; in the absence of water the NH engages instead in an intramolecular hydrogen bond that restricts the mobility of the phosphate group.[50] In the monomeric form the amide proton of the major conformer was found to be bound intramolecularly to one of the anionic or ester oxygens of the phosphate.[50] For sphingomyelin, the spectral trends indicated formation of an intramolecular hydrogen bond between the sphingosine hydroxyl and the phosphate ester oxygen, and the narrower linewidth and downfield position of the hydroxyl resonance relative to dihydrosphingomyelin indicated that this hydrogen bond is the stronger of the two.[51]

Two practical consequences follow. First, the position of the sphingomyelin NH and OH resonances is not fixed and should never be used as an identification criterion without accompanying concentration and water-content information; a shift of more than a part per million between two samples of the same material is unremarkable. Second, the presence of a resolvable exchangeable resonance in that region is itself evidence that the sample is a sphingolipid rather than a glycerophospholipid, and its disappearance on addition of deuterium oxide confirms the assignment cheaply and unambiguously. That deuterium oxide exchange test is a five-minute experiment and it is the single most efficient qualitative confirmation available in proton NMR of a putative sphingomyelin isolate.

5.4 Backbone, head group and acyl resonances

Table 5 collects representative proton assignments for sphingomyelin in deuterochloroform, drawn from the synthetic and conformational literature in which full assignment was established by two-dimensional correlation methods.[48,49,50,51] The values should be treated as indicative rather than definitive for the reasons already given: solvent, concentration, water content and temperature all move them, and the exchangeable positions move furthest.

The backbone protons occupy the region between approximately 3.8 and 4.3 ppm. The C1 methylene protons, which bear the phosphate ester, appear near 4.0–4.2 ppm and are diastereotopic. The C2 methine, adjacent to the amide nitrogen, appears near 3.9–4.0 ppm. The C3 methine, bearing the free hydroxyl and allylic to the C4–C5 olefin, appears near 4.0–4.2 ppm. In dihydrosphingomyelin the C2 methine was located at 3.88 ppm and the C4 methylene protons, which replace the olefinic pair, were resolved as two distinct bands at 1.33 and 1.49 ppm correlating to a single carbon.[50] The head-group protons comprise the phosphate-bearing choline methylene

near 4.2–4.3 ppm, the nitrogen-bearing choline methylene near 3.6–3.8 ppm, and the trimethylammonium singlet near 3.2–3.3 ppm, reported at 3.25 ppm in the dihydrosphingomyelin assignment work.[50]

The acyl chain contributes the α-methylene adjacent to the amide carbonyl near 2.1–2.2 ppm, the β-methylene near 1.55–1.60 ppm, the bulk methylene envelope between approximately 1.20 and 1.40 ppm, and the terminal methyl triplet near 0.85–0.90 ppm. Where the acyl chain is unsaturated the corresponding allylic and bis-allylic resonances appear near 2.0 and 2.8 ppm respectively, and the assignment of mono- and polyunsaturated fatty acids in tissue lipid extracts has been treated systematically.[45] Because sphingomyelin carries one acyl chain against the two of a diacyl glycerophospholipid, the ratio of the acyl envelope to the trimethylammonium singlet differs between the two classes by approximately a factor of two — a relationship that provides a useful consistency check on a proposed composition but is far too blunt to serve as a quantification in a mixture.

Table 5. Representative ^{1}H assignments for sphingomyelin in $CDCl_3$. Positions are indicative; solvent, concentration, water content and temperature displace them, and the exchangeable positions move furthest.[48,49,50,51]

Position	δ(^{1}H) / ppm	Multiplicity	Diagnostic value
Amide N–H	6.0–7.0	d (br)	Absent in all glycerophospholipids; exchanges with D_2O; strongly concentration-dependent[50,51]
H5 (=CH, sphingosine)	5.65–5.80	dt, J ≈ 15 Hz	SM-specific; absent in DHSM; trans coupling distinguishes from cis acyl olefins
H4 (=CH, sphingosine)	5.40–5.55	dd, J ≈ 15, 7 Hz	As above; partially overlapped by acyl olefinic envelope near 5.3 ppm
Glyceryl H2 (glycerophospholipids)	~5.20	m	Must be ABSENT in pure SM; its presence indicates glycerophospholipid contamination
Choline P–O–CH_2	4.20–4.35	m	Head group; shared with PC and all choline lipids
H3 (CH–OH)	4.00–4.20	m	Backbone; allylic to C4–C5 olefin
H1 (C–CH_2–OP)	3.95–4.20	m (diastereotopic)	Backbone; two-bond correlation to ^{31}P in 2D experiments
H2 (CH–N)	3.85–4.00	m	Backbone; correlates to C2 near 54 ppm[50]
Choline N–CH_2	3.60–3.80	m	Head group; shared with all choline lipids
$N(CH_3)_3$	3.20–3.35	s (9H)	NOT SM-specific — summed across PC, LPC, plasmalogens, DHSM and SM[44]
Acyl α-CH_2 (to amide C=O)	2.10–2.25	t	Amide-linked acyl chain
Allylic CH_2 (C6)	1.95–2.05	m	Adjacent to sphingosine olefin
Acyl β-CH_2	1.50–1.65	m	Acyl chain
Bulk $(CH_2)_n$	1.20–1.40	br s	Dominates the integral; obscures minor structural signals
Terminal CH_3	0.85–0.92	t	Chain terminus

5.5 Discrimination of sphingomyelin from glycerophospholipids by absence

One of the most reliable proton observations in this field is negative. Every diacyl glycerophospholipid carries a glycerol backbone whose C2 methine resonates near 5.2 ppm and whose C1 and C3 methylene protons resonate as characteristic multiplets in the region of 4.1–4.4 ppm. Sphingomyelin has no glycerol and therefore contributes nothing to those multiplets. In an isolated sphingomyelin the glyceryl pattern is absent entirely, and its appearance in a nominally pure sphingomyelin preparation is immediate evidence of glycerophospholipid contamination — most commonly phosphatidylcholine, which co-elutes readily with sphingomyelin on several chromatographic systems.

This test is particularly valuable in verifying the purity of sphingomyelin standards and enriched fractions. A material that gives a single ^{31}P resonance may still be impure, because ^{31}P resolution is limited and a phosphatidylcholine impurity is only 0.75 ppm away; a material that gives a single ^{31}P resonance and no glyceryl proton multiplets is much more securely pure. Since reference material purity propagates directly into every quantitative result calibrated against it, this five-minute check has disproportionate value in a laboratory establishing a sphingomyelin method.

The general proton analysis of membrane lipid mixtures has been developed on exactly this logic of structurally characteristic regions,[42] and applied to erythrocyte lipids,[43] to serum lipid extracts where up to thirty structural groups including sphingomyelin were identified and quantified,[46] and to the discrimination of animal species in raw and processed meat by multivariate treatment of lipid extract spectra.[47] In each case the proton spectrum functions as a structural fingerprint of the whole lipid pool rather than as a targeted assay for one class.

5.6 Quantitative ^{1}H NMR practice and its limits for sphingomyelin

Where a sphingomyelin isolate or a strongly enriched fraction is available, quantitative proton NMR is straightforward and precise. The trimethylammonium singlet provides a nine-proton integral of excellent signal-to-noise, the olefinic pair provides a two-proton structural confirmation, and an internal standard of known mass gives absolute concentration. Relaxation delays must again be set from measured T_1 values — the trimethylammonium protons of a phospholipid in solution commonly relax more slowly than the acyl envelope, and a delay adequate for the chain protons will under-integrate the head group. A combined proton and phosphorus approach to the relative and absolute quantification of lipid components has been described for liposomal systems and provides a workable template.[29]

In an unfractionated extract, however, quantitative proton NMR cannot deliver sphingomyelin. The trimethylammonium singlet is a class-summed signal, the olefinic resonances sit on a triacylglycerol background, the backbone protons overlap the head-group and glyceryl regions, and the exchangeable protons are not integrable. This is not a limitation that can be engineered away by higher field or better shimming; it is a consequence of the molecule sharing its most prominent functional group with several co-occurring classes. Any protocol proposing to quantify sphingomyelin in a food or tissue extract from the proton spectrum alone should be regarded as unsound unless it incorporates either a chromatographic pre-separation or a ^{31}P-derived apportionment of the choline signal.

The honest summary is that ^{1}H NMR earns its place in sphingomyelin analysis as the identification and confirmation nucleus and as the sensitive detector for enriched fractions, while the quantification of the class in a complex mixture belongs to ^{31}P.

6 ^{13}C NMR of Sphingomyelin

6.1 Sensitivity and relaxation constraints

Carbon-13 has a natural abundance of approximately 1.1% and a gyromagnetic ratio approximately one quarter that of the proton, and the resulting receptivity is roughly four orders of magnitude below that of ^{1}H. For a phospholipid at the concentrations typical of a lipid extract this places a natural-abundance carbon spectrum with usable signal-to-noise on the informative low-intensity carbons at hours rather than minutes of acquisition. Quaternary and carbonyl carbons relax slowly, with T_1 values that may reach tens of seconds, and under broadband proton decoupling the nuclear Overhauser enhancement varies from near-maximal for protonated carbons to small for non-protonated ones. Quantitative carbon spectra therefore require inverse-gated decoupling and very long relaxation delays, and the resulting experiment durations are rarely justified when ^{31}P is available and gives the same mole information in a fraction of the time.

The consequence is that ^{13}C is deployed in sphingomyelin work as a structural rather than a quantitative nucleus, and on isolates, standards and concentrates rather than on dilute extracts. Within that role it is extremely powerful, because it resolves structural distinctions that are invisible or marginal at the other two nuclei. The role of ^{13}C alongside ^{31}P and ^{1}H in the multinuclear fingerprinting of a phospholipid-rich material is illustrated by the krill oil work, in which all three nuclei were applied to the same product.[25]

6.2 Diagnostic carbon resonances

Table 6 collects representative carbon assignments. Several deserve individual comment.

The **C1 carbon**, bearing the phosphate ester, resonates near 66 ppm and — importantly — appears as a doublet through two-bond scalar coupling to phosphorus. That coupling is itself an assignment tool: in a proton-decoupled but phosphorus-coupled spectrum, the carbons within two or three bonds of the phosphorus are identifiable by their multiplicity alone, which locates the head-group attachment point unambiguously.

The **C2 carbon**, bearing the amide nitrogen, resonates near 54 ppm, where it coincides closely with the trimethylammonium carbons. In the dihydrosphingomyelin assignment work these two were incompletely resolved in the one-dimensional carbon spectrum, appearing together at 54.4 ppm, and were separated only by two-dimensional ^{1}H–^{13}C correlation, in which the 54.4 ppm carbon showed one cross peak to the nine-proton choline methyl signal at 3.25 ppm and a second to the H2 backbone proton at 3.88 ppm.[50] This is a clean illustration of why one-dimensional carbon assignment of sphingolipids is unsafe without heteronuclear correlation support.

The **olefinic carbons C4 and C5** of the sphingosine base appear in the region near 129 and 134 ppm respectively and are absent in dihydrosphingomyelin, where the corresponding C4 becomes an aliphatic methylene assigned at 34.0 ppm.[50] The **amide carbonyl** resonates near 173–174 ppm, well clear of the ester carbonyls of glycerophospholipids and triacylglycerols near 172–173 ppm, though the separation is small and should be treated as suggestive rather than conclusive without corroboration. The **choline carbons** appear near 59 ppm for the nitrogen-bearing methylene, near 66 ppm for the phosphate-bearing methylene, and at 54.4 ppm for the three equivalent methyls.

Table 6. Representative ^{13}C assignments for sphingomyelin and dihydrosphingomyelin in $CDCl_3$. The C3 position is the definitive discriminator between the two species.[50]

Carbon	δ(^{13}C) SM / ppm	δ(^{13}C) DHSM / ppm	Comment
Amide C=O	~173–174	~173–174	Distinguishable in principle from glycerophospholipid ester carbonyls near 172–173 ppm; small separation
C5 (olefinic)	~134	absent	Sphingosine unsaturation; absent in DHSM
C4 (olefinic)	~129	34.0	In DHSM this becomes an aliphatic methylene, assigned at 34.0 ppm[50]
C3 (CH–OH)	73.4	70.7	DEFINITIVE SM/DHSM discriminator; 2.7 ppm separation attributed to the C4–C5 unsaturation[50]
C1 (CH_2–OP)	~66	~66	Doublet through two-bond ^{31}P coupling; locates head-group attachment
Choline N–CH_2	~66	~66	Head group
Choline O–CH_2	~59	~59	Head group
$N(CH_3)_3$	54.4	54.4	Coincides with C2; separable only by 2D ^{1}H–^{13}C correlation[50]
C2 (CH–N)	~54	54.4	Overlapped with $N(CH_3)_3$ in 1D spectra[50]
Acyl α-CH_2	~36.7	~36.7	Adjacent to amide carbonyl
Bulk $(CH_2)_n$	29–30	29–30	Chain envelope
Terminal CH_3	14.1	14.1	Chain terminus

6.3 C3 as the definitive sphingomyelin–dihydrosphingomyelin discriminator

The single most valuable carbon observation in this field concerns C3. In sphingomyelin, C3 bears the secondary hydroxyl and is allylic to the C4–C5 double bond, and its resonance has been reported at 73.4 ppm. In dihydrosphingomyelin, where the double bond is absent, the same carbon was assigned at 70.7 ppm, and the difference was explicitly attributed to the absence of the C4–C5 unsaturation.[50]

The analytical significance of this 2.7 ppm separation becomes apparent when it is compared with the corresponding separation at the other nuclei. At ^{31}P the two species are separated by approximately 0.22 ppm, which at 121.4 MHz observation frequency corresponds to approximately 27 Hz and at 24.3 MHz to approximately 5 Hz. At ^{13}C the 2.7 ppm separation corresponds to approximately 204 Hz at 75.5 MHz carbon observation on a 300 MHz instrument. In relative terms the carbon separation is more than an order of magnitude more comfortable, and it is achieved without any manipulation of solvent, temperature, pH or chelation.

The trade is sensitivity and time. Where a laboratory is establishing the identity of a sphingomyelin standard, verifying the dihydrosphingomyelin content of an enriched dairy fraction, or resolving a dispute about whether a ^{31}P shoulder is dihydrosphingomyelin or phosphatidic acid, the carbon experiment settles the question definitively where the phosphorus experiment can only make it probable. Given the reported prevalence of dihydrosphingomyelin at approximately 20% of bovine milk sphingolipids,[33] that determination is not an

academic exercise, and a laboratory offering dairy sphingomyelin analysis should have made it at least once for each material type it handles.

6.4 Acyl chain characterization and two-dimensional carbon methods

Beyond the backbone and head group, the carbon spectrum reports on the amide-linked acyl chain. Chain-terminal and near-terminal carbons resolve according to chain length in the region between 14 and 32 ppm, olefinic carbons of unsaturated acyl chains appear near 128–130 ppm, and the α- and β-carbons to the amide carbonyl are resolved near 36.7 and 25.8 ppm. In principle this permits characterization of the acyl distribution; in practice, for a natural sphingomyelin comprising a dozen molecular species, the resulting resonances are heavily overlapped and the information recovered is coarse. Gas chromatography of the derived fatty acid methyl esters remains the appropriate method for acyl composition and should not be displaced by carbon NMR.

Two-dimensional heteronuclear methods are where carbon contributes most efficiently. A ^{1}H–^{13}C heteronuclear single quantum or multiple quantum correlation experiment recovers carbon-resolved information at proton sensitivity and completes in a time comparable with a one-dimensional carbon acquisition, and it was precisely such experiments — heteronuclear multiple quantum coherence together with correlation and total correlation spectroscopy — that were used to establish the complete proton and carbon assignment of dihydrosphingomyelin.[50] For a laboratory needing to confirm a sphingomyelin structure rather than to quantify it, the two-dimensional proton–carbon experiment is a considerably better use of instrument time than a one-dimensional carbon acquisition and should be the default choice.

Cross-polarization and heteronuclear correlation approaches under magic-angle spinning extend the same logic to the solid and bilayer states, where combined $^{1}H/^{31}P$ and $^{1}H/^{13}C$ correlation experiments have revealed a backbone–water interaction unique to sphingomyelin and not present in glycerol-backbone phosphatidylcholines.[56]

7 The Integrated Multinuclear Workflow

The preceding sections argue for a division of labor, and it is worth setting out the resulting workflow explicitly, since the value of the multinuclear approach lies in the sequence rather than in any individual experiment.

Stage one — establish the class profile and the quantification by ^{31}P. The extract is prepared in a chelated medium of demonstrated adequacy, an internal standard is added, and a one-dimensional inverse-gated proton-decoupled ^{31}P spectrum is acquired with a relaxation delay set from measured T_1 values. This yields mole fractions for every resolved class and, through the standard, absolute concentrations. It does not yet establish that the resonance integrated as sphingomyelin is sphingomyelin.

Stage two — confirm the assignment by two-dimensional ^{1}H–^{31}P correlation. A heteronuclear correlation experiment establishes which protons are attached to the head group giving rise to each phosphorus resonance, transferring the identification burden to the better-dispersed and less condition-sensitive proton dimension.[10,23,27] For sphingomyelin the diagnostic correlations are to the backbone C1 and C2 protons rather than to a glyceryl pattern.

Stage three — confirm the sphingoid base by ¹H. The olefinic pair near 5.4–5.8 ppm with a *trans* coupling near 15 Hz confirms a sphingosine base; the amide NH downfield of 6 ppm, and its disappearance on deuterium oxide exchange, confirms the amide linkage; the absence of glyceryl multiplets confirms the absence of a glycerol backbone.

Stage four — resolve the dihydro question by ¹³C where it matters. In dairy and lens-derived materials, and in any material where dihydrosphingomyelin may be significant, the C3 position at 73.4 ppm against 70.7 ppm settles the sphingomyelin–dihydrosphingomyelin apportionment definitively.[50] Where the sample will not support a carbon experiment, the ratio of the ¹H olefinic integral to the combined ³¹P sphingomyelin-plus-dihydrosphingomyelin integral provides an indirect estimate of the same quantity.

Stage five — verify by orthogonal chemistry. Spiking with authentic sphingomyelin should displace the assigned integral proportionately and should not generate a new resonance. Enzymatic treatment with sphingomyelinase, which cleaves the phosphocholine head group, should extinguish the sphingomyelin resonance and generate phosphocholine while leaving the glycerophospholipids intact; the analogous logic applies to phospholipase treatment of the glycerophospholipids, and the enzymatic modification of milk phospholipids has been followed directly by ³¹P NMR.[34] A selective enzymatic knockout is the most persuasive single piece of confirmatory evidence available and is inexpensive.

Stages one and two constitute the minimum defensible method for a novel matrix. Stages three through five are established once per material type and thereafter revisited only on change of matrix or reagent. For routine throughput on a characterized material, stage one alone is adequate, provided the validation record documents that stages two through five were completed and that the assignment has been fixed.

8 Sample Preparation and Extraction Chemistry

Extraction is the stage at which most of the systematic error in a sphingomyelin determination is introduced, and it deserves attention proportionate to that fact rather than the cursory treatment it usually receives.

The classical chloroform–methanol procedure of Folch and co-workers, in which tissue is homogenized in chloroform–methanol 2:1 and the extract washed with an aqueous salt solution to partition polar contaminants into the upper phase, remains the reference method and is the basis of the reagent systems described in Section 4.2.[58] The Bligh and Dyer modification reduces solvent consumption and is well adapted to high-moisture matrices,[59] and the methyl-*tert*-butyl ether procedure of Matyash and co-workers inverts the phase order so that the lipid-containing phase is uppermost, which simplifies automated handling and improves throughput.[60]

Sphingomyelin presents two specific extraction hazards. First, it is the most polar of the major phospholipid classes commonly encountered, and partial partitioning into the aqueous phase during washing is a real risk when the wash is aggressive or the methanol proportion is high. Recovery should be established for sphingomyelin specifically rather than assumed from total phospholipid recovery, and it should be established by spiking authentic sphingomyelin into the matrix and carrying it through the whole procedure. Second, sphingomyelin in dairy is concentrated in the milk fat globule membrane, and the physical accessibility of that membrane depends on processing history. Homogenization, pasteurization and spray drying all reorganize the membrane, and an extraction adequate for raw cream may be incomplete for a spray-dried powder.

Two developments deserve particular note for laboratories seeking to reduce solvent hazard or to increase throughput. The cholate micellar approach permits direct analysis of high-phospholipid dairy materials without prior extraction, eliminating extraction recovery as an error source entirely for those matrices.[30] Separately, an infant formula method has been described in which phospholipids are extracted with a heated ethanol–water mixture and the extract dispersed in surfactants with strong affinity for phospholipids, avoiding chloroform at both the extraction and the measurement stage while retaining absolute quantification of individual phospholipids by ^{31}P NMR.[38] For a laboratory operating under ISO/IEC 17025 with occupational exposure controls to satisfy, the elimination of chlorinated solvent from a routine method is a substantive benefit and not merely a convenience.

Whichever procedure is adopted, the following should be documented in the method: the recovery of sphingomyelin specifically, established by spike; the stability of the extract under the storage conditions used between preparation and acquisition; the absence of detectable hydrolysis to lysophospholipid or phosphocholine over that interval, which is directly observable in the ^{31}P spectrum and constitutes a built-in stability indicator; and the adequacy of chelation, established by demonstrating that further chelator addition does not move the resonances.

9 Quantitative Performance and Method Validation

The metrological standing of ^{31}P phospholipid NMR is better established than that of most food-analytical NMR methods, because it has been subjected to formal inter-laboratory testing. A proficiency exercise conducted on krill oil across fourteen laboratories, with samples prepared centrally by liquid–liquid extraction and analyzed and evaluated by the participants, returned relative standard deviations between 2.1% and 3.3% for phosphatidylcholine content and between 2.8% and 12.3% for other phospholipid classes.[26] That spread is informative in itself: the dominant, well-resolved class is determined with excellent reproducibility, while minor and less-resolved classes — the category into which sphingomyelin falls in a lecithin matrix — carry reproducibility several times worse. A laboratory quoting a sphingomyelin uncertainty should be quoting something closer to the upper figure than the lower one unless it has data of its own to justify otherwise.

Method comparison against independent techniques provides the other pillar of validation. The cholate-based dairy phospholipid method was validated against quantitative two-dimensional thin-layer chromatography, with the finding that the chromatographic method was the more sensitive and detected minor components not observed by NMR, while the NMR method was better suited to routine analysis on grounds of speed and versatility.[30] That is an honest characterization of the trade and should be reproduced in any comparison a laboratory undertakes: NMR is not more sensitive than chromatography for phospholipids and should not be represented as such.

Reported applications provide useful context for expected concentration ranges. Table 7 collects sphingomyelin and related phospholipid figures determined by ^{31}P NMR across a range of matrices, including the finding that sphingomyelin was the dominant phospholipid of human milk at approximately 35% of total phospholipid while phosphatidylcholine and phosphatidylethanolamine dominated infant formulas,[36] the species-comparative fingerprints in which human and camel milk were found richer in sphingomyelin than cow or mare milk,[31] the comparative study across human, cow, goat, yak and donkey milk,[37] and the brand-level differentiation of powdered infant formulas in which nine phospholipids were quantified and sphingomyelin ranged down to approximately 8.9 mg per 100 g in the lowest brand examined.[35] Comparable ^{31}P methodology

has been applied to cheese and fish,[27] to food emulsions including lysophospholipid determination,[28] and to the tracking of enzymatic phospholipid hydrolysis in milk and whey.[34]

The validation package for a sphingomyelin method should therefore comprise: measured T_1 values for every integrated resonance in the matrix of interest, with the relaxation delay justified against them; demonstration that inverse-gated decoupling has been used and that integrals are NOE-free, ideally by comparison against a fully relaxed non-decoupled or gated acquisition; linearity across the working range using authentic sphingomyelin; limit of detection and limit of quantification established by replicate low-level determination rather than by signal-to-noise extrapolation; spike recovery through the whole procedure; demonstrated resolution of sphingomyelin from dihydrosphingomyelin, phosphatidylserine and ethanolamine plasmalogen in the specific matrix, or an explicit statement that the reported value is a sum; intermediate precision across analysts, days and where applicable instruments; and a documented assignment confirmed by two-dimensional correlation or enzymatic knockout. Where the reported unit is mass rather than moles, the assumed molecular weight must be stated, because as shown in Section 2.2 the choice carries a systematic bias of up to approximately 15%.

Table 7. Selected sphingomyelin and phospholipid findings determined by ^{31}P NMR across food matrices, illustrating typical concentration ranges and the analytical questions addressed.

Matrix	Reported finding	Reference
Human milk vs infant formula	SM the dominant phospholipid of human milk at approximately 35% of total PL; PC and PE dominant in infant formulas	[36]
Milk across mammalian species	Species-specific fingerprints; human and camel milk richer in SM (78.3 and 117.5 µg/mL) and plasmalogens; total PL highest in camel (0.503 mM), lowest in mare (0.101 mM)	[31]
Human, cow, goat, yak, donkey milk	Comparative PL composition and fat globule structure; SM higher and acyls more unsaturated in human milk	[37]
Powdered infant formula (4 brands)	Nine PLs quantified; SM as low as 8.89 mg/100 g and PE 5.72 mg/100 g in one brand; multivariate brand differentiation	[35]
Dairy ingredient streams	PC, PE, PS, PI, SM and DHSM quantified in cholate; direct analysis without extraction for high-PL materials	[30]
Bovine milk sphingolipids	DHSM approximately 20% of total bovine milk sphingolipid; parallel LC–MS and ^{31}P NMR detection	[32,33]
Cheese and fish	2D ^{31}P,^{1}H profiling following liquid–liquid enrichment	[27]
Food emulsions	Quantification of phospholipids and lysophospholipids	[28]
Milk and whey	Enzymatic phospholipid hydrolysis followed directly by ^{31}P NMR	[34]
Human milk and infant formula digestion	SM highly resistant to upper gastrointestinal hydrolysis (~4% in human milk, up to ~16% in one formula) whereas glycerophospholipids exceeded 88% hydrolysis	[39]
Krill oil	Multinuclear ^{31}P, ^{1}H and ^{13}C fingerprinting; inter-laboratory proficiency testing	[25,26]

10 Solid-State and Bilayer NMR: Scope and Relevance

The foregoing has treated sphingomyelin as a solute. A substantial parallel literature treats it as a membrane component, and although that work is directed at biophysical rather than analytical questions, three of its findings bear on the analytical problem and are worth noting briefly.

First, the ^{31}P resonance of a phospholipid in an intact bilayer is not narrow. It is dominated by chemical shift anisotropy, producing the characteristic powder lineshape from which bilayer phase and headgroup orientation are inferred, and the analytical requirement for narrow lines is met only by destroying that organization through dissolution in an organic medium or solubilization into small, rapidly tumbling micelles. This is why the solvent systems of Section 4.2 exist, and it is why an attempt to acquire a high-resolution ^{31}P spectrum of an intact milk fat globule membrane preparation will fail. The behavior of sphingomyelin headgroup motion and its ^{31}P lineshape consequences have been modeled explicitly,[57] and the early characterization of sphingomyelin in bilayer systems by NMR established the framework.[53] The continuum from micellar to membrane states, and the corresponding transition from narrow isotropic lines to anisotropic powder patterns, has been reviewed comprehensively.[18]

Second, magic-angle spinning recovers isotropic resolution without dissolution, and under those conditions the sphingomyelin and phosphatidylcholine headgroup resonances of a raft-forming mixture can be resolved and their individual mobilities and phase transitions followed.[55] This offers a route to sphingomyelin measurement in an intact membrane preparation that solution-state methods cannot provide, at the cost of specialized probe hardware.

Third, the interaction of sphingomyelin with cholesterol — the interaction underlying raft behavior and of direct interest in dairy membrane fractions — has been characterized by solid-state NMR in sphingomyelin–cholesterol binary systems,[54] and conformational analysis of sphingomyelin in bicelles has provided a bridge between the solution and membrane states.[52] The unique backbone–water interaction detected in sphingomyelin bilayers by combined $^{1}H/^{31}P$ and $^{1}H/^{13}C$ heteronuclear correlation under magic-angle spinning, and absent in glycerol-backbone lipids, is a direct spectroscopic expression of the structural distinction on which this entire paper turns.[56]

For the analytical laboratory the practical message is limited but real: sphingomyelin quantification requires disruption of membrane organization, the completeness of that disruption is an assumption that should be tested, and residual undissolved or aggregated material will be under-represented in the integral rather than absent from it, since its signal is broadened into the baseline rather than eliminated.

11 Orthogonal and Complementary Techniques

NMR is not the only route to sphingomyelin, and an honest account of its position requires comparison against the alternatives. Table 8 summarizes the comparison.

High-performance liquid chromatography with evaporative light scattering or charged aerosol detection separates phospholipid classes physically and is the established routine method in much of the dairy industry. A widely used HPLC method for phospho- and sphingolipids in dairy products provides the reference point.[41] Chromatography is more sensitive than NMR, requires far less sample, and is cheaper per determination. Its disadvantages are that the detectors named are not mass-proportional in a simple linear fashion and require class-specific calibration against authentic standards, that sphingomyelin and phosphatidylcholine can be

difficult to resolve on some systems, and that dihydrosphingomyelin is not separated from sphingomyelin at all on most normal-phase systems and is therefore silently summed.

Liquid chromatography–mass spectrometry delivers molecular species resolution that NMR cannot approach, identifying individual combinations of sphingoid base and acyl chain. It was the basis of the demonstration that dihydrosphingomyelin constitutes approximately 20% of bovine milk sphingolipids and of the characterization of the sphingoid base distribution within that fraction.[32,33] Shotgun approaches with lithium-ion-induced fragmentation have been applied specifically to sphingomyelin quantification in dairy.[40] The limitation is quantitative: ionization efficiency varies with molecular species, so absolute quantification requires isotopically labeled internal standards for each species class and remains vulnerable to matrix suppression. The observation advanced in the parallel-detection work — that ^{31}P NMR may be superior to mass spectrometry for the quantification of sphingolipid *classes* while mass spectrometry is unmatched for *species* identification — remains the correct summary of the relationship, and the two techniques are complements rather than competitors.[33]

Thin-layer chromatography, particularly in its two-dimensional quantitative form, retains a role as an independent reference method and was used as such in the validation of the cholate NMR method, where it proved the more sensitive of the two.[30]

A qualification is owed to the claim, made repeatedly above, that ^{31}P NMR resolves classes but not molecular species. Partial resolution of phospholipid molecular species by ^{31}P NMR has in fact been demonstrated under favorable conditions, the small residual shift differences between species of differing acyl composition becoming observable at high field with narrow lines.[9] The effect is real but slight, it has been shown principally for glycerophospholipids rather than for sphingomyelin, and it does not constitute a practical route to sphingomyelin species distribution in a food matrix. The operational claim — that a routine ^{31}P determination reports a class total — stands, but it should be stated as a limitation of the routine method rather than as a property of the nucleus.

The distinguishing advantages of NMR remain: it is non-destructive, it requires no chromatographic separation and therefore no recovery correction, it is inherently molar and requires no class-specific response calibration, it simultaneously quantifies every phosphorus-containing species present rather than only those targeted, and it provides structural confirmation alongside quantification. The distinguishing disadvantages are its cost, its sensitivity, and its inability to resolve molecular species. For an ingredient specification expressed as total sphingomyelin, NMR is the stronger method. For a nutritional or metabolic study requiring species-level detail, mass spectrometry is indispensable. For routine quality control at high throughput and low cost, chromatography usually wins on economics. The reviews of NMR in lipid and phospholipid analysis provide fuller comparative treatment.[20,21,22]

Table 8. Comparison of analytical approaches to sphingomyelin determination.

Attribute	^{31}P NMR	HPLC–ELSD/CAD	LC–MS	2D-TLC
Basis of quantification	Molar; one P per molecule	Detector response; class calibration required	Ion current; species-dependent efficiency	Densitometry
Response factor needed	No	Yes, per class	Yes, per species	Yes
Molecular species resolution	No	No	Yes	No

Attribute	³¹P NMR	HPLC–ELSD/CAD	LC–MS	2D-TLC
SM/DHSM resolution	Yes, if optimized	Generally not	Yes	Partial
Sensitivity	Low	High	Very high	High
Sample consumption	High (mg)	Low (µg)	Very low	Low
Structural confirmation	Yes (with ¹H/¹³C)	No	Yes (fragmentation)	No
Non-destructive	Yes	No	No	No
Cost per determination	High	Low	Moderate–high	Low
Key reference	[5,30,38]	[41]	[32,33,40]	[30]

12 Chemometric Treatment of Phospholipid NMR Datasets

Phospholipid ³¹P spectra lend themselves naturally to multivariate treatment, since each spectrum reduces to a small number of well-defined integrals whose relative proportions constitute a compositional fingerprint. Multivariate analysis of ³¹P data has been used to differentiate powdered infant formula brands,[35] and proton lipid-extract spectra combined with multivariate statistics have been used to discriminate the animal species of raw and processed meat.[47] The broader question of whether the phospholipid fraction is being adequately exploited for food authentication has been posed directly in the recent review literature.[22]

Three cautions apply with particular force to this data type, and they are worth stating because the failure modes are not obvious.

First, **compositional closure**. Mole percentages of phospholipid classes sum to one hundred by construction. Such closed data occupy a simplex rather than a Euclidean space, and correlations between components are induced by the closure itself rather than by any chemical relationship. Principal component analysis applied directly to closed compositional data will generate structure that is partly an artifact of the constraint, and any apparent negative correlation between sphingomyelin and phosphatidylcholine in such a dataset must be regarded as uninterpretable until the closure has been addressed, most straightforwardly by a log-ratio transformation prior to modeling.

Second, **shift instability as a hidden variable**. Because phospholipid ³¹P chemical shifts respond to temperature, pH, cation loading and lipid concentration, a spectral dataset acquired over months will contain systematic peak-position drift correlated with acquisition date rather than with sample identity. Where spectra are binned or treated as full profiles rather than reduced to integrals, that drift becomes a dominant source of variance and models will classify on it. Alignment prior to modeling, or reduction to integrals over generously defined regions, is not optional for this data type.

Third, **the sample-to-variable ratio**. Phospholipid class datasets typically contain between six and twelve variables and, in food authentication studies, frequently fewer than fifty samples. Discriminant models built on such data are prone to optimistic error estimates unless validated on genuinely held-out material, and cross-validation within a single production batch or a single sampling campaign does not constitute independent validation. Where a sphingomyelin-based authentication claim is advanced — for instance that a declared human-milk-mimetic formula can be distinguished from a conventional one by its phospholipid profile — the

claim should rest on independent validation samples and on a stated error rate, not on a well-separated scores plot.

None of this argues against chemometric treatment of phospholipid data, which is a natural and productive application. It argues that the specific structure of this data type — closed, small, and subject to instrumentally induced drift — demands specific precautions that generic multivariate practice does not supply.

13 Recommended Protocols

The following are offered as defensible starting points for a laboratory establishing sphingomyelin capability, to be adapted against the matrix at hand and validated as described in Section 9. The parameters assume a mid-field superconducting instrument observing ^{31}P near 121 MHz; scaling to other fields follows Table 4.

Protocol A — quantitative ^{31}P class profile in a chelated organic medium. Approximately 10–50 mg of lipid extract is dissolved in 2 mL of chloroform; 1 mL of a reagent comprising one part 0.2 M EDTA in deuterium oxide titrated to pH 6 with cesium hydroxide and four parts methanol is added; the mixture is vortexed to a single optically clear phase and transferred to a 5 mm tube.[5] An internal standard of known mass, resonating clear of the phospholipid envelope, is added at a level giving an integral comparable with the analyte of interest. The spectrum is acquired with inverse-gated proton decoupling, a 90° excitation pulse, a spectral width sufficient to encompass the standard, a relaxation delay of at least five times the longest measured T_1, and sufficient transients for a signal-to-noise ratio of at least 150:1 on the sphingomyelin resonance. Temperature is regulated and recorded. Processing employs modest exponential apodization, careful manual phasing, and a baseline correction applied over a region excluding all analyte signals.

Protocol B — direct ^{31}P analysis of high-phospholipid dairy material without extraction. The material is dispersed in an aqueous sodium cholate–EDTA medium and analyzed as above.[30] This is appropriate for buttermilk protein concentrates, beta serum and comparable ingredient streams and eliminates extraction recovery from the error budget. Temperature and pH must be regulated and recorded, since the micellar medium places some classes in a pH-responsive regime even though sphingomyelin and phosphatidylcholine are not among them.[13]

Protocol C — chlorinated-solvent-free infant formula analysis. Phospholipids are extracted with a heated ethanol–water mixture and the extract dispersed in a phospholipid-affine surfactant for measurement, permitting absolute quantification of individual phospholipids without chloroform at either stage.[38]

Protocol D — assignment confirmation. A two-dimensional ^{1}H–^{31}P correlation experiment is acquired on a representative sample, and the sphingomyelin phosphorus resonance is confirmed by correlation to sphingoid backbone protons rather than to a glyceryl pattern.[23,27] The confirmation is repeated on any change of matrix or reagent system.

Protocol E — sphingomyelin/dihydrosphingomyelin apportionment. On an isolate or enriched fraction, a ^{13}C spectrum or a two-dimensional ^{1}H–^{13}C correlation is acquired and the C3 region examined; resonance at approximately 73.4 ppm indicates sphingomyelin and at approximately 70.7 ppm dihydrosphingomyelin.[50] Where carbon sensitivity is inadequate, the ratio of the ^{1}H sphingosine olefinic integral to the summed ^{31}P sphingomyelin-plus-dihydrosphingomyelin integral provides an indirect estimate.

Protocol F — enzymatic confirmation. A portion of the sample is treated with sphingomyelinase and re-analyzed by ^{31}P; extinction of the assigned resonance with concomitant appearance of phosphocholine confirms the assignment, and the analogous experiment with phospholipase confirms the glycerophospholipid assignments. Enzymatic modification of milk phospholipids has been followed by ^{31}P NMR directly and the approach is well precedented.[34]

14 Limitations, Pitfalls and Common Errors

The following enumerates the failure modes most likely to be identified by a technical reviewer, an accreditation assessor or an opposing expert. Table 9 summarizes them with their remedies.

Treating the trimethylammonium singlet as sphingomyelin. As set out in Section 5.1, this signal is a summed measure of all choline-bearing phospholipids. Any sphingomyelin value derived from it in an unfractionated extract is overstated by the phosphatidylcholine content.

Unresolved dihydrosphingomyelin. In dairy and lens-derived materials, dihydrosphingomyelin may represent a fifth of the sphingolipid.[33] Where it is not resolved, the reported value is either a sphingomyelin-plus-dihydrosphingomyelin sum reported as sphingomyelin, or a sphingomyelin value from which the dihydro species has been misassigned to phosphatidic acid or ethanolamine plasmalogen.

Unresolved phosphatidylserine. At approximately 0.04 ppm from sphingomyelin, phosphatidylserine is the tightest overlap in the profile, and its position is strongly cation-dependent.[8] In a calcium-rich matrix with inadequate chelation the two may be irretrievably merged.

Continuous rather than inverse-gated decoupling. Nuclear Overhauser enhancements of 50–60% have been reported for phospholipid phosphorus,[5] and they are not uniform across classes. Integrals acquired under continuous decoupling are not mole fractions.

Truncated relaxation delay. With T_1 values of the order of 3.3 s,[5] a delay of less than approximately 17 s following a 90° pulse produces differential saturation. The common practice of using a 2–5 s delay to gain throughput is a silent, systematic and class-dependent error.

Chemical shifts transferred from published tables. Positions are conditional on solvent, water content, chelator, cation, temperature, pH and concentration.[5,7,8,13,14,16] Assignment must be anchored internally.

Mass reporting without a stated molecular weight assumption. The molecular weight spread across natural sphingomyelin species is approximately 15%, and a mass-based result is meaningless without the assumption stated.

Extraction recovery assumed rather than measured for sphingomyelin specifically. Sphingomyelin is the most polar major phospholipid and is the most vulnerable to loss during aqueous washing.

Benchtop resolution claims unsupported by arithmetic. As Table 4 shows, a 60 MHz instrument cannot resolve sphingomyelin from phosphatidylserine and can only marginally resolve it from dihydrosphingomyelin. Claims to the contrary should be met with a request for the spectrum.

Chemometric models on closed compositional data without transformation, and validated internally. See Section 12.

Beyond these specific items, two general limitations should be acknowledged in any report. NMR does not resolve sphingomyelin molecular species, so a sphingomyelin value is a class total and any statement about chain-length or base distribution requires mass spectrometric or chromatographic support. And NMR is not a sensitive technique; where sphingomyelin is present at trace levels the appropriate method is chromatographic or mass spectrometric, and an NMR non-detection should be reported with a stated limit of detection rather than as an absence.

Table 9. Principal failure modes in sphingomyelin determination by NMR and their remedies.

Failure mode	Consequence	Remedy
$N(CH_3)_3$ singlet treated as SM	Result overstated by the PC content	Quantify by ^{31}P; use the ^{1}H singlet only as a total-choline denominator
DHSM unresolved or misassigned	Up to ~20% error in dairy; or DHSM attributed to PA/EPlas	Resolve at adequate field, or apportion by ^{13}C at C3 (73.4 vs 70.7 ppm)
PS unresolved from SM	SM overstated by the PS content	Demonstrate chelator excess; acquire a temperature series; adjust pH in micellar media
Continuous proton decoupling	Class-dependent NOE distortion of integrals	Inverse-gated decoupling
Relaxation delay $< 5 \cdot T_1$	Differential saturation; systematic class bias	Measure T_1 by inversion recovery; set delay accordingly, or use a calculated reduced flip angle
Shifts taken from published tables	Misassignment when conditions differ	Internal standard, spiking, 2D ^{1}H–^{31}P correlation
Mass reported without stated MW assumption	Systematic bias up to ~15%	Report as moles, or state the assumed average molecular weight
Extraction recovery assumed	SM lost preferentially to the aqueous phase	Spike authentic SM through the whole procedure
Benchtop resolution overstated	SM–PS and SM–DHSM claims unsupportable	Apply the arithmetic of Table 4; confirm the profile at higher field
Closed compositional data modeled directly	Spurious inter-class correlation	Log-ratio transform before multivariate modeling
Internally validated chemometric model	Optimistic error rate	Validate on independent held-out material

15 References

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16 Summary

Sphingomyelin analysis by NMR is a problem in which the quantitative and the structural burdens fall on different nuclei, and methods fail when one nucleus is asked to carry both.

Phosphorus-31 provides the quantification. Its single phosphorus per molecule makes the integral a direct mole census requiring no response factor, no calibration for chain length and no chromatographic recovery correction, and the technique has demonstrated inter-laboratory reproducibility of approximately 2–3% for a dominant, well-resolved class and approximately 3–12% for minor classes.[26] What ^{31}P does not provide is identification. The whole phospholipid class dispersion is compressed into roughly 1.15 ppm; sphingomyelin sits 0.04 ppm from phosphatidylserine, 0.22 ppm from dihydrosphingomyelin and 0.75 ppm from phosphatidylcholine, and those positions migrate with solvent, water content, chelator, counter-cation, temperature, pH and concentration. Resolution is won principally by chemistry — chelation, solvent selection, temperature and pH manipulation — and only secondarily by magnetic field, which is the central practical finding of the multiparametric optimization literature.[16,17]

Proton NMR provides identification and confirmation. The sphingosine C4–C5 olefinic pair near 5.4–5.8 ppm with its *trans* coupling near 15 Hz, the exchangeable amide NH downfield of 6 ppm, and the absence of the glyceryl multiplets that every diacyl glycerophospholipid must display, together establish that a material is a sphingomyelin rather than a phosphatidylcholine. What proton NMR does not provide is quantification in a mixture: the trimethylammonium singlet near 3.2 ppm is shared by every choline-bearing class and is not a sphingomyelin signal, and any protocol quantifying sphingomyelin from it in an unfractionated extract is wrong by the amount of phosphatidylcholine present.

Carbon-13 resolves the distinction the other two nuclei find hardest. The C3 resonance at 73.4 ppm in sphingomyelin against 70.7 ppm in dihydrosphingomyelin[50] separates the two species by an interval more than an order of magnitude more comfortable, in relative terms, than the corresponding ^{31}P separation, and it does so without any manipulation of the sample medium. Given that dihydrosphingomyelin has been reported at approximately 20% of bovine milk sphingolipids,[33] this is a determination that a laboratory offering dairy sphingomyelin analysis should have performed at least once for each material type it handles. The cost is sensitivity and time, which confines ^{13}C to isolates and concentrates and makes two-dimensional ^{1}H–^{13}C correlation the more efficient choice in most circumstances.

The resulting workflow is sequential rather than parallel: quantify by ^{31}P; confirm the assignment by two-dimensional ^{1}H–^{31}P correlation, which transfers the identification burden to the better-dispersed and less condition-sensitive proton dimension;[23] confirm the sphingoid base by ^{1}H; apportion sphingomyelin against dihydrosphingomyelin by ^{13}C where the matrix demands it; and verify by spiking or by selective enzymatic cleavage. The first two stages constitute the minimum defensible method for an unfamiliar matrix.

On instrumentation, the arithmetic is decisive and is set out in Table 4. Because the ^{31}P observation frequency is 40.48% of the proton frequency, a 60 MHz benchtop system separates sphingomyelin from phosphatidylcholine by approximately 18 Hz — comfortably resolvable against the 2–3 Hz linewidths attainable in a well-chelated medium, and sufficient for the dairy, infant formula and lecithin determinations that constitute the bulk of commercial demand. The same instrument separates sphingomyelin from dihydrosphingomyelin by approximately 5 Hz, which is marginal and not a basis for a specification, and from phosphatidylserine by approximately 1 Hz, which is unattainable. Benchtop ^{31}P is therefore a legitimate quantitative tool for a defined determination in a controlled matrix and is not a general-purpose phospholipid

profiler; the sound practice is to establish the full profile and the assignment at mid or high field and to transfer only the demonstrated determination to the benchtop for routine throughput.

Finally, two limitations should appear in every report rather than being left for a reviewer to raise. NMR determines phospholipid classes, not molecular species, so a sphingomyelin value is a class total and any claim about acyl chain or sphingoid base distribution requires chromatographic or mass spectrometric support.[32,33,40] And where a result is expressed as mass rather than as amount of substance, the assumed molecular weight must be stated, because the spread across naturally occurring sphingomyelin species is approximately 15% and the choice of average silently determines the answer.