Principles of multiparametric optimization for phospholipidomics by ^{31}P NMR spectroscopy

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Abstract Phospholipids have long been known to be the principal constituents of the bilayer matrix of cell membranes. While the main function of cell membranes is to provide physical separation between intracellular and extracellular compartments, further biological and biochemical functions for phospholipids have been identified more recently, notably in cell signaling, cell recognition and cell–cell interaction, but also in cell growth, electrical insulation of neurons and many other processes. Therefore, accurate and efficient determination of tissue phospholipid composition is essential for our understanding of biological tissue function. ^{31}P NMR spectroscopy is a quantitative and fast method for analyzing phospholipid extracts from biological samples without prior separation. However, the number of phospholipid classes and subclasses that can be quantified separately and reliably in ^{31}P NMR spectra of tissue extracts is critically dependent on a variety of experimental conditions. Until recently, little attention has been paid to the optimization of phospholipid ^{31}P NMR spectra. This review surveys the basic physicochemical properties that determine the quality of phospholipid spectra, and describes an optimization strategy based on this assessment. Notably, the following experimental parameters need to be controlled for systematic optimization: (1) extract concentration, (2) concentration of chelating agent, (3) pH value of the aqueous component of the solvent system, and (4) temperature of the NMR measurement. We conclude that a multiparametric optimization approach is crucial to obtaining highly predictable and reproducible ^{31}P NMR spectra of phospholipids.

Keywords Phospholipidomics · Tissue extracts · Phospholipid quantification · ^{31}P NMR spectroscopy · Multiparametric optimization · Spectral resolution

Introduction

Phospholipids (PLs) are polar lipids that occur in a wide range of biological tissues, but are also found in many food sources, notably eggs, and in plants. PLs form the matrix of human and animal cell membranes. The main function of cell membranes is mechanical. PL bilayers provide membranes with the appropriate balance of mechanical stability for physical separation between intracellular and extracellular compartments on the one hand, and sufficient fluidity and permeability for interactions between cells and organelles on the other. These interactions also include exchange of molecules. Moreover, neutral lipids, proteins, glycoproteins and glycolipids are imbedded in membrane PLs, e.g., cholesterol, sphingolipids, enzymes, transporters, receptors, and cell recognition proteins. PL multilayers are crucial components of myelin which provides electrical insulation in the nervous system, in particular in the brain. In cells and in biofluids such as blood and milk, PLs stabilize lipid droplets by coating these with a monolayer, thus forming stable oil-in-water emulsions. Besides these physicochemical features, PLs have many biological and biochemical functions (London and Feigenson 1979). Most importantly, they are a rich source of cell signaling molecules (Tome et al. 2003), and play an important role in cell recognition and cell–cell interaction.

Many diseases are associated with aberrations of membrane PL composition and metabolism (Aoki et al. 2008). The chemical PL composition of membrane monolayers, bilayers and multilayers affects their physical, chemical and biological properties. Therefore, analysis of PL profiles

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is used in basic research, but has also been proposed for differential diagnosis, e.g., for characterizing tumors and other diseases (Fuchs et al. 2005; Merchant et al. 1999; Solivera et al. 2009; Belouèche-Babari et al. 2010; Delikatny et al. 2002; Glunde et al. 2006; Lutz and Cozzone 2010a, b). Several methods are available for PL profile measurements:

- thin layer chromatography (TLC), for fast qualitative identification of the PL classes present in a sample (Peterson and Cummings 2006). Analysis is performed against appropriate standards.
- high-performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD), for separation and quantification of PLs (Ibrahim et al. 2010; Deschamps et al. 2001). Extensive quantitative calibration with multiple standards.
- wet-chemistry methods, for measuring specific entities associated with PLs. Quantitative analysis: elemental phosphorus by ultraviolet/visible (UV/VIS) spectrophotometry, and water content by Karl Fischer titration. Semi-quantitative analysis (Avantlipids <http://www.avantilipids.com>).
- Fourier transform infrared (FT-IR) spectroscopy, for qualitative analysis of functional groups (Gómez-Fernández and Villalán 1998).
- mass spectrometry (MS), notably with electrospray ionization (ESI-MS). Quantitative and extremely sensitive (Cui and Thomas 2009); can provide structural information when used as a tandem technique (MS-MS). Extensive quantitative calibration with multiple standards.
- nuclear magnetic resonance spectroscopy (NMR) spectroscopy, for fast and reliable quantitation of PL classes and sub-classes from crude mixtures without separation. Efficient analysis at moderate sensitivity by phosphorus-31 (^{31}P) NMR spectroscopy (Lutz and Cozzone 2010a, b). Simple quantitative calibration with a single standard compound.

Although significantly less sensitive and not appropriate for identification of individual molecular PL species, NMR spectroscopy is more quantitative (under suitable experimental conditions) and requires less sample preparation than more intricate methods such as MS. In addition, NMR is non-destructive and *intrinsically* quantitative (Günther 1995; Sanders and Hunter 1993). For commonly used NMR samples, the area under a specific signal is strictly proportional to the number of nuclei generating this signal. Consequently, there is a linear relationship between signal intensity and the amount of the corresponding chemical compound. In addition, the sensitivity of the signal detection system does not depend on the molecule in question. For these reasons, NMR signal intensities can be used to directly

determine molar quantities in mixtures by way of a single standard compound, if the experimental conditions are chosen properly.

Although ^{31}P NMR spectroscopy is well suited for comprehensive PL analysis based on crude tissue extracts (aka *phospholipidomics*; Lutz 2005), widespread application was hampered for many years by a lack of reproducibility and robustness. More recently, it has been shown that this method can be employed as a very reliable tool for studying PL classes and subclasses provided that all relevant experimental parameters are optimized (Lutz and Cozzone 2010a, b, 2013). Here, we review (1) the physicochemical foundations underlying multiparametric optimization of PL profile analysis by ^{31}P NMR spectroscopy, and (2) practical aspects of this optimization method.

Molecular basis of phospholipid profile analysis by ^{31}P NMR spectroscopy

Identification of PL classes and subclasses based on molecular structure

The most basic prerequisite for quantifying individual PL classes and subclasses by ^{31}P NMR spectroscopy is, apart from sufficient signal strength, an adequate separation (resolution) of the corresponding NMR signals. Several factors influence the spectral resolution: molecular structure and mobility of the PLs to be analyzed, the microscopic (intermolecular) environment of the observed molecule, and the experimental conditions chosen. The role of molecular structure will be discussed in this paragraph. In ^{31}P NMR spectra, PLs are generally distinguishable based on differences in the molecular environment of the observed nucleus (^{31}P , representing virtually 100 % of the phosphorus nuclei in the phosphate groups). In almost all PLs, the phosphate moiety is esterified both to the PL backbone and to an alcohol in the polar head. The PL backbone is further linked to one or two lipid moieties. The chemical environment of ^{31}P in PLs is determined by the polar head and the PL backbone, and by chemical bonds right close to backbone carbons, e.g., glycerol C-1 and C-2 for glycerophospholipids such as phosphatidylcholine (PtdC). Variations in any of these molecular environments can shift the position of the ^{31}P signal.

The position of an NMR signal of a particular compound relative to that of a reference is referred to as chemical shift, δ , measured in parts per million (ppm). Phosphodiester ^{31}P NMR resonances lie in the high-field region of the spectrum (range ca. 1.5–2.5 ppm), while phosphomonoester resonances occur at lower fields (2.5–6.5 ppm), as shown in a typical PL ^{31}P NMR spectrum of a rat brain extract (Fig. 1, top right). In general, the PL backbone is glycerol,

but sphingomyelin (SM) is an important exception (sphingosine backbone). This difference in backbones results in distinct ^{31}P NMR signals for SM and PtdC, although both PL have identical choline-containing polar heads. In the polar head of a phosphodiester PL, choline may be substituted with ethanolamine (in phosphatidylethanolamine, PtdE), *myo*-inositol (in phosphatidylinositol, PtdI), serine (in phosphatidylserine, PtdS), glycerol (in phosphatidylglycerol, PtdG), or with a glycerol bridge linked to a second phosphatidic acid moiety (in cardiolipin, CL). These substitutions result in downfield shifts relative to the PtdC signal by 0.1 to 1.4 ppm. Phosphatidic acid (PtdA) is a phosphomonoester PL; the phosphate moiety is only esterified with the glycerol backbone and produces a ^{31}P NMR peak in the low-field region of the spectrum (range 2.7–3.4 ppm). Phosphatidylinositol 4-phosphate (PtdIP or PI(4)P) and phosphatidylinositol 4,5-diphosphate (PtdIP₂ or PI(4,5)P₂) have one or two phosphate moieties, respectively, monoesterified with the inositol moiety, in addition to the phosphate moiety diesterified with the glycerol backbone and the inositol moiety. These additional phosphate groups generate one and two low-field ^{31}P NMR resonances, respectively, in addition to a high-field peak from the diesterified phosphate group. The chemical shifts of these low-field peaks range between 3.1 and 6.3 ppm, and are very sensitive to experimental conditions, because complex-forming cations have relatively free access to the monoesterified phosphate residues. In the following, observed phosphorus nuclei will be underlined in abbreviations when necessary to distinguish between monoester and diester phosphate $^{31}\mathbf{P}$: PtdIP or PtdIP; and PtdIP₂ or PtdIP₂.

The absence of a fatty-acid moieties (FA) at the *sn*-1 or *sn*-2 position of the glycerol backbone results in a significant downfield shift of the ^{31}P NMR signal when compared to the fully acylated phospholipid (distinct signals for 1- and 2-lysophosphatidylcholine). Moreover, replacing one of the acyl bonds (= ester bonds) with an O-alkyl bond (= ether bond) results in smaller (0.05–0.1 ppm) but characteristic downfield shifts, e.g., for 1-alkyl-2-acyl-phosphatidylcholine, AAPtdC, versus PtdC (Fig. 1, bottom right), and for 1-alkyl-2-acyl-phosphatidylethanolamine, AAPtdE, versus PtdE (Fig. 1, bottom left). In ether-linked alkenes, a double bond exists next to the ether link at the *sn*-1 position. These PLs are vinyl ether lipids known as plasmalogens (e.g., ethanolamine plasmalogen, PtdE_{plasm}), and generate ^{31}P NMR signals that lie between those of the corresponding diacylated and alkyl-acylphospholipids (e.g., between PtdE and AAPtdE; Fig. 1, bottom left).

The influence of molecular mobility on the resolution of PL ^{31}P spectra

Besides molecular structure, molecular mobility also affects the capability of ^{31}P NMR spectroscopy to show distinguishable

signals for different PL classes and subclasses. This is due to the effects of molecular mobility on NMR linewidth (LW). LW affects the signal-to-noise ratio (SNR) of NMR signals, and both LWs and chemical shifts of NMR resonances determine the resolution of individual peaks in a given spectrum. Optimal peak separation, in turn, is required for reliable PL identification and quantification. The separation of two adjacent signals whose peak maxima are located at a given distance, $\Delta\delta$, increases with decreasing LW, because of decreasing signal overlap. LW is usually measured at half maximum intensity ($\nu_{1/2}$ = full width at half height, FWHH), and is directly related to processes determining the loss of coherence of magnetization following an excitation pulse (Farrar and Becker 1971). Apart from magnetic-field inhomogeneity, sample properties such as molecular mobility and exchange phenomena affect observed LW (Farrar and Becker 1971). The LW contribution originating from molecular processes is termed natural linewidth. The link between molecular motions and natural linewidth, LW_n, is established through so-called spin–spin relaxation mechanisms (processes reducing the spin–spin relaxation time, T₂), where T₂ ∝ 1/LW_n.

Both high and low-frequency motions may cause T₂ relaxation, i.e. contribute to a decrease in T₂. The motional frequencies and their intensities are determined in correlation functions, also known as spectral density functions. The latter relate reorientational or translational molecular motions to the relaxation time, T₂ (but also to the spin–lattice relaxation time, T₁, which is discussed below). PLs located in membranes have very limited, anisotropic mobility, and thus result in lines far too broad for analysis of individual PLs. For this reason, all ^{31}P NMR spectroscopic methods for PL analysis suggested in the literature are based on appropriate mobilization of membrane PLs, be it in an aqueous environment (by using detergents) or in organic media (by using solvent molecules). These and other factors pertaining to molecular aggregation and the intermolecular environment strongly depend on the solvent system used. Besides the rotational and translational molecular motions mentioned above, fast and intermediate chemical exchange at the phosphate moiety can also induce relaxation. Exchange involves solvent protons that form hydrogen bonds with phosphate oxygen, and cations that form complexes with the PL phosphate group.

Tissue extract solvents for PL analysis

Detergent-containing solvents

Membrane PLs need to be solubilized to produce sufficient resolution in ^{31}P NMR spectra for the measurement of individual PL classes and subclasses. However, it is noteworthy that complete dissolution into individual PL

Fig. 1 Principle of the one-phase and two-phase phospholipid systems used in ^{31}P NMR analysis (*top left*), and regions of a typical PL spectrum of a rat brain extract (*top right* and *bottom*; based on a one-phase sample). The two-phase system hampers correct PL quantitation because most of the upper phase is located outside the sensitive volume of the NMR coil. *Top right*: Complete range of the ^{31}P NMR PL spectrum. For better visibility of weak signals (PtdIP, PtdG), exponential line broadening (LB = 3 Hz) was applied. In this representation, several PL signals are not well resolved, notably in the PtdC and PtdE regions. For PLs generating more than one ^{31}P NMR signal, observed nuclei are *underlined* (PtdIP, PtdIP₂, PtdIP₂, PtdIP₂). Currently unassigned signals are denoted by Un (where $n=1, 2, \dots$). *Bottom left* PtdE

and PtdS regions of the same spectrum. For better peak resolution, Lorentzian-Gaussian line shape transformation was applied (LB = −1 Hz, GB = 0.3). Because of these processing parameters, many very weak PL signals are difficult to detect. However, at least two peaks can be discerned for each PtdE, PtdE_{plasm}, AAPtdE, and PtdS. *Bottom right* PtdC region obtained with the same processing parameters as the PtdE region. Several signals at the base of the dominating PtdC resonance were detected unambiguously, while they cannot be discerned in the *upper right panel* (spectrum generated with exponential line broadening). PtdC1u is a currently unassigned PtdC analog. (From *Anal. Chem.* 2010, 82, 5433–5440. Copyright 2010 American Chemical Society. Reprinted with permission)

molecules is not required; under appropriate conditions, good spectral resolution can be achieved with small micelles. Since the 1970s, an admixture of detergents such as cholate or Triton X-100 to aqueous PL suspensions has been used to generate small mixed micelles that ensure narrower spectral linewidths than would be achieved in the absence of detergents (London and Feigenson 1979). This is due to the fact that in the absence of detergents, PLs spontaneously form vesicles in water, resulting in relatively immobile PLs. Thus, vesicle-to-micelle transformation is essential to PL solubilization by detergents (Lopez et al. 1998; Muller et al. 2004; Puppato et al. 2007; Schiller et al. 2001; Pearce and Komoroski 1993; Fuchs et al. 2007a, b; Sullentrop et al. 2002; Jakop et al. 2009; Renner et al. 2005; Dannenberger et al. 2010; Bauer et al. 2009). A number of arguments speak in favor of PL solubilization by detergents, e.g., the possibility to prepare samples by directly dissolving membranes or biological tissue without the need for extraction, and to achieve narrow LWs even at high PL concentrations (London and Feigenson 1979). However, for good signal resolution, initial extraction with organic solvents is

needed before dissolving the extracted lipids in a detergent solution (Schiller and Arnold 2002). In addition, there are two major disadvantages of the detergent method that are frequently mentioned in the literature: (1) samples cannot be used for ^1H or ^{13}C NMR spectroscopy: for the detergent to be effective, it must be present at concentrations exceeding those of the PLs, thus “contaminating” large spectral regions with its ^1H or ^{13}C resonances; and (2) recovery of PLs for further experiments is virtually impossible after mixing with detergents. In view of these disadvantages, detergent-free tissue extracts based on organic solvents are generally preferred.

Organic solvent systems

Almost all published examples are based on ternary solvent systems consisting of chloroform, methanol and an aqueous solution. However, the relative proportions of these solvents vary significantly. As a consequence, some of these solvent mixtures form two phases, while others form one single phase when the solvents are mixed. To produce a one-

phase, mostly chloroform-containing, solvent that also dissolves significant amounts of water (up to 10 vol.%), the methanol content must be almost 40 vol.%. The physicochemical conditions for the transition from one-phase to two-phase mixtures in the ternary system chloroform/methanol/water have been determined by way of a phase diagram based on molar fractions (Branca et al. 1995). The good spectral resolution that can be obtained from the chloroform-containing phase of certain two-phase extract preparations has led many researchers to opt for this method, despite issues concerning the precision of PL quantitation (Lutz and Cozzone 2010a, b, 2013). In any event, the solvent composition of the chloroform-containing phase in the widely used two-phase system was found to be very similar to the composition of an optimized one-phase system, and lies near the “tie line” in the ternary phase diagram (Branca et al. 1995; Metz and Dunphy 1996). Since the optimal solvent composition is temperature-dependent, a methanol concentration slightly above the tie-line value (40 vol.%) guarantees homogeneous one-phase extracts over the temperature range of interest for optimization (Lutz and Cozzone 2010a, b). Further optimization of ^{31}P NMR PL analysis is described below for such a one-phase system; examples of two-phase applications have been summarized previously (Lutz and Cozzone 2010a, b, 2013).

Optimization strategy

Role of experimental parameters in optimization of PL ^{31}P NMR spectra

Multiple instrumental factors (e.g., magnetic field strength and spectroscopic sequences available) and other experimental parameters influence SNR, linewidth, and absolute or relative peak positions in ^{31}P NMR spectra (Lutz and Cozzone 2010a, b, 2013; Günther 1995; Sanders and Hunter 1993). These parameters are best optimized together once the solvent system has been chosen (see preceding paragraphs). Subsequently, data processing parameters are optimized (Lutz and Cozzone 2010a, b, 2013). Two categories of experimental parameters can be distinguished: (1) the chemical composition of the NMR sample (besides the solvent composition discussed above), and (2) the physical conditions of the NMR measurement. Among the latter, sample temperature is of particular importance because it affects chemical shift and linewidth that directly determine the separation of NMR peaks (Fig. 2, right panels).

The composition of a chemical solution is primarily defined by the concentrations of its dissolved components. In a one-phase chloroform-d/methanol/water (50:40:10 vol.%) solvent system, these components are extracted lipids and added chelating agent (trans-1,2-cyclohexyldiaminetetraacetic

acid, CDTA) (Lutz and Cozzone 2010a, b, 2013). In addition, the physicochemical characteristics of the sample include the pH value of the aqueous component of the solvent mixture. Since the acid form of CDTA is used to prepare the aqueous component, the pH needs to be adjusted by addition of a base (CsOH). The effects on chemical shift and linewidth caused by altering pH, lipid extract concentration, CDTA concentration and sample temperature are not necessarily additive (Lutz and Cozzone 2010a, b). Therefore, all four parameters should be optimized together (Fig. 2). In the following paragraphs, we provide an overview of the chemical-shift and linewidth changes produced by each of these parameters, as a function of the remaining parameters. This information is essential for efficient and successful optimization of PL analysis by ^{31}P NMR spectroscopy. The numerical parameter values given in the following sections refer to databases included in Supplementary Informations of previously published papers (Lutz and Cozzone 2010a, b). These empirical values are based on an analysis of rat brain extracts, but are also useful as baseline data for the optimization of PL analysis of other tissues.

Effects of temperature on chemical shift and linewidth of PL signals

The sample temperature chosen for PL analysis should be sufficiently high to ensure complete lipid solubility, and sufficiently low to avoid significant evaporation of organic solvents during the spectrum acquisition which takes at least 5–6 h if low-concentration PLs are to be quantified. Thus, the optimal temperature ranges from 277 to 297 K. PtdC chemical shifts increase linearly with temperature (Fig. 3, top left, filled circles), and slopes are not correlated with pH, extract concentration, or CDTA concentration (Lutz and Cozzone 2010a). Chemical shifts of other PLs by and large follow the trends exhibited by PtdC. However, in some cases, chemical shifts have a tendency to level off at higher temperatures. These measurement points are often fitted somewhat better by logarithmic curves or second-order polynomials than they are by straight lines, as shown for SM (Fig. 3, right, “+” symbols). The temperature sensitivity of chemical shifts varies as a function of the PL investigated, notably as a function of their head groups. These differences in temperature sensitivity result in changes in relative peak positions over a given temperature range. Consequently, signals that are relatively distant from each other at one temperature, may approach or even overlap at a different temperature (see also Fig. 2). Even the order in which peaks occur in the spectrum changes with temperature for some signals; the crossing temperature depends on the extract and CDTA concentrations, as well as on pH. For SM and PtdE of brain extracts, the lowest crossing temperature is ca. 277 K (Fig. 3, right), and the highest is ca. 287 K (Lutz and Cozzone 2010a).

Fig. 2 Effects of extract and CDTA concentration, temperature and pH on the resolution of ^{31}P NMR PL spectra of rat brain extracts. *Top left* Extract concentration, 236 mg/mL; CDTA concentration and pH in the aqueous component of the solvent, 200 mM and 7.33, respectively; measurement temperature, 297 K. The PtdE_{plasm} and SM signals are well-resolved. *Bottom left* Extract concentration, 236 mg/mL; CDTA concentration and pH in the aqueous component of the solvent, 1,000 mM and 7.36, respectively; measurement temperature, 297 K. The PtdE_{plasm} and SM signals overlap entirely; they cannot be resolved although the increased CDTA concentration resulted in reduced linewidths compared with the top left spectrum. *Top right* Extract

concentration, 118 mg/mL; CDTA concentration and pH in the aqueous component of the solvent, 50 mM and 7.14, respectively; measurement temperature, 297 K. The PtdE and SM signals are well-resolved. *Bottom right* Extract concentration, 118 mg/mL; CDTA concentration and pH in the aqueous component of the solvent, 50 mM and 7.14, respectively; measurement temperature, 277 K. The PtdE and SM signals overlap entirely; they cannot be resolved although the decrease in temperature resulted in reduced linewidths compared with the top right spectrum. (From *Anal. Chem.* 2010, 82, 5433–5440. Copyright 2010 American Chemical Society. Reprinted with permission)

Apart from PtdC, most PL resonances partially overlap, or are too weak for precise LW measurements in ^{31}P NMR spectra. Therefore, LW effects can be best analyzed by studying PtdC. PtdC linewidths increase with temperature for different extract and CDTA concentrations and for different pH values (Fig. 4). PtdC linewidths for samples of different extract concentrations converge to low values as the sample temperature decreases (Fig. 4). The linear correlation coefficients, r , for the temperature dependence of LW are close to unity.

Effects of extract concentration on chemical shift and linewidth of PL signals

The extract concentration chosen for PL analysis should be sufficiently high to allow the detection all PLs of interest, without the need to resort to excessively long measurement times to lower the detection threshold. The upper concentration limit is given by the PL solubility in the solvent mixture, but also by the formation of PL aggregates that may significantly reduce spectral resolution through line broadening, and by the resulting salt concentration that

may significantly reduce the sensitivity of the NMR experiment. The optimal concentration range is obtained by extracting between 100 and 1,000 mg tissue, and by dissolving the lipid extract in 1 mL solvent mixture. Generally, PtdC chemical shifts decrease moderately with increasing extract concentration in an approximately linear fashion. For all combinations of pH and CDTA concentration, plots of chemical-shift decreases for different temperatures result in almost equidistant lines (Lutz and Cozzzone 2010a), which reflects the linear temperature dependence described in the previous paragraph. The dependence of chemical shift on extract concentration is less pronounced for low sample temperatures and high CDTA concentrations than it is for high sample temperatures and low CDTA concentrations.

The PtdC linewidth is positively correlated with extract concentration. There is a linear correlation for samples that contain 100–1,000 mM CDTA (linear correlation coefficients, r , close to unity); for lower CDTA concentrations a sigmoid curve is obtained (Lutz and Cozzzone 2010b). For all measurement temperatures, LWs tend to converge to low values at low extract concentrations (lower limit for LW $\approx$ 1 Hz).

Fig. 3 ^{31}P NMR chemical shifts of PL peaks from rat brain extracts, as a function of measurement temperature (individual series measured for five different temperatures). In this figure, shortened abbreviations are used due to space restrictions: *PC* PtdC (phosphatidylcholine); *PCpl* PtdC_{plasm} (choline plasmalogen); *AAPC* AAPtdC (alkyl-acyl-phosphatidylcholine); *PIP₂* PtdIP₂ (phosphatidylinositol diphosphate); *PI* PtdI (phosphatidylinositol); *PS* PtdS (phosphatidylserine); *PE* PtdE (phosphatidylethanolamine); *PEpl* PtdE_{plasm} (phosphatidylethanolamine plasmalogen); *AAPE* AAPtdE (alkyl-acyl-phosphatidylethanolamine); *PA* PtdA (phosphatidic acid); *PG* PtdG (phosphatidylglycerol).

Effects of CDTA concentration on chemical shift and linewidth of PL signals

The CDTA concentration chosen for PL analysis should be sufficiently high to significantly reduce LWs for satisfactory separation of all PL peaks of interest. The upper CDTA concentration limit is given by the CDTA solubility in the solvent mixture, but also by the salt concentration that may significantly reduce the sensitivity of the NMR experiment. The chemical-shift ranges for all temperatures taken together vary as a function of CDTA concentration: within narrow limits (0.15 ppm) for choline-containing PLs; within wider limits (0.3 ppm) for individual ethanolamine-containing PLs and *PtdIP₂*; and within still wider limits (0.4–0.5 ppm) for further PLs containing moieties other than choline or ethanolamine in the polar head (Lutz and Cozzone 2010a).

Currently unassigned peaks are denoted by *Un* (where *n*=1, 2, 3 ...). Peaks that are not currently identified but most probably represent analogs of phosphatidylcholine are denoted by *PC1u* and *PC2u*. Less-confident assignments are followed by question marks (e.g. *lyPC?* indicates a possible lyso-phosphatidylcholine peak). Conditions: extract concentration, 236 mg brain tissue per mL solvent; CDTA concentration, 50 mM; pH 7.14 in the aqueous component of the extract solvent used. (From *Anal. Chem.* 2010, 82, 5433–5440. Copyright 2010 American Chemical Society. Reprinted with permission)

Adding up to 200 mM CDTA to a one-phase PL sample causes a major drop in the PtdC linewidth, but a further increase to 1,000 mM has much less effect. This indicates that virtually all paramagnetic ions may be masked at 200 mM CDTA so that further CDTA addition has little effect on LWs. At low temperatures, CDTA has much less effect on LW than at high temperatures.

Effects of pH on chemical shift and linewidth of PL signals

Effects of pH changes on the ^{31}P NMR chemical shifts of PtdC and analogs are rather small within the limited sample pH range recommended. Values much larger or smaller than pH 7 should be avoided as certain PLs may hydrolyze. The pH dependence of PL chemical shifts is complex and varies as a function of the PL class in question, but also of extract

Fig. 4 PtdC linewidth as a function of measurement temperature, obtained by ^{31}P NMR spectroscopy of rat brain tissue extracts at 162 MHz (9.4 T). Each diagram shows the data obtained for a particular extract preparation protocol. Linewidths obtained for different concentrations (by serial dilution) are color-coded. Lines tend to be particularly narrow for low extract concentrations and low temperatures, notably at high CDTA concentrations in the aqueous component of the solvent. Linewidths below 1 Hz are only observed for 1,000 mM CDTA, and for 200 mM CDTA at pH 7.33. Temperature-dependent variations increase with extract concentrations, and are largest for samples with the lowest CDTA concentrations. (From *Anal. Chem.* 2010, 82, 5441–5446. Copyright 2010 American Chemical Society. Reprinted with permission)

and CDTA concentrations. Generally, monoesterified phosphate moieties are characterized by larger changes in ^{31}P chemical shift than diesterified phosphate moieties (Lutz and Cozzone 2010a). Also, the pH effects on PtdC linewidths are rather complex and considerably smaller than the effects of temperature, extract concentration or CDTA concentration.

Criteria-based approach to optimizing PL analysis

Rationale

Before designing an optimization strategy, it is indispensable to define the criteria according to which optimization is to be performed. The optimal choice of parameters does not only depend on the sample type but also on the information needed. For instance, if specific PLs are of particular interest, experimental parameters can be optimized to achieve the best signal separation for the relevant spectral regions. In addition, the variation of an experimental parameter often changes linewidths and chemical shifts simultaneously.

Therefore, parameters have to be optimized for both effects *together*. In many cases, a compromise between minimizing linewidths and maximizing chemical-shift differences between peaks needs to be found for best peak separation. The crucial factors to be considered for optimization will be presented in this section.

Speed versus precision

If a quick analysis of the most abundant PLs is desired, without the need for precise absolute quantities, relatively high sample concentrations that allow for short data acquisition times are adequate. By contrast, if accurate, absolute quantitation of a large number of PLs (even the less abundant) is required, longer acquisition times for less concentrated extracts are preferable. In particular, longitudinal relaxation times, T_1 , have to be considered for optimizing the data acquisition time. If simple and accurate PL quantitation is a priority, TR should be $>5 \times T_1$. In this way, virtually complete relaxation is achieved between individual transients acquired for signal averaging, thus avoiding the

problem of partial saturation of magnetization. Under these conditions, the area under a peak is strictly proportional to the concentration of the underlying compound and to the number of spins represented, irrespective of the molecular structure of this compound (as long as molecules are mobile). However, if short measurement times are a priority, T_1 should be considerably reduced. Subsequently, the area under each peak has to be multiplied by an appropriate correction factor for accurate PL quantitation.

Choice of optimal sample composition and temperature

As detailed above, a solvent mixture consisting of 50 vol.% CDCl_3 , 40 vol.% CH_3OH and 10 vol.% of an aqueous solution is useful for many applications. This well-defined, robust mixture forms a single homogeneous phase over a wide range of temperatures, pH values, extract and CDTA extractions (Lutz and Cozzone 2010a). This system permits more reliable and efficient PL quantification than two-phase systems, and it provides high spectral resolution as well as sufficient sensitivity for the quantitation of less-abundant PLs. However, in cases where best signal separation is of much higher priority than efficient and precise quantitation, the use of a higher percentage of chloroform-d in the solvent mixture may be attempted, although this easily leads to phase separation in the sample. If this is the case, the volume of the lower phase must be determined to obtain absolute PL amounts (mmol or mg).

High extract and low CDTA concentrations should generally be avoided because they result in very broad PL lines. A CDTA concentration of 200 mM in the aqueous component of the solvent mixture is generally sufficient. Increasing the CDTA concentration to 1,000 mM results in slightly narrower lines, but also in more overlap for some signals. Signal resolution also depends, to a minor extent, on the pH of the aqueous component of the solvent mixture. Particular attention should be paid to the measurement temperature. The temperature can easily be adapted to minimize overlap for peaks of interest; in particular, crossing points of temperature-dependent curves should be avoided. The temperature at which curves cross also depends on the choice of the other experimental parameters (extract and CDTA concentrations, pH). The parameters given above may serve as a starting point for optimization. Further details and examples for optimization protocols have been described elsewhere (Lutz and Cozzone 2010a, b).

Summary

The linear response, high resolution and relative speed of ^{31}P NMR spectroscopy allows for accurate and selective analysis of many phospholipid classes and subclasses

(phospholipidomics). Highly reproducible spectra can be obtained, provided that the experimental conditions are well defined and optimized. The optimization strategy to be employed must consider the effects of all experimental parameters involved, some of these not being additive. Ultimately, the objectives of the underlying application must define the criteria for optimization, i.e. the desired balance of spectral resolution, sensitivity, precision of metabolite quantification, speed, and other factors. Recent developments, also including a comprehensive database (Lutz and Cozzone 2010a, b), greatly facilitate the optimization process. As a consequence, the analysis of phospholipid profiles from tissue extracts can be expected to gain increasing popularity, notably in the field of metabolomics.

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Conflicts of interest None

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