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Phase Behavior of Polar Porcine Brain Lipid Extract Using Multinuclear Solid-State NMR

by

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Abstract

Polar lipid extract, often sourced from brain matter, are commonly used in permeability assays. The phase behavior of their constituent phospholipids have long been widely studied at various levels of hydration, both in isolation and in prepared mixtures with other lipids commonly present in extracts, such as cholesterol. In this study, the phase behavior of whole porcine polar brain lipid extract evaluated using ^{13}C -, ^{31}P - and ^1H -MAS NMR as well as static ^{31}P - and ^2H -NMR, the latter enabled by D_2O addition. Focus was put on phase behavior evaluation using comparative signal enhancements from ^{13}C DP, CP and INEPT techniques, a procedure called dynamics based spectral editing due to the techniques' different dependences on fatty acid chain segment reorientation time and C-H bond angles. By supporting results from dynamics based spectral editing with results from the long-established static NMR-experiments, it was found that dynamics based spectral editing alone can distinguish four phases in porcine polar brain lipid extract. A phase map spanning 264.0-348.3 K and 5-90% water content by total molar water content was established for the whole extract using the primary apparent phases at each temperature and water content. The main known limitation of the phase map is the presence of unresolved NMR information in the results. This information may pertain to individual phase behaviors of the constituent phospholipids in the extract. Recoupling NMR experiments are recommended for future work to obtain phospholipid-by-phospholipid phase behavior information.

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Structural information about the fats that make up the blood-brain barrier via the magnetism of atomic cores

Screening for drugs that can pass the blood-brain barrier prompts interest in the structure of the fatty material that builds it. That information can be found using intricately designed magnets.

The brain is largely made up of fat. Specifically, much of the fat is cholesterol and phospholipids. These are molecules that mostly contain atoms that can be analyzed magnetically using Nuclear Magnetic Resonance (NMR) spectroscopy (hydrogen, carbon and phosphorous specifically). This study aims to tell if NMR applied only to carbon can be used to reveal the structure of fats. Fats notoriously have several solid, liquid and semi-solid forms rather than the usual trio of solid, liquid and gas.

NMR relies on the precessing (spinning-at-an-angle) motion that the magnetic moments of atomic cores perform when they return to magnetic equilibrium after exposure to a resonant magnetic field. The spinning happens at a frequency specific to the set of atomic bonds that the atom finds itself in. With NMR, molecules are an enormous cohort of hobby analog radio broadcasters to tune into. Chemists can gain a picture of how the molecules in a sample look by checking which NMR frequencies exist and how intense their signals are compared to each other.

Normally, atoms in solids experience too many competing magnetic interactions other than atomic bonds for NMR to work. As is, NMR must be done on samples in liquid solution where fast molecular motions average-out these interactions when viewed across time, like spinning car tires seeming to lose their side patterns. In fact, spinning solids rapidly at a specific angle can average-out the interactions as well. This way, solids can also be studied with NMR. Spin fast and almost all solid interactions go away; the side patterns simply appear

as circles. Spin slowly and some interactions remain; potentially revealing patterns re-emerge.

Many specialized NMR techniques rely on transferring polarization (basically transferring magnetic moment) from one type of atom to another to improve signal. INEPT is one such technique (NMR and abbreviations get along well). It transfers polarization through atomic bonds and relies on fast molecular motion to frequently orient the bond properly for transfer. Imagine it as a township that is heavily into astrology and can somehow communicate telepathically every time the planets align. The faster the solar system spins, the more often that happens. In contrast, CP (Cross Polarization), improves signal by transferring polarization from one atom to another directly through space. It is favored by slow molecular motion that lets atoms remain physically close over long periods of time. Think of a quiet countryside village where every neighborly encounter is a monologue face-off; at least you learn something. Finally, experiments without enhancement are called DP (direct polarization). This is more akin to workday stairwell greetings; polite and comfortably distanced.

Building on previous work, this study shows that DP, CP and INEPT applied to carbon while transferring polarization from hydrogen can be used to detect one solid- and three semi-solid states in brain extract fat. This is done by observing the relative signal intensities from these techniques at certain NMR frequencies to determine if the fatty acid chains are moving freely or not. Ensuring that carbon NMR can do this task required verification via other established NMR techniques. Though experiments on other materials may still need such verification, hopefully this study may help extend the usefulness of carbon-only NMR in organic materials science. With carbon neighborhoods present in most-if-not-all organic materials, magnetic hospitality abounds.

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1. Introduction

Since their inception, methods like Parallel Artificial Membrane Permeability Assay (PAMPA) have been used as high-throughput screening processes to evaluate the ability of potential drug molecules to pass through biological membranes.¹ The typical modus of the method is to immobilize the contents of a relevant animal lipid extract, expose it to the drug molecule and note, by means of indicators on the opposite of the lipid layer, whether the layer is permeable to the drug molecule. The blood-brain barrier (BBB) is one of the areas investigated using PAMPA with specific methods continuing to be refined² long after the advent of high-efficacy cell-based designs such as organ-on-a-chip devices³. Commonly, polar porcine brain lipid extract (PBL) is used in these PAMPA assays. Other than the desired surfactant chemical properties, material properties in the form of lamellar liquid crystalline structures akin to that of a lipid bilayer are desired when formulating the assays.⁴

Though the desired structural properties are known, the structural properties of the PBL as a whole have not been widely investigated. In this study, the overall phase behavior at different levels of hydration and a range of temperatures of the extract will be evaluated using solid state nuclear magnetic resonance (ssNMR) techniques. In terms of nuclei, ^{13}C , ^{31}P , ^1H and ^2H will be evaluated using previously established methods.⁵ ⁶ ⁷ ⁸ Dynamics based spectral editing provides mobility information for each bond configuration of ^{13}C via mobility dependent signal generation techniques. ^{31}P chemical shift anisotropy measurement gives characteristic spectral pattern shapes depending on the symmetry of the analyzed phase. D_2O quadrupolar splitting measurement (^2H) detects the presence of phases with considerable level of order in terms of bond angles via their ability to affect the D-O bond angle freedom through hydrophobic interactions. Additionally, changes in pattern width in general will be used to detect the occurrence of phase changes. Simple sample preparation procedures will be followed in the interest of testing the possibility of gaining liquid crystalline structures with few and easily executed process steps. Powdered PBL, referred to as pPBLe throughout the report, will be used as the starting point. Aside from evaluating material properties of hydrated pPBLe, additional attention will be paid to the acquisition and verification of ^{13}C ssNMR results corresponding to known phases adopted by the lipids present in the pPBLe. The purpose is to add to the reference base of ^{13}C NMR spectra for the purpose of material studies.

Considering the above, the objectives of this study are:

- Determining the general phase behavior of the extract across a wide temperature- and water content range using only information from ssNMR experiments.

- Drawing a phase map for the extract in question using only information from ssNMR experiments.

- Determining whether ^{13}C dynamics based spectral editing alone could provide enough information to draw the phase map.

- Using simple sample preparation methods to determine whether they are sufficient for allowing the extract to adopt liquid crystalline structures (assuming that adequate NMR information can be obtained).

2. Theory and background

2.1 Chemical characteristics of the investigated extract

2.1.1 Phospholipids – structure and dynamics

Formally, phospholipids (PLs) are any lipids that contain phosphorus atoms. Naturally, many subclasses of this group of lipids exist in turn, several of which are present in mammalian brains to some extent. This thesis, however, concerns almost exclusively glycerophospholipids (GPLs). Thus, in the following sections of this report, the term phospholipids will be used to refer to glycerophospholipids unless stated otherwise. The only case in this work where a need arises to distinguish PLs and GPLs is in the comparison of the latter with sphingomyelin. The structure of sphingomyelin is shown in Figure 1 to the right.

Phospholipids are comprised of a glyceride backbone, two fatty acid groups and a phosphate group to which some additional head group is attached. The general structure of a GPL is shown in Figure 2. PL fatty acid chains prominently featuring in this thesis are shown in Figure 3. Finally, the head groups featured in this thesis are shown in Figure 4 below. Anionic GPLs without a head group are called phosphatidic acids (PA).

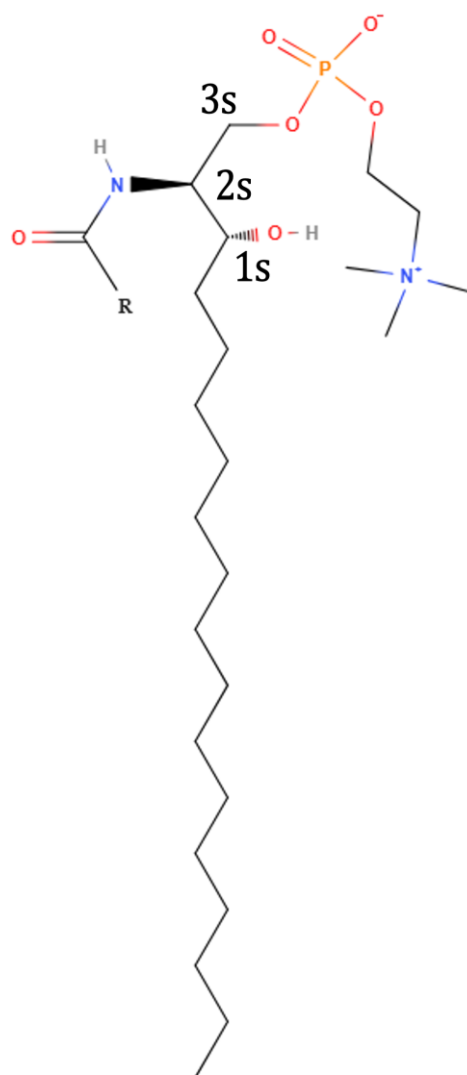


Figure 1: The structure of sphingomyelin, a non-glycerophospholipid present in mammalian brains. 'R' denotes a second fatty acid chain. Indexing that will be used throughout the report for three carbon atoms of sphingomyelin (1s, 2s and 3s) is shown. Bond angles are not exact.

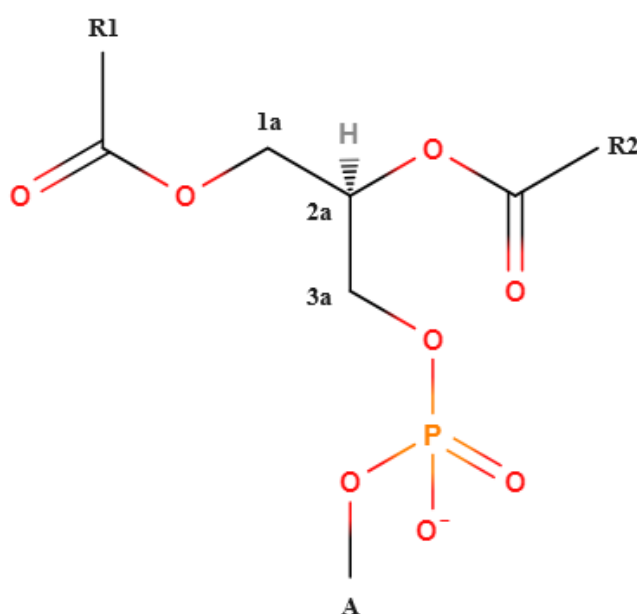


Figure 2: General structure of a glycerophospholipid. R1 and R2 denote fatty acid chains while A denotes a head group. The carbon indexes will be used throughout this report. Bond angles are not exact.

Common GPL nomenclature uses a combination of shorthand names for the respective molecular segments and organic chemical position numbers (such as 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphoserine) where numbers 1 and 2 are occupied by fatty acid chains and number 3 is occupied by a phosphate head group (see Figure 2 for carbon numbers). Additionally, the term *phosphatidyl*- (such as phosphatidylcholine) is used as a compact name omitting the glyceride backbone.

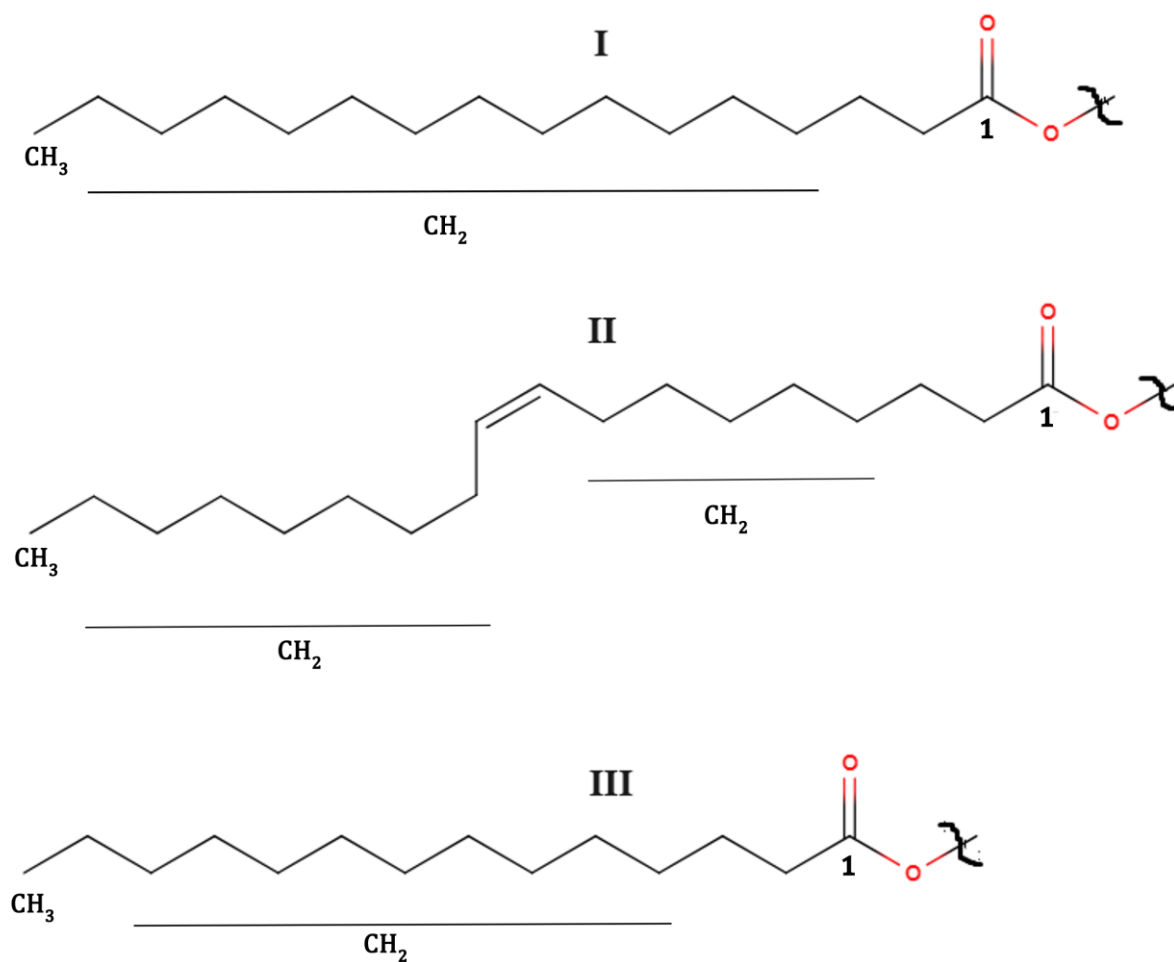


Figure 3: Prominent fatty acid groups of GPLs studies in this work. The groups are palmitoyl (I), oleoyl (II) and myristoyl (III). Carbon numbering begins at the carboxyl group. CH_3 and CH_2 groups will be designated as such throughout the report for all fatty acid chains unless stated otherwise.

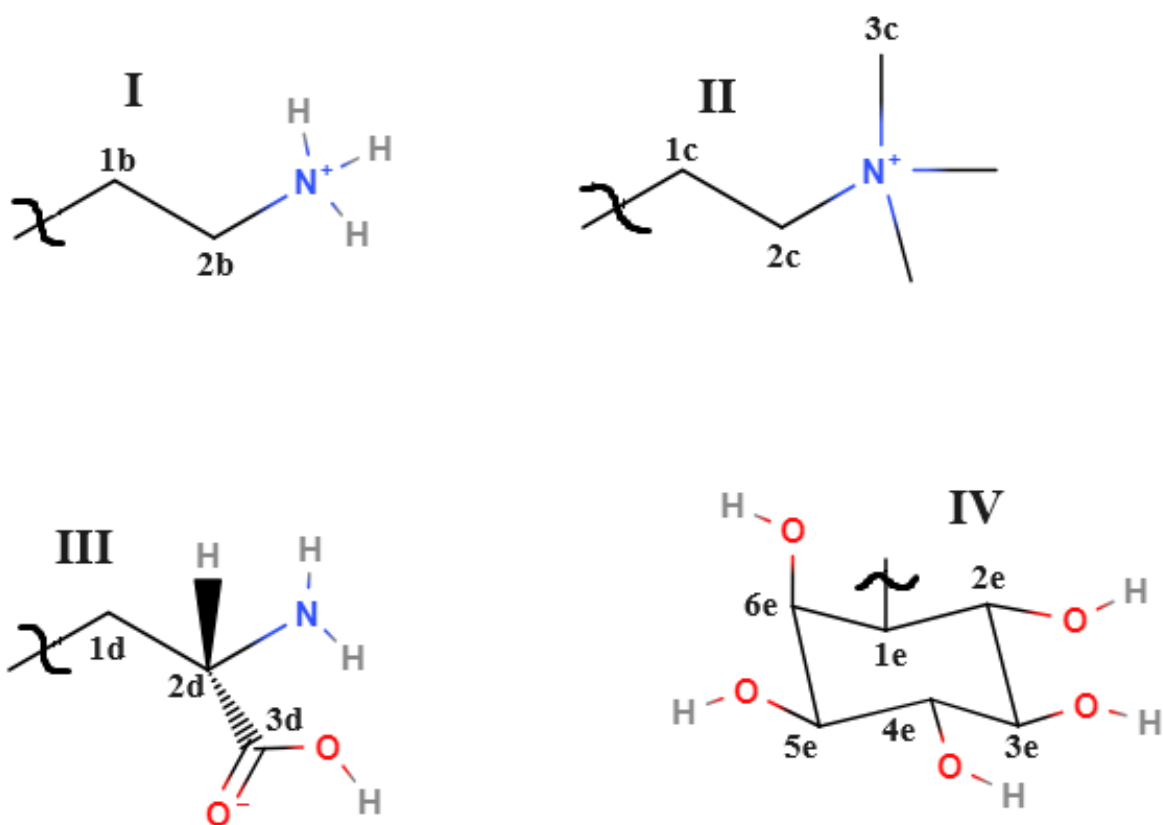


Figure 4: PL head groups featured in this report: ethanolamine (I), choline (II), serine (III) and inositol (IV). The carbon indexes in the figure will be used throughout the report. Bond angles are not exact.

Cholesterol is a known major lipid component of the porcine brain.⁹ Its carbon atoms and, by extension, the associated hydrogen atoms are labeled in Figure 5 below. Cholesterol carbon atoms will be referred to using the index “-C” throughout the report.

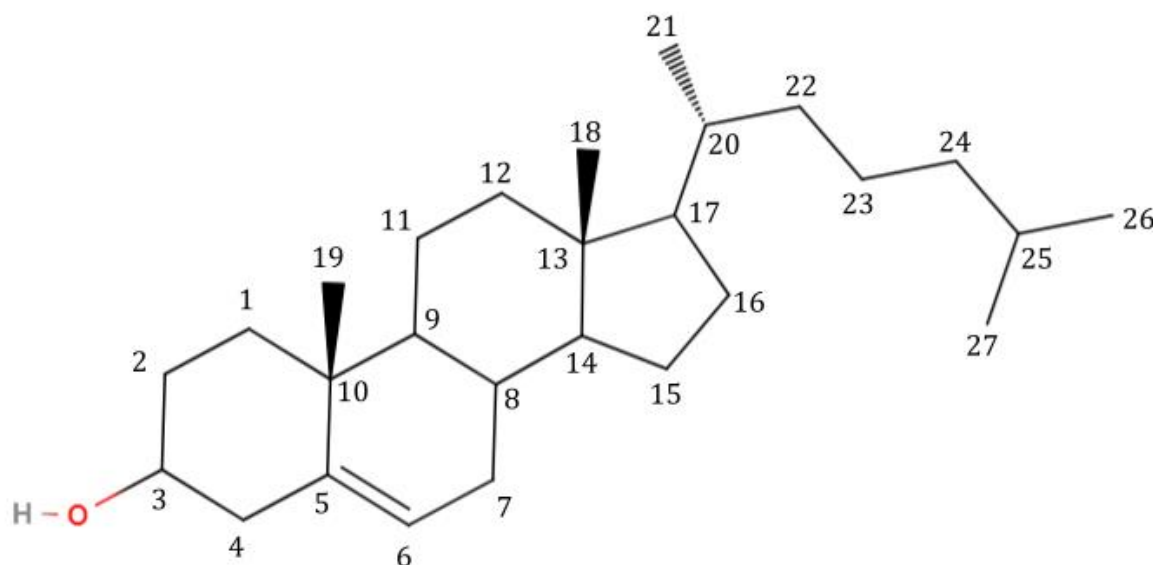


Figure 5: The molecular structure of cholesterol with carbon atoms numbered 1 through 27.

In terms of molecular dynamics in GPL bilayers, headgroups diffuse very slowly and it is instead proposed that they mainly perform a conical, wobbling motion as their main contribution to molecular movement.¹⁰ As a result, the fatty acid groups tilt as the head group responds to changes in surface tension.¹¹ As for carbon atoms in the fatty acid groups, their orientational freedom is often expressed as the order parameter with respect to the bilayer direction seen in eq.(1)

$$S = \frac{\langle 3\cos^2(\theta) - 1 \rangle}{2} = \overline{P_2(\cos \theta)} \quad (1)$$

where S is the order parameter, θ is the C-H bond angle to the bilayer normal and the overbar indicates an averaging across the sample. For other phases, the order parameter is analogously defined with a corresponding relevant structure surface. The fractional expression is also equal to the second order Legendre polynomial with notation shown in the right-hand equality. From the perspective of order parameters, there is considerable freedom of movement on the terminal CH_3 -groups, the last CH_2 -group on the chain and the first CH_2 -group on the chain. The intermediate CH_2 -groups, in contrast tend to have relatively restricted movement.^{12 13}

2.1.2 Known contents of the polar brain lipid extract

The producer of the pPBLe reports its contents according to the Table 1 below.¹⁴ The production process is described as a total lipid extraction via precipitation with acetone followed by extraction of the acetone-insoluble material with diethyl ether. Thus, content of any lipids from the total lipid extraction that are soluble in diethyl ether is also anticipated.

Table 1: Declaration of contents in the powdered Polar Brain Lipid extract (pPBLe). The abbreviations are phosphatylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and anionic phospholipid (PA)
Reproduced from <https://avantilipids.com/product/141101>

Brain Polar Extract Phospholipid Profile	
Component	wt/wt%
PC	12.6
PE	33.1
PI	4.1
PS	18.5
PA	0.8
Unknown	30.9
Total	100

2.1.2 Notation for phases

Table 2 below shows the notation used for various phases relevant to the lipid systems of this study. Figure 6 shows an illustration of the configuration of the PL molecules in each phase.^{15 16 17 18}

Table 2: Notations used for the phases of interest in this report.

Phase	Notation
Lamellar liquid crystal	L_{α}
Lamellar gel	L_{β}
Hexagonal liquid crystal	H_1
Reverse hexagonal liquid crystal	H_{II}
Micellar	L_1
Reverse micellar	L_2
Amorphous solid	L_s
Crystalline solid	L_c
Liquid ordered	L_o

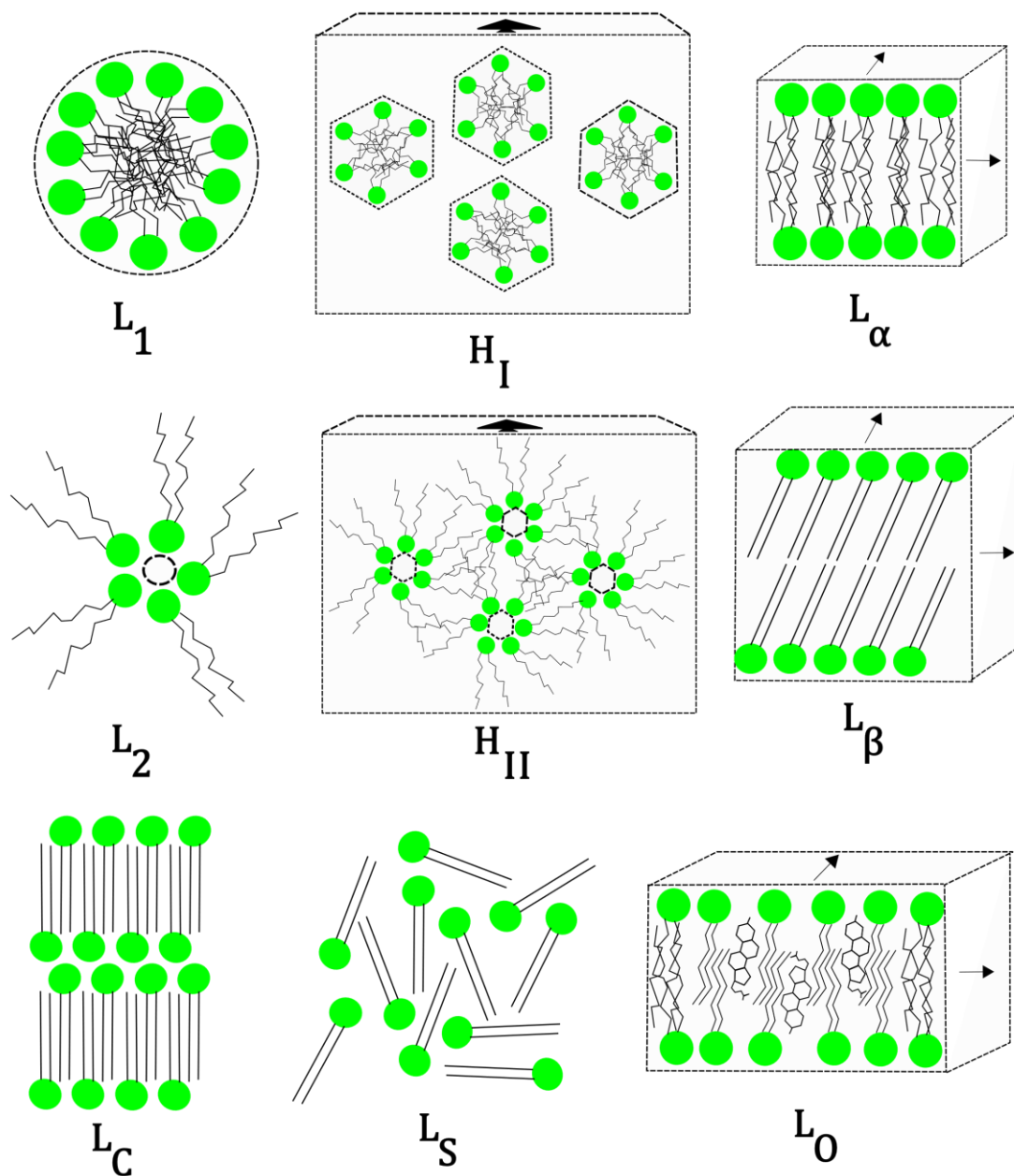


Figure 6: Arrangement of PL molecules within the phases outlined in Table 2. Green circles represent polar head groups and attached black lines represent non-polar tail groups. The latter are represented by straight lines if highly ordered (see order parameter section) and increasingly curved and/or twisted as their order decreases further. Arrows denote approximate directions of pattern repetition in space.

2.2 Previously documented phase behavior and related research results for the known constituent phospholipids in the pPBLe

The largest constituents, i.e. phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylcholine (PC) will be considered primarily. It is important to note that no information on the specific fatty acid chain content of these phospholipids as they appear in the pPBLe, nor on possible methylation of the PE, is available. In addition to reports detailing experiments on pure phospholipids, reports of experiments performed on mixtures between the aforementioned phospholipids and cholesterol will be considered. Furthermore, sphingomyelins are a known constituent of many cell membranes¹⁹ and will be given consideration.

2.2.1 Phosphatidylethanolamine (PE)

Previously, DSC and XRD experiments along with literature review have been used to construct a phase diagram for di-oleoyl-phosphatidylethanolamine (DOPE) covering temperatures of 258-303 K and water contents of 0-34 percent of total weight (see Figure 7).²⁰ Lamellar phase was reported up to about 297 K for 16 weight percent and up to slightly lower temperatures for about 12-20 wt% water. Reverse hexagonal phase was reported from 283 K and higher for water contents below 8 wt% and above 30 wt%. For water contents of 8-30 wt% overall, two-phase behavior of lamellar and reverse hexagonal phase was reported up to various temperatures between 283-297 K. For sub-zero temperatures, lamellar phase was reported to occur at temperatures as low as 268 K for 16 wt% water and above. Below this temperature, only mixtures of solid phospholipid crystals, rigid lamellar phase and ice was reported. As for results using NMR-techniques, previous work has reported ³¹P CSA values for 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) hydrated at 75% weight at the temperatures 273, 303 and 348 K respectively. The values indicate that PE adopts a lamellar structure at 273 and 303 K and a hexagonal structure at 343 K.²¹ A study of POPE at an unspecified level of hydration yielded spectra indicating lamellar phase behavior between 273 K and 298 K, a hexagonal phase behavior at 333 K

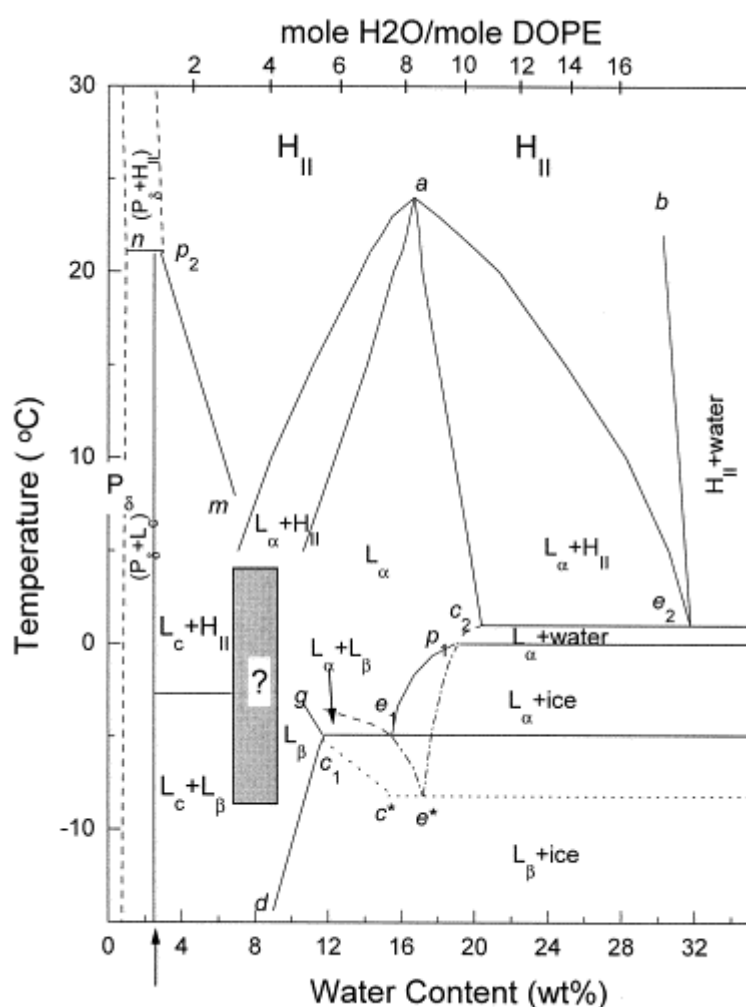


Figure 7: Phase diagram for di-oleoyl-phosphatidylethanolamine (DOPE). Lamellar structures (phases of index 'L') occur below temperatures of about 324 K to various extents. Above 24°C (297 K), reverse hexagonal phase (H_{II}) is prevalent. Pure lamellar liquid crystalline phase (L_α) occurs in a triangle-shaped domain around 16 wt% water content at temperatures above 5°C (278 K). Original image by Shalaev E.Y. and Steponkus P.L. doi: 10.1016/S0005-2736(99)00068-1, reprinted used under the CC BY-NC-ND 4.0 licence (<https://creativecommons.org/licenses/by-nc-nd/4.0/legalcode.en>), retrieved 2024-06-03)

and two-phase behavior in between 298 and 333 K, gradually changing from lamellar to hexagonal phase.²² Another study using DSC measurements reported strong fatty acid chain dependence of phase transition temperatures for PE. According to the report, for fully hydrated PE, unsaturated fatty acid chains yield solid-to-lamellar phase transitions at about 268-310 K with lamellar-to-hexagonal transitions occurring at about 288-338 K, depending on positional configuration with respect to the glyceride backbone. However, for fully saturated chains, transitions to hexagonal phase were absent, while transitions from solid-to-lamellar phase or rippled lamellar phase to liquid crystalline lamellar phase occurred anywhere between 305 K and 349 K. The report also notes though, that a special preparation procedure was required to produce both the lamellar liquid crystal and solid lamellar phases as it does not readily form for saturated fatty acid side chains on PE.²³

2.2.2 Phosphatidylserine (PS)

Reports on the phase behavior of pure PS are somewhat scarce. Previously reported chain-melting transition temperatures and diffraction patterns indicate that pure, fully hydrated PS forms rigid lamellar bilayers at temperatures of 283-318 K depending on the length and type of its fatty acid chains. A liquid crystal lamellar phase was reported to occur at even higher temperatures. The report also states that PS has a particularly high capacity for water uptake.²⁴ In terms of mixtures, a study using static ³¹P-NMR reported spectra with peak shapes indicating lamellar phase for temperatures 296-318 K for 60:40 mole percent mixture of PC/PS in which PS had two different combinations of fatty acid chains.²⁵ Yet another report showed static ³¹P static NMR spectra for di-palmitoyl-phosphatidylserine (DPPS) indicating lamellar phase at temperatures as high as 353 K when mixed with stearylamine (which appears to bolster the lamellar phase) and 313 K when mixed with sphingosine (which appears to disrupt the lamellar phase). Chain-melting transition temperatures recorded were similar to those recorded in the previously mentioned study.²⁶

2.2.3 Phosphatidylcholine (PC)

PC is likely the most widely studied GPL. A study of hydrated (58-72 weight percent) di-myristoyl-phosphatidylcholine (DMPC) yielded static ²H-NMR spectra indicating rigid lamellar phase and/or a rippled transition phase for temperatures of about 263-295 K. For temperatures of about 295-308 K, liquid crystal lamellar phase was reported.²⁷ A DSC study found that di-palmitoyl-phosphatidylcholine (DPPC) of water contents of about 26-88 wt% adopts solid crystal structure below about 290 K, rigid lamellar phase between 290-305 K, rippled gel phase between about 305-315 K and lamellar phase between about 315-390 K. At water contents of about 10-26 wt% the phase boundaries shift until, below 10 wt% water content, the transition temperature to lamellar liquid crystal phase increases sharply and only an unspecified solid phase exists instead at most temperatures.²⁸ The latter was also found by a DSC study on dehydrated PC.²⁹ Additionally, the aforementioned reports found that specific fatty acid chain content could change the primary transition temperature of PC (i.e. the initial break-up of the solid phase) within an interval of about 20 K.²⁸⁻²⁹ The former report also found that chain length of the palmitoyl chains in DPPC can change the primary transition temperature by as much as 48 K for chain lengths ranging from 14 to 22 carbon atoms.²⁸

2.2.4 Mixtures of individual phospholipids with cholesterol

Mixtures of PC and cholesterol have garnered enough attention that MD simulations have been carried out, devising models for lipid bilayer organization based on a wealth of experimental results. Reports show that cholesterol in PC increases order and thickness of bilayer structures. That then leads to the formation of a liquid bilayer phase rich in cholesterol, labeled L_O while the transition temperature to L_α -phase increases.³⁰ ³¹ The first report also concluded that the specific fatty acid content of the phospholipid affects the extent of the ordering effect (in terms of acyl chain confirmation) that cholesterol has on it.³⁰ Later work performed simulations based on those reports, predicting that the L_O phase occurs at about 18 mol% cholesterol and higher and that the transition temperature to L_α -phase increases approximately linearly from about 295 K to about 335 K as the mol% of cholesterol increases from 5 to 50. The fidelity of the simulation was estimated by calculating lipid tail order parameters (the defining angle was the angle between lipid tail C-C bond vectors and the bilayer normal) within it, yielding values similar to those of the experimental work it was based on.³² Another report found that for DPPC, L_O -phase occurs from the temperature of 293 K for as low as 5 mol% cholesterol to then transition to L_α -phase at higher temperatures. The report also found that the L_α -phase is largely replaced by L_O -phase at about 30 mol% cholesterol. One report featured an extensive investigation of chain segment properties yielded by NMR- and MD simulation techniques for hydrated pure POPC bilayers as well as hydrated POPC bilayers of various mol% contents of cholesterol. Its experiments were carried out at 300 K and showed results largely congruent with the above statements. The order parameters for the main fatty-acid CH_2 -segments ranged from about 0.2 to 0.4 as the mol% of cholesterol increased and an initial increase in order parameter value took place somewhere between 15 and 34 mol% cholesterol, indicating the onset of L_O phase formation. Additionally, the report featured ^{13}C -NMR spectra showing a notably change in the relationship between CP- and INEPT-signal enhancement for considerable molar percentages of cholesterol; the main fatty acid CH_2 -groups enjoy much less INEPT signal enhancement while maintaining similar CP signal enhancement compared to that of pure POPC.³³

For PE mixtures with cholesterol, reports suggest that the influence of cholesterol varies with the fatty acid chains, similarly to the case of PC. A common effect is a decrease in the lamellar liquid crystal to hexagonal phase transition temperature for molar percentages of cholesterol up to 30%, an effect suggested by two reports.³⁴ ³⁵ One of the reports further suggests a plateau in transition temperature for 30-50 mol% cholesterol, followed by an increase in transition temperature for higher molar percentages. ³⁴

For PS mixtures with cholesterol, a decrease of about 5 K in primary transition temperature for POPS and SOPS was reported up to 5 mol% cholesterol via DSC experiments, followed by a plateau of only slight further decrease. A second transition temperature was shown to occur between 30-50 mol% cholesterol for POPS and 30-80 mol% cholesterol for SOPS. XRD experiments were performed to show that the additional phase occurring was most likely a two-phase region featuring a separate phase of immiscible cholesterol.³⁶

2.2.5 Mixtures of multiple phospholipids with Cholesterol and Sphingomyelin (SM)

A study performing experiments on phospholipid bilayers at 50 wt% hydration in found that cholesterol at 31 mol% had an ordering effect on 1:2 (by mol) mixtures of SM and POPC, POPE as well as POPS by

means of ^2H -NMR and fluorescence spectroscopy on deuterated phospholipid species. The studied temperatures were 293-323 K and the effect appeared as an increase in quadrupolar splitting distance for patterns congruent with lamellar liquid crystal phase. Fluorescence spectroscopy and further ^2H -NMR results then suggested that cholesterol also has an ordering effect on bilayers consisting of 22:44:17:17 molar amounts of SM, POPC, POPE, POPS and an additional 31 mol% cholesterol out of which cholesterol preferentially interacts with SM at low cholesterol mol%. The ^2H -NMR spectra featured 50 kHz wide patterns containing many peak pairs (i.e. several quadrupolar splittings from individual chains) rather than a smooth curve between the highest intensity peaks, indicating lateral heterogeneity within the bilayer, though the structure remains lamellar.³⁷

As for NMR studies, previously reported ^{31}P -spectra at 313 K featured a broad albeit jagged shoulder pattern, indicating a lamellar phase with regions of less-ordered or isotropic phospholipid and possibly unresolved peak patterns of individual phospholipid species. The study was also able to produce well-resolved ^{13}C DP-MAS spectra at the same temperature. Notably, the study was performed using porcine total lipid extract from the same manufacturer as in this work. Relative contents of GPLs compared to each other were reportedly similar to the pPBLe used in this work while the amount of cholesterol, sphingomyelins, cerebrosides and gangliosides compared to GPLs was higher. Sample preparation also differed. The report used a procedure of chloroform solvation, evaporation, overnight lyophilization, heated water suspension and freeze-thaw cycling as treatment of the powder, as compared to the simpler approach taken in this work.³⁸

2.2.5 Considerations regarding the L_α -phase

The L_α -phase is typically associated with PC, a PL that is a sizeable minority in the pPBLe that is the subject of this study. This phase has also been shown to share many NMR-measurement responses and characteristics with the L_α -phase, the only readily available identifier appearing to be reduced line broadening due to stabilization of liquid phases compared to pure PL as the molar content of cholesterol increases. This feature cannot be utilized in this study since the pPBLe is a mixture of multiple PLs. Another notable identifier compared to the latter is resolved splittings of about 40 kHz within ^2H quadrupolar splitting patterns of deuterated PL species, an aspect beyond the scope of this study since D_2O is the only source of ^2H -signal present.³⁹ Under these considerations and under the condition that a sufficiently high cholesterol content can be estimated within the pPBLe, the L_α -phase will be assumed to occur to some extent at conditions where L_α -phase occurs.

2.3 Solid-State NMR

2.3.1 The vector model and the NMR signal

Samples, whether they are solid or liquid, consist of atomic nuclei which have an intrinsic nuclear spin. Since the nuclei contain a charge distribution, this motional feature causes them to have a magnetic moment. In an undisturbed sample, random movement causes the net magnetic moment of the entire sample to be zero. NMR acts on the sample by subjecting the sample to a strong magnetic field, commonly labeled $\mathbf{B}_0$. This causes the magnetic moments of the nuclei to orient themselves preferentially along a direction parallel to the magnetic field. This gives the sample a net magnetic moment along said direction, which is set as the spatial z-axis and it offers the starting point of the vector model for NMR. Figure 8 illustrates the main magnetic fields, magnetic moments and frequencies of interest in NMR as shown in the vector model. This framework is usually referred to as the *laboratory frame*.⁴⁰

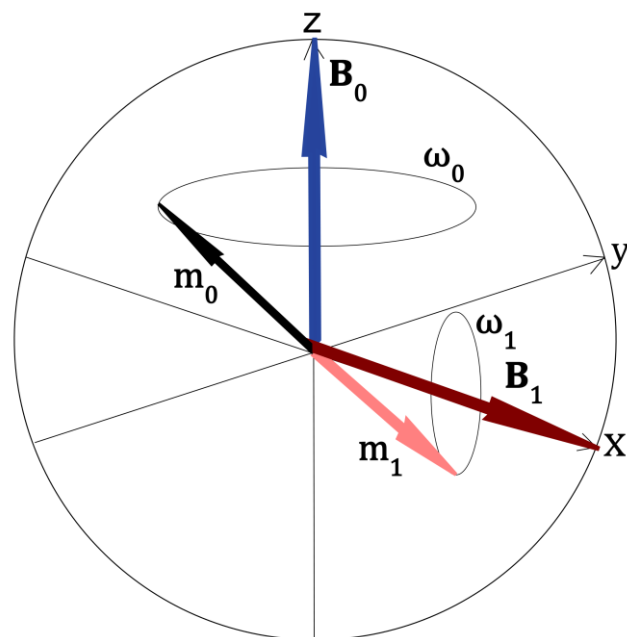


Figure 8: The vector model of NMR. The magnetic moments m_0 and m_1 are two hypothetical magnetic moments precessing about $\mathbf{B}_0$ and $\mathbf{B}_1$ respectively. The ω_0 and ω_1 frequencies are placed next to their associated precessive motions (illustrated by circles). Vector lengths are not proportional to magnetic field strength.

When a sample is in the spectrometer and not being experimented on, the sample largely only experiences $\mathbf{B}_0$. Observable signal is generated by subjecting the sample to a second, oscillating magnetic field $\mathbf{B}_1$ placed along the x-axis. This puts $\mathbf{B}_1$ in the *transverse plane* perpendicularly to $\mathbf{B}_0$. When $\mathbf{B}_1$ is switched off, the magnetic moment spins at an angle (i.e it "precesses") around the z-axis at the frequency $\omega_0/2\pi = \gamma\mathbf{B}_0$. This frequency is called the *Larmor frequency*. γ is the gyromagnetic ratio of the nucleus being observed. After some period of time, the magnetic moment will once again point along the z-axis, a state commonly referred to as *equilibrium magnetization* in an NMR context.^{40 41}

To make $\mathbf{B}_1$ appear static despite oscillating and thus make it possible to comprehensibly view the movement of the magnetic moment during experiments, a *rotating frame* of viewing is introduced. The frame rotates about the z-axis at the frequency of the transmitter that generates $\mathbf{B}_1$, here called ω_c or the *carrier frequency*. This frequency is set to be as close as possible to the resonance frequency of the species of nucleus to be excited. The result is that in the rotating frame, the Larmor precession instead occurs around an effective field axis $\mathbf{B}_{\text{eff}}$ which is the vector sum of the reduced $\mathbf{B}_0$ field (defined as $\mathbf{B}_0(1-\omega_c/\omega_0)$) and $\mathbf{B}_1$ as is shown in Figure 9 to the right.⁴¹

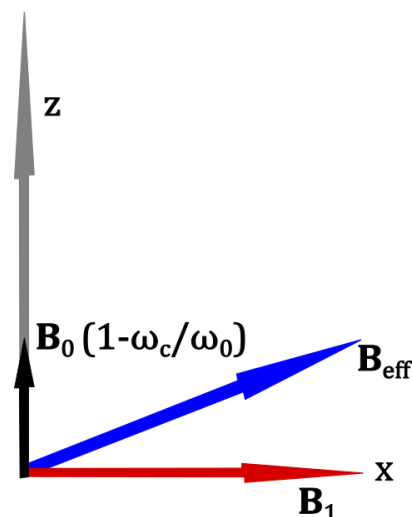


Figure 9: The effective axis of precession in the rotating frame

Analogously to how the magnetic moment precesses about the z-axis after a pulse of $\mathbf{B}_1$, the magnetic moment precesses about the axis of $\mathbf{B}_{\text{eff}}$ during the pulse itself. The frequency of this precession is $\omega_1/2\pi = \gamma\mathbf{B}_1$ (parameter definitions consistent with that of the Larmor frequency) and it is called the *nutation frequency*. Depending on its duration and transmitter wattage, a $\mathbf{B}_1$ pulse may or may not bring the magnetic moment to a $\pi/2$ angle with $\mathbf{B}_0$. Regardless, the

resulting angle of the magnetic moment after the pulse is called the *nutaton angle* or the *flip angle*. The term $\pi/2$ -pulse refers to a pulse calibrated to achieve the $\pi/2$ nutaton angle.⁴⁰

A pulse that meets the condition $\omega_0 = \omega_c$ is said to be *on resonance* with respect to the observed nucleus and a pulse which does not is analogously said to be *off resonance*. Under on-resonance conditions, $\mathbf{B}_{\text{eff}}$ becomes the $\mathbf{B}_1$ axis.⁴¹ A further implication that follows with the on/off-resonance dichotomy, due to the viewing of the experiment in a rotating frame, is that the magnetic moment of any nucleus for which the pulse is off resonance will not be pointing along the transverse axis of the pulse. This is because the rotating frame will either spin away from or fall behind the frequency of the nucleus in question (the rotating frame is spinning at on-resonance speed).

2.3.2 Signal generation using a basic pulse sequence

A schematic for the most basic NMR experiment, the pulse-acquire experiment, often referred to as a so-called “zg-experiment” is shown in Figure 10 to the right. The figure includes descriptions of several important time-parameters in NMR experiments. The amplitude of the precession as seen in the transverse plane is on the t-axis. The sign of the amplitude is defined along the x-axis. As can be seen, its amplitude gradually decays as the angle of the magnetic moment with respect to the z-axis in 3D space decreases.⁴¹ As the magnetic moment performs this motion it induces a current in a coil. This current is digitally transformed to the NMR signal and is called the FID (Free Induction Decay). In the experiment, the dwell time has the impact of determining the *spectral width* of the scan, i.e. the span of frequencies in Hz that are sampled. The lower the dwell time, the greater the spectral width. This parameter is separate from the *bandwidth*, which is a measurement of the frequency span of nuclei that are excited by the $\mathbf{B}_1$ -pulses and thus will give rise to spectral peaks. Bandwidth is proportional to the amplitude of $\mathbf{B}_1$; the stronger the pulse, the greater the bandwidth.⁴²

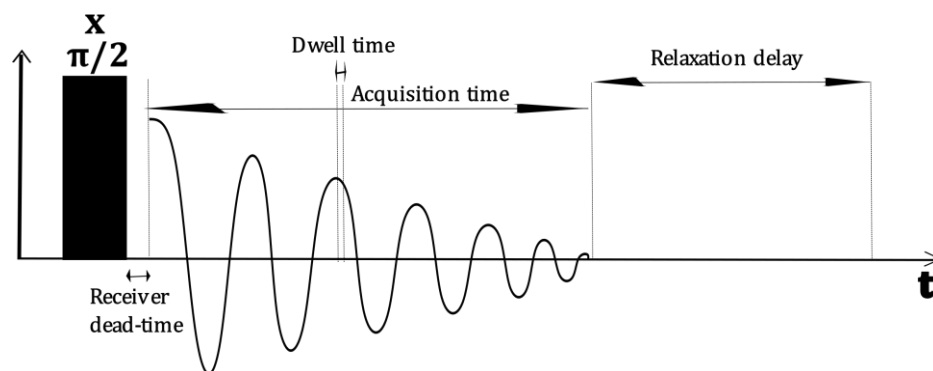


Figure 10: The pulse-acquire experiment. Pulses are commonly displayed with their alignment axis and nutation angle, here x and $\pi/2$ respectively. Receiver dead time is a delay introduced after the end of a $\mathbf{B}_1$ pulse to avoid registering lingering $\mathbf{B}_1$ -pulse as signal, which would damage the receiver. The dwell time is the time-interval between signal registrations (signal is not registered continuously). The relaxation delay is the time allowed between successive experiments if a series of identical experiments is needed to gain adequate signal. The acquisition time is the total time of signal acquisition and may need to be adjusted to be at least as long as the portion of the FID that is readily distinguishable from noise.

2.3.3 Chemical shift and the ppm scale

Covalent bonds, asymmetric bond environments and molecular interactions cause most nuclei to have slightly different ω_0 . This in turn causes inequivalent nuclei to appear as different peaks in NMR spectra. The difference in frequency compared to a standard peak, which may be set according to a calibration

sample or simply referenced internally in the spectrum, is called *chemical shift*. It is reported in Hz or, more frequently, in *ppm*. The latter is a fraction of the $\mathbf{B}_0$ frequency and allows for ease of comparison of results between different NMR spectrometers.⁴¹

The origin of chemical shift is *chemical shielding* induced by $\mathbf{B}_0$ which makes the electrons in the system circle around it. This makes the electrons produce their own magnetic fields which oppose $\mathbf{B}_0$ at their centre of motion, i.e. at their corresponding nucleus. This effect decays rapidly with distance according to a $1/r_i^3$ dependence on r_i , the electron-nucleus distance. Thus, it is chiefly the low electron shell electrons that contribute to the field. This is called the *diamagnetic contribution* to the *chemical shielding* and is nearly constant for many species of nuclei regardless of their chemical environment. Additionally, $\mathbf{B}_0$ distorts the electron distribution around each nucleus and mixes the energetic quantum levels of the electrons. This means that more electron quantum states will be populated. If any of these produce paramagnetism, a magnetic field that supports $\mathbf{B}_0$ at the nucleus appears. Since this field supports $\mathbf{B}_0$, it acts to deshield the nucleus. This is called the *paramagnetic contribution* to the chemical shielding and is, in contrast to the diamagnetic contribution, strongly dependent on the species of nucleus.^{40 42}

2.3.4 Basic data processing

The idealized FID in Figure 10 is generally not representative of reality. A total FID from an actual NMR experiment consists of stacked frequencies from the different chemical species of the observed nucleus present in the system, i.e. their individual FIDs. Via multiplication with test waveforms, these individual frequencies can be extracted to be displayed on a frequency axis. This process is called *Fourier Transformation* (FT) and is central to generating spectra in NMR analysis. To improve the results of the FT in terms of either resolution or noise suppression, it is common to multiply the FID with a so-called *window function* to achieve a desired level of *line broadening*. The most common

window function is an exponential. An increasing exponential enhances peak resolution but increases noise and a decaying exponential reduces noise but may render closely spaced peaks unresolved. A common procedure is to use a *matched filter* which is to choose the exponential so that the line broadening in Hz becomes equal to the inverse of the acquisition time. This is commonly seen to achieve the highest resolution possible without also enhancing noise to the point that it may be mistaken for sample peaks.⁴¹ Multiplication of test waves with an FID of a period that does not begin at an angle of 0° with respect to the receiver phase distorts the lineshape of the spectral peaks as shown in Figure 11 above. This is counteracted by

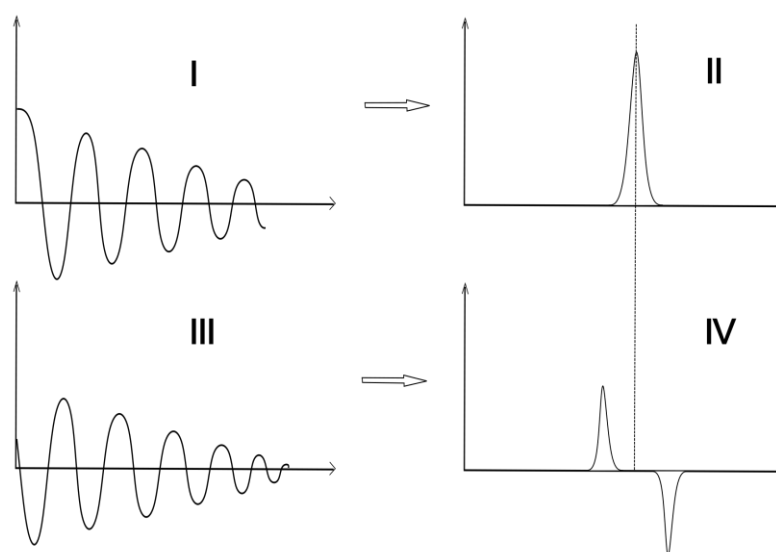


Figure 11: Acquiring signal at a $\pi/2$ phase angle relative to the receiver (III) distorts the desired absorption mode lineshapes (II) into dispersion mode lineshapes (IV). The two resulting peaks have an opposing sign and each have half the area of the peak in (II). The phase of the FID in (III) is not exact. In (I), a representation of an FID that is in-phase with the receiver is shown.

mathematically shifting the period of the FID to begin at 0°, returning the spectral peaks to the desired absorption mode lineshape. This procedure is called *phase correction*.⁴¹

2.3.5 Predicting the effect of pulses using operators

Sequences of multiple pulses (as opposed to a single pulse in the pulse-acquire experiment) can be used to manipulate the magnetic moment in such a way that it gives observable signal under sample conditions where the pulse-acquire experiment fails. For such proposed pulse sequences, it is necessary to be able to predict whether the components of the magnetic moment will point in a direction that will generate a useful FID at the end of the pulse sequence. This is done via the *Hamiltonian* operator used to extract values for spin energy levels from the time-dependent Schrödinger equation.⁴⁰

The Hamiltonian for a pulse-acquire experiment without sample-internal interactions present is shown in eq.(2).⁴¹

$$\hat{H} = \Omega \hat{I}_z + \hat{I}_x \omega_1 \quad (2)$$

where $\hat{I}_z$ is the z-component of the magnetic moment averaged across the sample density (i.e. $\hat{I}_z = (0,0,1)$) and $\hat{I}_x$, conversely, represents magnetic moment along the x-axis. These will be referred to as *axis operators* for convenience from now on. Ω is the offset frequency, i.e. the difference between Larmor frequency and the frequency of the rotating frame. The first term accounts for free evolution of the magnetic moment in the z-direction and the second term accounts for flipping of a magnetic moment by a pulse along the x-axis by B_1 . If the pulse is on resonance, the first term is null. Otherwise, it can be thought of as accounting for the chemical shift of the nucleus.^{41 42}

To evaluate the magnetization at time t from the start of a pulse sequence, the Hamiltonian propagates the density operator as in⁴¹

$$\hat{\rho}(t) = e^{-i\hat{H}t} \hat{\rho}(0) e^{i\hat{H}t} \quad (3)$$

where $\hat{\rho}(0)$ is the density operator at t=0. It shall be noted that the density operator can be expressed as a linear combination of the axis operators.⁴¹ As can be seen, each term in the Hamiltonian will give rise to a factor in the density operator. The power of each factor will contain a product of an axis operator (or a *product operator* which is the product of axis operators on different nuclei), a chosen time period and a relevant transverse angular frequency. The result is an angle which is then applied to $\hat{\rho}(0)$. A perhaps obvious feature of Hamiltonians is that the effect of any distinct time period (a pulse or a period of free evolution) can be represented by summarizing the Hamiltonians for each interaction present during said time period, giving a new $\hat{\rho}(0)$ from which to calculate the $\hat{\rho}(t)$ of the next time period. This procedure is then repeated until one arrives at the point of signal acquisition in the pulse sequence to yield the magnetic moment that will be available for signal detection.^{40 41 42}

To simplify working with product operators, a couple of known results of pulses are usually employed to streamline noting the effects of on-resonance $\pi/2$ and π pulses.⁴¹ These are:

- An on-resonance $\pi/2$ pulse along an axis rotates the magnetic moment $\pi/2$ about that axis in a positive direction (positive angle of rotation). For instance, an on-resonance pulse along x positive sets the magnetic moment to $-\hat{I}_y$ if the magnetic moment is $\hat{I}_z$ at the start of the experiment. A pulse along y negative sets the magnetic moment to $-\hat{I}_z$ if it is $-\hat{I}_x$ at the start of the experiment.
- An on-resonance π pulse along an axis inverts the signs of the axis operators of affected magnetic moments unless they are parallel to the axis of rotation at the time of the pulse.
- At the point of signal acquisition, only in-phase terms represent observable signal (further explained below)

Coupling effects between nuclei (explained in later sections) give rise to product operators, i.e. terms that are products of the axis operators belonging to the individual nuclei. If index 1 denotes nucleus 1 and index 2 denotes nucleus 2, then the product operators are (see table 1):⁴¹

Table 3: Product operators for two nuclei or "two spins" sorted by their phase nature.

Anti-phase on 1	Anti-phase on 2	Multiple quantum	Non-equilibrium
$2\hat{I}_{1x}\hat{I}_{2z}$	$2\hat{I}_{1z}\hat{I}_{2x}$	$2\hat{I}_{1x}\hat{I}_{2x}$	$2\hat{I}_{1z}\hat{I}_{2z}$
$2\hat{I}_{1y}\hat{I}_{2z}$	$2\hat{I}_{1z}\hat{I}_{2y}$	$2\hat{I}_{1x}\hat{I}_{2y}$	
		$2\hat{I}_{1y}\hat{I}_{2x}$	
		$2\hat{I}_{1y}\hat{I}_{2y}$	

In contrast to the anti-phase terms in the table, the axis operators are *in-phase terms* and give observable signal if present at the end of a pulse sequence. The anti-phase terms, however, evolve into in-phase terms on the opposing nucleus over time, a fact exploited by some pulse sequences using well-selected "delays" of free evolution before acquisition.^{41 42} Non-equilibrium terms account for sample population not excited by pulses, which may still be able to receive magnetization from the excited population. Multiple quantum coherences are mainly of interest in 2D-NMR but also play a role in dipolar couplings. To understand their evolution over time, they are broken down into *double-quantum* and *zero-quantum* parts.⁴¹ These are defined in table 2 below.

Table 4: Definition of raising- and lowering operators and double- and zero-quantum components of multi-quantum coherences

Raising(+) or lowering (-) operator	Reading in terms of axis operators	Multi-quantum operator	Double-quantum component	Zero-quantum component
$\hat{I}_+$	$\hat{I}_x + i\hat{I}_y$	$2\hat{I}_{1x}\hat{I}_{2x}$	$\frac{1}{2}(\hat{I}_{1+}\hat{I}_{2+} + \hat{I}_{1-}\hat{I}_{2-})$	$\frac{1}{2}(\hat{I}_{1+}\hat{I}_{2-} + \hat{I}_{1-}\hat{I}_{2+})$
		$2\hat{I}_{1x}\hat{I}_{2y}$	$\frac{1}{2i}(\hat{I}_{1+}\hat{I}_{2+} - \hat{I}_{1-}\hat{I}_{2-})$	$\frac{1}{2i}(-\hat{I}_{1+}\hat{I}_{2-} + \hat{I}_{1-}\hat{I}_{2+})$
$\hat{I}_-$	$\hat{I}_x - i\hat{I}_y$	$2\hat{I}_{1y}\hat{I}_{2x}$	$\frac{1}{2i}(\hat{I}_{1+}\hat{I}_{2+} - \hat{I}_{1-}\hat{I}_{2-})$	$\frac{1}{2i}(\hat{I}_{1+}\hat{I}_{2-} - \hat{I}_{1-}\hat{I}_{2+})$
		$2\hat{I}_{1y}\hat{I}_{2y}$	$-\frac{1}{2}(\hat{I}_{1+}\hat{I}_{2+} + \hat{I}_{1-}\hat{I}_{2-})$	$\frac{1}{2}(\hat{I}_{1+}\hat{I}_{2-} + \hat{I}_{1-}\hat{I}_{2+})$

2.3.6 Spin systems and spectra resulting from J-couplings

Nuclear spin is a quantum mechanical property and its measurable energy is quantized into discrete levels (while its real energy can be any quantity in a continuum of superposition states of these levels). It also has an angular momentum. A feature of quantized angular momenta is that degenerate states, i.e. spin states of equal energy, cease to be degenerate when under the effect of magnetic fields; they are split into *Zeeman sublevels* in a *Zeeman split*. The field that provides this effect in the NMR spectrometer is $\mathbf{B}_0$. Magnetic pulses from $\mathbf{B}_1$ bring the sublevels together again, allowing them to be populated freely (for on-resonance pulses) by the affected nuclei until the pulse is turned off and the Zeeman split returns.^{40 41} A quantum mechanical interpretation of NMR is thus that the observed signal is the process of returning to the equilibrium populations of the sublevels under $\mathbf{B}_0$.

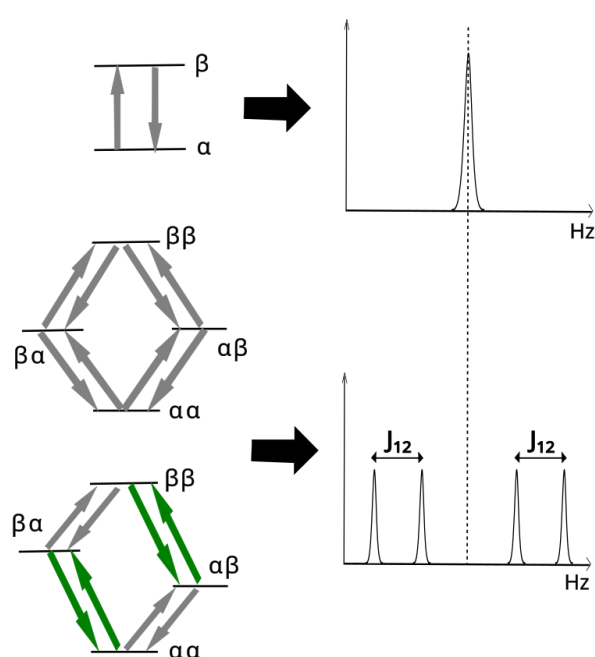


Figure 12: Spin system of single (top right), homonuclear coupled (middle right) and heteronuclear coupled (bottom right) spins and their resulting spectra. The identities of the energy levels are also indicated. "aa" indicates both nuclei being in the α -state and the rest of the energy levels are named analogously. In each energy level diagram, equally energetic transitions are colored the same.

Spin systems illustrate the Zeeman sublevels that exist in a system and provide an apt explanation for the concept of *J-coupled nuclei* and why they result in double peaks in NMR spectra. J-coupling occurs between covalently bonded nuclei. The spin of one of the nuclei influences the spin of a delocalized electron, which in turn influences the spin of the other nucleus. Figure 12 below shows the spin systems and resulting spectra of a lone spin (i.e. a lone nucleus), two coupled spins from identical nuclei (homonuclear coupling) and two coupled spins from different nuclei (heteronuclear coupling). Homonuclear- and heteronuclear coupled spins both produce two doublets of peaks positioned at frequency values about the frequency value of an uncoupled spin. The *coupling constants* J_{12} between the nuclei are illustrated in Figure 12 below. In the spin system, transitions between top or bottom levels and the middle levels are called *single-quantum transitions*, transitions directly between the top- and bottom levels are called *double-quantum transitions* and transitions directly between middle levels are called *zero-quantum transitions*.⁴¹

As mentioned, *J-coupling*, is mediated through atomic bonds. Its resulting split peak patterns thus provide information the bond configurations present in the system. When an observed nucleus is coupled to many other nuclei however, the resulting abundance of split peaks may make the spectrum confusing. To counteract this, a *decoupling sequence* of $\mathbf{B}_1$ pulses can be applied to the spin of the nucleus that is not being observed during acquisition. The aim is to lock its magnetic moment in the transverse plane where it will not generate observable signal while the magnetic moment of the observed nucleus is allowed to evolve freely. This effectively reduces the spin system back to that of a single spin, making heavily coupled nuclei easier to observe. Such decoupling sequences are often complex and performed with ramped pulse power, the latter to be on resonance with as many chemical species of the unobserved nucleus as possible.^{40 41}

The Hamiltonian for free evolution of a system of coupled spins is ⁴¹

$$\hat{H} = \Omega_1 \hat{I}_{1z} + \Omega_2 \hat{I}_{2z} + 2\pi J_{12} 2\hat{I}_{1z}\hat{I}_{2z} \quad (4).$$

2.3.7 Anisotropic interactions and their appearance in solid state NMR

J-coupling is an isotropic interaction and perhaps the prevailing interaction that affects spectra in solution state NMR. In solid state NMR, molecules are not mobile enough to enjoy isotropically averaged motion. As a result, anisotropic interactions come into effect. These are orientationally dependent and as a result, cause many molecular orientations to become magnetically unique even when viewed over long periods of time. When viewed through the lens of spin systems, the result is the introduction of a myriad of energy levels into the system, enabling transitions corresponding to frequencies of a wide span of energy differences to occur. The spectral result is a continuous band of frequencies as opposed to well defined peaks (see figure 13 below). As can be seen in Figure 13, the spectral width of an experiment may be insufficient to capture the complete span of anisotropic interactions. Setting an encompassing spectral width is a point of interest for experiment calibration in solid state NMR.

In the following paragraphs, the anisotropic interactions of greatest importance to this study will be explained. Before that, it is worth mentioning that orientationally dependent interactions such as these usually are represented mathematically by second rank cartesian tensors as in ⁴⁰

$$\sigma = \begin{pmatrix} \sigma_{xx} & \sigma_{xy} & \sigma_{xz} \\ \sigma_{yx} & \sigma_{yy} & \sigma_{yz} \\ \sigma_{zx} & \sigma_{zy} & \sigma_{zz} \end{pmatrix} \quad (5).$$

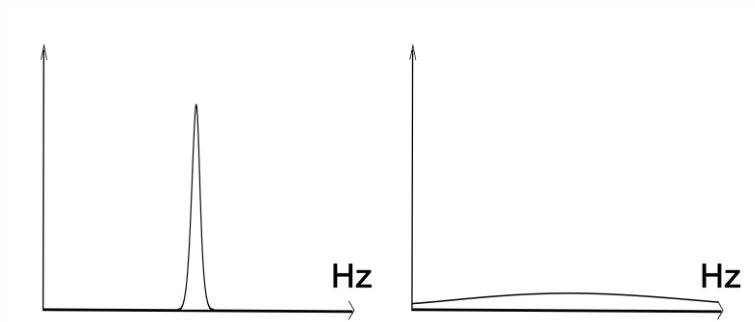


Figure 13: Comparison between spectra of NMR performed on the same nucleus in solution state (left) and solid state (right). The peak and the band have the same area (not rendered to exactness in the figure).

Depending on the studied interaction, the x-y-z axis frame may be defined in a way that facilitates analysis of the interaction, often along the main symmetry axis of the interaction tensor. This is referred to as switching to a *principal axis frame* (PAF). ⁴⁰

2.3.7.1 Chemical shift anisotropy (CSA)

Chemical shielding tensors for pairs of nuclei take on ellipsoid forms centered around the covalent bond vector. The tensors may or may not be axially symmetric with respect to the x- and y-axis. Regardless of symmetry, it is common to define a PAF set within the ellipsoid (see Figure 14 to the right).⁴⁰

Note the angle θ between the principal z-axis of the tensor and the field vector of $\mathbf{B}_0$ and the angle φ defined in the principal x-y-plane with $\varphi=0$ along the principal x-axis. In such a PAF, it can be shown that the resulting frequency relative to the Larmor frequency due to chemical shielding is⁴⁰

$$\omega_{cs}(\theta) = -\omega_0 \Delta \frac{1}{2} (3\cos^2(\theta) - 1) \quad (6)$$

for an x-y symmetrical shielding tensor and⁴⁰

$$\omega_{cs}(\theta, \phi) = -\omega_0 \sigma_{iso} - \omega_0 \Delta \frac{1}{2} (3\cos^2(\theta) - 1 + \eta \sin^2(\theta) \cos(2\phi)) \quad (7)$$

for an x-y asymmetrical shielding tensor. The product $\omega_0 \sigma_{iso}$ is equal to ω_{iso} which is the isotropic chemically shifted frequency of the nucleus. The parameters σ_{iso} , Δ and η are called the *isotropic shielding value*, *anisotropy* and *asymmetry* respectively. They are defined as⁴⁰

$$\sigma_{iso} = \frac{1}{3} (\sigma_{xx}^{PAF} + \sigma_{yy}^{PAF} + \sigma_{zz}^{PAF}) \quad (8)$$

$$\Delta = \sigma_{zz}^{PAF} - \sigma_{iso} \quad (9)$$

$$\eta = \frac{\sigma_{xx}^{PAF} - \sigma_{yy}^{PAF}}{\Delta} \quad (10)$$

Where σ_{xx}^{PAF} , σ_{yy}^{PAF} and σ_{zz}^{PAF} are the maximum values of the chemical shielding tensor in the PAF along the x-, y- and z-axis respectively.

For samples that possess all molecular orientations, often called *powdered samples* as the condition is usually fulfilled by powders, the results of the shielding tensor are parameters directly measurable in an NMR spectrum. The drawback is that when observing NMR spectra, the positions in the chemical shielding PAF that lead to the results are not necessarily known. To account for this ambiguity, it is common to switch back to matrix row-column-indexing when noting CSA results acquired from spectra. With these notations, the chemical shifts shown in Figure 15 below are used to define anisotropy and asymmetry analogously to the above, as in⁴⁰

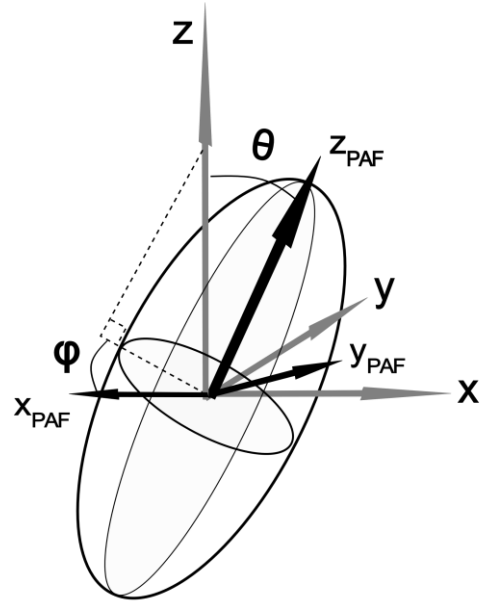


Figure 14: Sketch of a principal axis frame for an anisotropic interaction set within its interaction tensor ellipsoid. The ellipsoid is the tensor, x,y,z are the laboratory frame axis and x,y,z-PAF are the PAF axis. Both frames are internally orthogonal. If $\phi=0$, the tensor is axially symmetrical.

$$\Delta_{cs} = \delta_{11}^{PAF} - \delta_{iso} \quad (11)$$

$$\eta_{cs} = \frac{\delta_{11}^{PAF} - \delta_{22}^{PAF}}{\Delta_{cs}} \quad (12).$$

An obvious application of this effect is to detect if the macroscopic arrangement of shielding tensors changes for some species of nuclei in a sample due to changes in macroscopic conditions. Changes in arrangement can be with respect to axis of symmetry (if the direction of the pattern changes but its form remains roughly constant) or its level of symmetry (if the form of the pattern changes from axially symmetric to axially asymmetric).

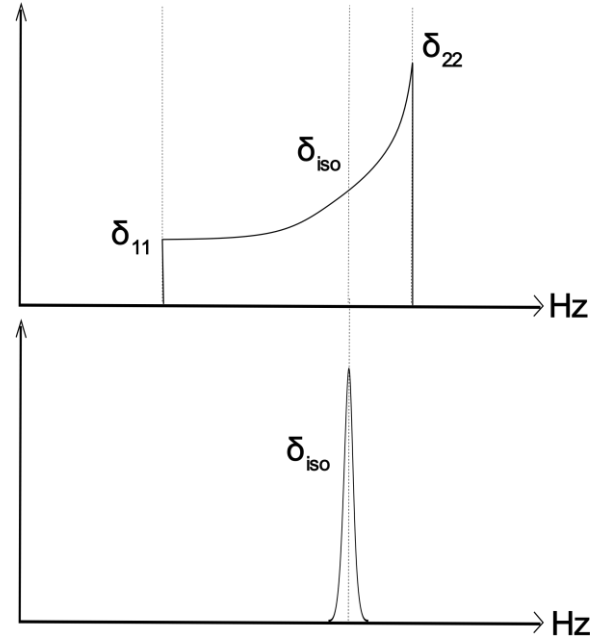


Figure 15: The chemical shifts defining the anisotropy- and asymmetry parameters for chemical shift anisotropy. The top spectrum is a powder pattern for an axially symmetric chemical shift anisotropy tensor. The bottom spectrum shows the location of the corresponding isotropic peak for the same nucleus.

2.3.7.2 Dipolar coupling

In contrast to influencing spin states through covalent bonds via electrons, nuclei can also influence each other directly through space via alignment of their magnetic moments. This is called *dipolar coupling* and requires close proximity in space (lengths on the order of a few Å) as well as slow molecular motion, since different angles of a segment with respect to $\mathbf{B}_0$ attains different efficacies of the dipolar coupling and fast motion will cause motional averaging to zero. ⁴⁰

The dipolar coupling constant is seen in eq.(13)

$$d = \hbar \left(\frac{\mu_0}{4\pi} \right) \frac{1}{r^3} \gamma_I \gamma_S \quad (13)$$

where $\hbar = h/2\pi$, μ_0 is the strength of the dipole moment, r is the internuclear distance and γ_x are the gyromagnetic ratios of the nuclei involved. ⁴⁰ The dipolar coupling constant is once again utilized within a principal axis frame with angles defined in Figure 16 below to give the tensor seen in eq.(14)

$$\mathbf{D} = \left(-\frac{d}{2}, -\frac{d}{2}, d\right) \quad (14).$$

For the influence of dipolar coupling on the Larmor frequency in terms of angular frequency units, the Hamiltonian is (see eq.(15) below). Importantly, spin S acts on spin I in this framework.⁴⁰

$$\hat{H}_{dd} = -2\hat{\mathbf{I}} \cdot \mathbf{D} \cdot \hat{\mathbf{S}} \quad (15)$$

where the vectorial product operators for the spins of the respective nuclei, $\hat{\mathbf{I}}$ and $\hat{\mathbf{S}}$, can be separated into terms that describe the effect of the dipolar coupling on individual *coherences* (also explained in greater detail in a later section) in the series of equations (16) below:⁴⁰

$$\begin{aligned} \hat{H}_{dd} &= -d[A + B + C + D + E + F] \\ A &= \hat{I}_z \hat{S}_z (3\cos^2(\theta) - 1) \\ B &= -\frac{1}{4}[\hat{I}_+ \hat{S}_- + \hat{I}_- \hat{S}_+] (3\cos^2(\theta) - 1) \\ C &= \frac{3}{2}[\hat{I}_z \hat{S}_+ + \hat{I}_+ \hat{S}_z] \sin\theta \cos\theta e^{-i\phi} \\ D &= \frac{3}{2}[\hat{I}_z \hat{S}_- + \hat{I}_- \hat{S}_z] \sin\theta \cos\theta e^{i\phi} \\ E &= \frac{3}{4}[\hat{I}_+ \hat{S}_+] \sin^2(\theta) e^{-2i\phi} \\ F &= \frac{3}{4}[\hat{I}_- \hat{S}_-] \sin^2(\theta) e^{+2i\phi} \end{aligned} \quad (16)$$

where *heteronuclear notation* has been introduced. This notes spins belonging to ^1H with the letter *I* and spins belonging to other nuclei (i.e. *heteronuclei*) with the letter *S*. Homonuclear dipolar coupling simply leads to line broadening and heteronuclear dipolar coupling leads to powder patterns (i.e. spectral patterns resulting from samples which contain all molecular orientations) resembling two opposing axially symmetric CSA patterns seen in Figure 17. Measuring techniques exist to

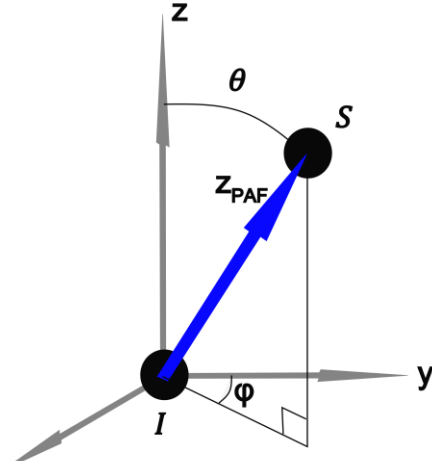


Figure 16: For the dipolar coupling tensor, the z_{PAF} axis and θ angle are defined as for chemical shift anisotropy. Notably, the ϕ angle is defined differently. *I* and *S* are nuclei defined as per heteronuclear notation. The x-y-z-axi are the laboratory frame.

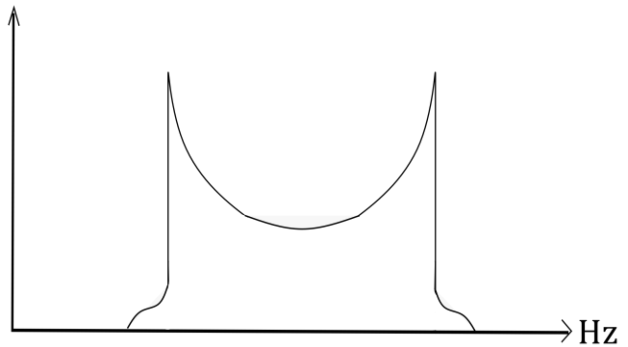


Figure 17: The powder pattern resulting from heteronuclear dipolar coupling. The pattern can be thought of as two opposing chemical shift anisotropy patterns.

measure both these types of dipolar couplings but those techniques are beyond the scope of this study.⁴⁰

Naturally, dipolar couplings may need to be decoupled just like J-couplings. In such a situation, frequencies of roughly three times the decoupling constant are required from the $\mathbf{B}_1$ pulses. In a decoupling context, heteronuclear dipolar interactions are decoupled while homonuclear dipolar couplings mediate the decoupling by causing interactions between on- and off resonance nuclei of the decoupled nuclear species.

2.3.7.3 Quadrupolar coupling

This section warrants a brief explanation of the spin order of nuclei. ^1H and ^{13}C are part of a rare group of nuclei called *spin-1/2 nuclei* which contain charge distributions that do not have electric quadrupole moments. Most NMR-active nuclei (i.e. nuclei which gain Zeeman sublevels under an applied magnetic field) are quadrupolar nuclei. These have a spin number higher than $1/2$, giving them more Zeeman sublevels under $\mathbf{B}_0$. As an example, the Zeeman levels of a spin-1 nucleus is shown in Figure 18 below. Quadrupolar nuclei also possess electric quadrupole moments capable of interacting with any other electric field gradients, i.e. the distribution of other nuclei and electrons around them. This is called *quadrupolar coupling* and inherently grants the nuclei additional sets of energy levels corresponding to these interactions, also shown in Figure 18 below, with notations of the various coherences also indicated.⁴² The interaction can be on the order of MHz and can thus greatly reduce the efficacy of $\mathbf{B}_1$ pulses. A notable exception to this is ^2H which has a fairly weak quadrupole moment.⁴⁰

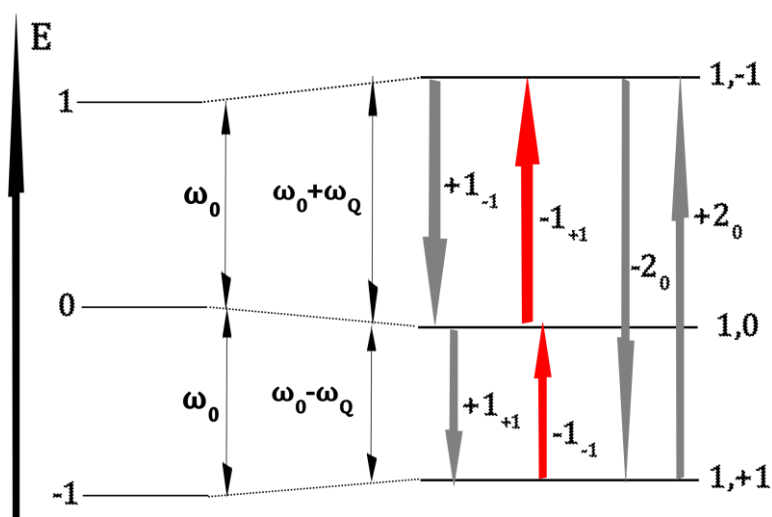


Figure 18: The Zeeman energy levels (far right) and first-order quadrupolar energy levels (far left) for spin-1 nuclei. Energy gaps are indicated by black arrows and their corresponding frequencies are shown to their left, ω_0 is the Larmor frequency and ω_Q is the frequency change due to quadrupolar coupling in the rotating frame. Quadrupolar energy transitions are indicated by grey arrows with their labels to their right. Red arrows indicate transitions resulting in signal-providing coherences.

The Hamiltonian for quadrupolar coupling is (see eq.(17))

$$\hat{H}_Q = \frac{eQ}{2I(2I-1)\hbar} \hat{\mathbf{I}} \cdot \mathbf{V} \cdot \hat{\mathbf{I}} \quad (17)$$

where e is the electron charge, Q is the nuclear quadrupole moment, I is the spin number and $\mathbf{V}$ is the electric field gradient tensor from the surrounding environment at the nucleus.⁴⁰ The principal axis frame for the case of quadrupolar coupling has the z-axis placed along the main direction of the electric field gradient at the nucleus and angles θ and φ defined in the same manner as for dipolar coupling (see Figure (13) above). In this frame, the anisotropy (eq.(18)) and asymmetry (eq.(19)) of the quadrupole moment are

$$eq = V_{zz}^{\text{PAF}} \quad (18)$$

$$\eta_Q = \frac{V_{xx}^{\text{PAF}} - V_{yy}^{\text{PAF}}}{V_{zz}^{\text{PAF}}} \quad (18)$$

Returning to the laboratory frame armed with these parameters, it can be shown⁴⁰ that the full angular dependence of the quadrupolar coupling Hamiltonian can be accounted for by the lengthy expression seen in eq.(20)

$$\begin{aligned} \hat{H}_Q = & \frac{e^2 q Q}{4I(2I-1)\hbar} \left\{ \frac{1}{2} (3\cos^2(\theta) - 1) (3\hat{I}_z^2 - \hat{I}^2) + \frac{3}{2} \sin\theta \cos\theta [\hat{I}_z(\hat{I}_+ + \hat{I}_-) + (\hat{I}_+ + \hat{I}_-)\hat{I}_z] \right. \\ & \left. + \frac{3}{4} \sin^2\theta (\hat{I}_+^2 + \hat{I}_-^2) \right\} \\ & + \eta_Q \frac{e^2 q Q}{4I(2I-1)\hbar} \left\{ \frac{1}{2} \cos 2\phi [(1 - \cos^2\theta)(3\hat{I}_z^2 - \hat{I}^2) + (\cos^2\theta + 1)(\hat{I}_+^2 + \hat{I}_-^2)] \right. \\ & + \frac{1}{2} \sin\theta [(\cos\theta \cos 2\phi - i \sin 2\phi)(\hat{I}_+ \hat{I}_z + \hat{I}_z \hat{I}_+) \\ & \left. + (\cos\theta \cos 2\phi - i \sin 2\phi)(\hat{I}_- \hat{I}_z + \hat{I}_z \hat{I}_-)] + \frac{i}{4} \sin 2\phi \cos\theta (\hat{I}_+^2 - \hat{I}_-^2) \right\} \quad (20) \end{aligned}$$

which, similarly to chemical shift anisotropy, contains a term that is equal to zero if $\eta_Q = 0$ (i.e. if the gradient field tensor is symmetrical in the principal axis frame x-y-plane). The cause for this intricate relation is that quadrupolar coupling of the second order is also governed by a fourth rank cartesian tensor.⁴⁰

For a spin-1 nucleus such as ^2H , this property results in powder patterns similar to that of heteronuclear dipolar coupling shown in Figure 17 above. The distance between the high intensity peaks is equal to $3/4\chi$ where eq.(21)

$$\chi = \left(\frac{eQ}{\hbar} \right) \mathbf{V} \quad (21)$$

is called the quadrupolar coupling constant.⁴⁰

2.3.8 Magic Angle Spinning (MAS)

Solid state NMR is able to mimic the conditions of solution state NMR by rapidly spinning the sample at the particular angle of 54.74° relative to the direction of B_0 in a procedure referred to as *Magic Angle Spinning* (MAS). The choice of angle is due to the $(3 \cos^2 \theta - 1)$ factor seen in many of the terms outlined in the explanations of the anisotropic interactions above. The interactions all have principal axis frames with angles defined relative to the direction of B_0 and are governed by second rank cartesian tensors; $3 \cos^2(54.74) = 1$ which makes the factor go to zero for interaction vectors oriented at-or-near this angle under MAS due to motional averaging. The greater the spinning rate, the greater the efficacy.⁴² Notably, higher order quadrupolar interactions are governed by higher-than-second rank Cartesian tensors which are not affected by the averaging described above.⁴⁰ Quadrupolar interaction is thus only partially averaged by MAS and cannot be eradicated by this method. This is the basis for a number of more intricate MAS experiments using doubly rotating cylinders (beyond the scope of this study) designed to resolve the higher order terms.⁴⁰

2.3.9 Relaxation

The source of the decay seen in the FID is interactions of the local magnetic fields of nuclei with each other and B_0 after B_1 has been switched off. Interactions cause transfer of magnetization which lead to return to equilibrium populations of the sublevels and the decay of coherences, i.e. the magnetic components represented by operators described in a previous section. Principally two forms of relaxation exist: *longitudinal relaxation* where aligning z-components of local magnetic moments resonate and go toward equilibrium z-magnetization and *transverse relaxation* where transverse components of local magnetic moments align to produce or remove coherence on neighboring nuclei. Both these relaxation mechanisms are mediated by dipolar interactions, where transverse relaxation especially is aided by dipolar coupling.⁴²

Relaxation is closely related to molecular motion, particularly molecular rotation as it serves to orient the local magnetic fields. The *rotational correlation time* is the time that it takes for a molecule (approximated as a sphere for the purpose of illustration) to rotate to a 1 rad angle away from its starting position in any direction. The average local magnetic field of a nucleus averages toward zero when viewed over increasingly longer time intervals. This averaging as a function of time interval is described by a *correlation function*. FT performed on the correlation function then gives the *spectral density*, a measurement of how many molecules in a sample rotate at various frequencies. It may take the form

$$J(\omega) = 2\langle B_x^2 \rangle \frac{\tau_c}{1 + \omega^2 \tau_c^2} \quad (22)$$

where B_x is the average mean-square amplitude of a relevant magnetic field.⁴² The spectral density shown above actually corresponds to a simple, exponentially decaying correlation function and is not representative of solids and liquid crystal. Nevertheless, it illustrates the relation with τ_c . Finally, the spectral density gives the *time constants*, i.e. the frequency of occurrence of the relaxation events that transfer magnetization. The time constants are⁴²

$$T_1^{-1} = \frac{3}{10} b^2 \{J(\omega_0) + 4J(2\omega_0)\} \quad (23)$$

$$T_2^{-1} = \frac{3}{20} b^2 \{3J(0) + 5J(\omega_0) + 2J(2\omega_0)\} \quad (24)$$

For longitudinal (T_1) and transverse (T_2) relaxation respectively. Their dependence on the rotational correlation time is illustrated in Figure 19 below. As can be seen T_1 goes through a minimum (i.e. the longitudinal relaxation has a maximum frequency) while T_2 approaches zero for very long correlation times (i.e. transverse relaxation occurs increasingly frequently the slower the molecule rotates).⁴²

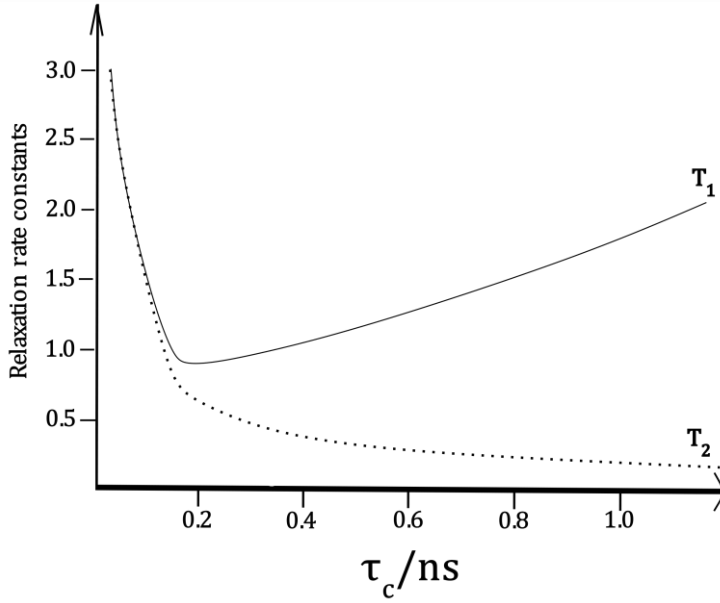


Figure 19: Sketch of the behaviors of the longitudinal relaxation rate constant T_1 and the transverse relaxation rate constant T_2 as a function of the rotational correlation time τ_c .

In eq.(23) and (24), b is the dipole constant between the relevant nuclei, which is the relevant magnetic interaction in this case. It is worth noting that relaxation depends not only on the spectral density of oscillations at the Larmor frequency but also twice the Larmor frequency and zero frequency. This is due to the relationship of rotation of various things and the rotation of the magnetic moment on spin 2 under dipolar coupling. Rotation of the molecule in which the nucleus find themselves rotate the magnetic moment on spin 2 by twice the rotation angle of the molecule (giving a relationship at double the Larmor frequency). Rotation of spin 1 rotates the magnetic moment on spin 2 by the same rotation angle (giving a relationship at the Larmor frequency). Finally, nuclei at equilibrium spin state can still interact with the transverse magnetic moment of excited nuclei (giving a relationship at zero frequency).⁴²

2.3.10 The DP-, INEPT, CP-, and quadrupolar echo pulse sequences

For the writing of Hamiltonians below, it is assumed that $\pi/2$ and π pulses are on-resonance with the nucleus (i.e. the spectrometer channel) that they are applied to. As mentioned previously, this assumption removes the free-evolution term in the Hamiltonians.

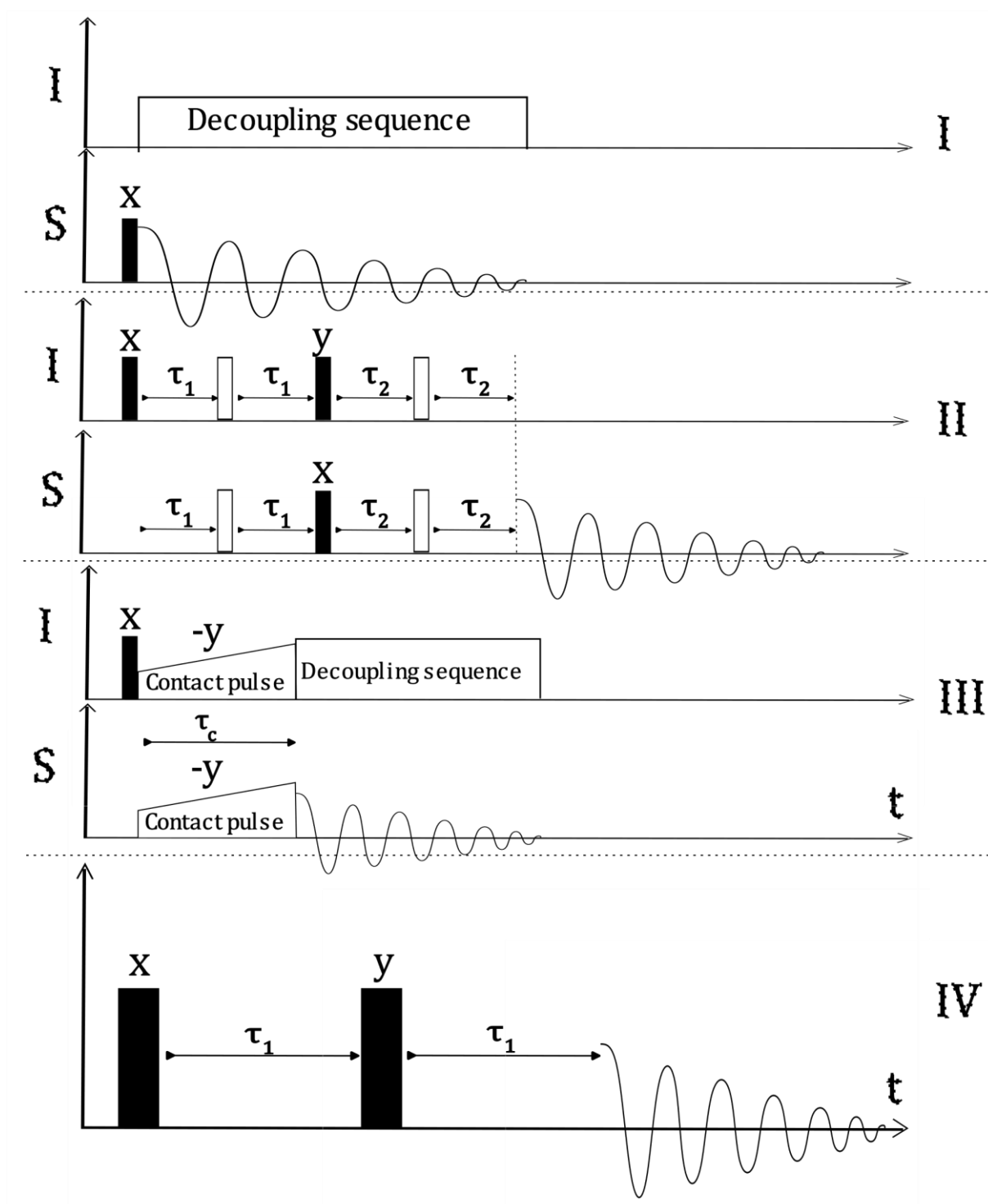


Figure 20: The DP (I), INEPT (II), CP (III) and quadrupolar echo (IV) pulse sequences. Shaded rectangles indicate $\pi/2$ pulses, unshaded rectangles indicate π pulses. Pulse axis is indicated above each pulse. τ -parameters indicate durations. The time axis are not uniformly scaled.

2.3.10.1 Direct Polarization (DP)

The DP-pulse sequence is shown in Figure 20 above. In terms of Hamiltonians, what it accomplishes is to remove the coupling-term seen in eq.(4), reducing the Hamiltonian to that seen in eq.(2) at the time of acquisition. This Hamiltonian contains only an in-phase term and will give observable signal. The result is, depending on the efficacy of the pulse sequence, absence of J-coupling doublets and reduced line broadening due to heteronuclear dipolar coupling.

2.3.10.2 Insensitive Nuclei Enhancement by Polarization Transfer (INEPT)

The INEPT sequence is shown in Figure 20 above. It exploits the evolution of anti-phase terms on one spin into in-phase terms on an opposing spin to generate higher amounts of magnetization than would be possible through pulse-acquire experiments on nuclei that are either dilute, have low gyromagnetic ratios or both. ^{41 42}

It can be shown ⁴¹ that, after the initial $\pi/2$ pulse to the I-nucleus and spin echo (a π -pulse preceded- and follow by equally long time delays), the state of the spin system after expanding the density operator containing the J-coupling Hamiltonian is

$$k_I \cos(2\pi J_{IS}\tau_1) \hat{I}_y - k_I \sin(2\pi J_{IS}\tau_1) 2\hat{I}_x \hat{S}_z \quad (25)$$

where k_I denotes the strength (in arbitrary units) of the initial magnetization on the I nucleus produced by the x - $\pi/2$ -pulse on I. From there, a y - $\pi/2$ -pulse is applied to the I-nucleus and a x - $\pi/2$ -pulse is applied to the S-nucleus. This does not affect the in-phase term of the I-nucleus as it is currently along the same axis, but it does change the I-component of the anti-phase term. Knowing that only in-phase terms on the S nucleus are of interest and that the in-phase term on the I-nucleus can only evolve into anti-phase terms on the S-nucleus, which are not observable anyway, this term can be discarded. The effect of the two different $\pi/2$ -pulses on the anti-phase term is: ⁴¹

$$-k_I \sin(2\pi J_{IS}\tau_1) 2\hat{I}_x \hat{S}_z \xrightarrow{\pi/2 \hat{I}_y} k_I \sin(2\pi J_{IS}\tau_1) 2\hat{I}_z \hat{S}_z \xrightarrow{\pi/2 \hat{S}_x} -k_I \sin(2\pi J_{IS}\tau_1) 2\hat{I}_z \hat{S}_y \quad (26).$$

Starting from the right-hand anti-phase term and letting it evolve under coupling, two new terms are generated after expansion of the density operator:

$$-k_I \cos(2\pi J_{IS}\tau_2) \sin(2\pi J_{IS}\tau_1) 2\hat{I}_z \hat{S}_y + k_I \cos(2\pi J_{IS}\tau_1) \sin(2\pi J_{IS}\tau_2) \hat{S}_x \quad (27)$$

where, notably, one of them is an in-phase term on nucleus S with a k_I -coefficient, meaning that it will possess the magnetic strength of the very first transverse magnetization on the I-nucleus. ⁴¹

2.3.10.3 Cross Polarization (CP)

The CP-pulse sequence is shown in Figure 20 above. It acts by transferring magnetization from one species of nucleus to another via dipolar couplings. Its mode of action is most easily understood when viewing it through a *doubly rotating frame* where both nuclei are simultaneously viewed through a rotating frame with the frequency of their own B_1 pulses. The $\pi/2$ pulse along the x-axis creates transverse magnetization along the -y-axis on the transferring nucleus. Next, -y-axis pulses are applied to both the transferring (I) and receiving nucleus (S) with different, carefully selected pulse powers so as to give them the same nutation frequency, fulfilling what is known as the *Hartman-Hahn condition*. The choice of the -y-axis locks the transferring magnetization along this axis and causes the receiving magnetization to rotate about it, placing the magnetizations perpendicularly to each other. The S-nucleus then experiences the transferring magnetization as an additional B_1 field. This phase of the pulse sequence is called the *contact pulse*. During it, the magnetization created during the initial $\pi/2$ pulse decays into the surroundings due to it being too great for the weaker contact pulse to sustain. The Hartman Hahn condition sets the energy gap for internal spin state transitions equal between the two nuclei, causing a preference for distributing the decaying magnetization onto the receiving nuclei via the dipolar couplings. Using the same notation as in the dipolar coupling sections, the deceptively simple Hamiltonian for the polarization transfer during the contact pulse for an individual pair of dipoles in a doubly rotating frame is

$$\hat{H}_{\text{ddRF}} = -d\hat{I}_z\hat{S}_z(3\cos^2(\theta) - 1) \quad (28).$$

Note that transverse magnetization on I is disregarded as it is not the nucleus of interest for the experiment and that transverse magnetization on S has also been disregarded as it is assumed to be miniscule compared to the enhancement received from the cross-polarization. Generally, the transfer of magnetization improves with increasing *contact time* (contact pulse duration) until a duration of 1-14 ms.⁴⁰ The way in which magnetization transfer efficiency largely increases with time as opposed to having a well-defined optimal contact time is perhaps best understood by viewing the problem within the rotating frame of the I-nucleus. In this frame, the magnetic moment of S not only precesses about the -y-axis at its own ω_1 but also precesses in a negative direction about the z-axis at a frequency which is the difference in frequency between the two respective rotating frames. Thus, with respect to the magnetic field that influences it (the B_1 pulse and the lingering magnetization on I), the receiving magnetic moment performs a complex motion where the z-magnetization of the respective nuclei occasionally align, giving cumulative time-dependent dipolar coupling action at the end of the contact pulse. Keeping in mind that 1-14 ms is thousands of times longer than the durations of $\pi/2$ -pulses (which are on the order of microseconds), the time for the above described magnetization to take place is very long and leaves many chances for alignment of z-magnetizations. The described efficiency increase stops when the cumulative polarization transfer is superseded by a combination of cumulative decay of the transferring magnetization and $T_{1\rho}$ -relaxation, i.e. relaxation mediated by surrounding specimens of nucleus I.

To bridge the problem of sensitivity to imperfect Hartman Hahn matching and the existence of a multitude of receiving nuclei with different ω_0 , a contact pulse of ramped frequency is generally used (also illustrated in Figure 20 above). The ramping should ideally span all ω_0 belonging to nuclei that are expected to be dipole coupled to the transferring species of nucleus. Another important consideration is to choose moderate MAS-speeds since very fast MAS will average the interactions necessary for the signal enhancement.⁴⁰

Both the introduction of a ramped pulse and the introduction of MAS affect the Hamiltonian in eq.(28).⁴⁰ For the purpose of this thesis however, the semi-qualitative description above is deemed sufficient.

2.3.10.4 Quadrupolar echo

The quadrupolar echo-pulse sequence is shown in Figure 20 above. This particular echo sequence is specific for spin-1 nuclei.⁴² The main problem with FIDs from quadrupolar nuclei is that the strong interactions cause them to decay rapidly; they often do not extend past the receiver dead time. Using equally long delay periods lasting at least as long as the receiver dead time before and after the second pulse, an effect similar to that of spin-echo sequences for spin- $1/2$ nuclei is achieved. The difference is that it acts on quadrupolar coherences. The pulse sequence refocuses the desired $-1+$ and $-1-$ coherences of the quadrupolar energy levels, which contain orientational information. Accounting for the coherences can be made more effective by using a combination of arithmetic and matrix notations of the density operators.

In matrix notation, the coherences are sorted as ⁴²

$$\hat{\rho} = \begin{pmatrix} \rho_{|+1\rangle} & \rho_{+1+1} & \rho_{+2_0} \\ \rho_{-1-1} & \rho_{|0\rangle} & \rho_{+1-1} \\ \rho_{-2_0} & \rho_{-1+1} & \rho_{|-1\rangle} \end{pmatrix} \quad (29)$$

It can be shown⁴² that after an initial $\pi/2$ pulse, the state of the entire spin system is

$$\hat{\rho} = \frac{1}{3} \begin{pmatrix} 1 & i\mathbb{B} \frac{1}{\sqrt{2}} & 0 \\ -i\mathbb{B} \frac{1}{\sqrt{2}} & 1 & i\mathbb{B} \frac{1}{\sqrt{2}} \\ 0 & -i\mathbb{B} \frac{1}{\sqrt{2}} & 1 \end{pmatrix} \quad (30)$$

where $\mathbb{B}$ is a Boltzmann factor of

$$\mathbb{B} = \frac{\hbar\gamma B^0}{k_B T} \quad (31)$$

where in turn, B^0 is the local magnitude of $\mathbf{B}_0$, γ is the gyromagnetic ratio of the quadrupolar nucleus, k_B is the Boltzmann constant and T is the system temperature.⁴² Under the assumption that the ensemble of nuclei have the same chemical shift, the effects of the pulse sequence on the Zeeman sublevels can be neglected, setting the matrix diagonal to zero. It can be shown that after a subsequent period τ of free evolution, the state of the spin system in matrix notation is

$$\hat{\rho} = -i \frac{1}{3\sqrt{2}} \mathbb{B} \exp\left(i \frac{1}{4} \omega_Q^{(1)} \tau\right) \begin{pmatrix} 0 & 0 & 0 \\ 1 & 0 & -1 \\ 0 & 0 & 0 \end{pmatrix} - i \frac{1}{3\sqrt{2}} \mathbb{B} \exp\left(-i \frac{1}{4} \omega_Q^{(1)} \tau\right) \begin{pmatrix} 0 & -1 & 0 \\ 0 & 0 & 0 \\ 0 & 1 & 0 \end{pmatrix} \quad (32)$$

where $\omega_Q^{(1)}$ is the frequency of the first order quadrupolar interaction in the rotating frame.⁴² Rotation by the $\gamma\text{-}\pi/2$ pulse yields the subsequent, similar density operator ⁴²

$$\hat{\rho} = -i \frac{1}{3\sqrt{2}} \mathbb{B} \exp\left(i \frac{1}{4} \omega_Q^{(1)} \tau\right) \begin{pmatrix} 0 & -1 & 0 \\ 0 & 0 & 0 \\ 0 & 1 & 0 \end{pmatrix} - i \frac{1}{3\sqrt{2}} \mathbb{B} \exp\left(-i \frac{1}{4} \omega_Q^{(1)} \tau\right) \begin{pmatrix} 0 & 0 & 0 \\ 1 & 0 & -1 \\ 0 & 0 & 0 \end{pmatrix} \quad (33)$$

Now focusing on the -1-coherences exclusively, their individual density operators in arithmetic notation at this stage are ⁴²

$$\rho_{-1-1} = -i \frac{1}{3\sqrt{2}} \mathbb{B} \exp\left(-i \frac{1}{4} \omega_Q^{(1)} \tau\right) \quad (34)$$

$$\rho_{-1+1} = -i \frac{1}{3\sqrt{2}} \mathbb{B} \exp\left(i \frac{1}{4} \omega_Q^{(1)} \tau\right) \quad (35)$$

Multiplication with another free-evolution exponential yields the density operators for these two signal-generating coherences at the time of acquisition as ⁴²

$$\rho_{-1-1} = -i \frac{1}{3\sqrt{2}} \mathbb{B} \exp\left(-i \frac{1}{4} \omega_Q^{(1)} \tau\right) * \exp\left(i \frac{1}{4} \omega_Q^{(1)} \tau\right) = -i \frac{1}{3\sqrt{2}} \quad (36)$$

$$\rho_{-1+1} = -i \frac{1}{3\sqrt{2}} \mathbb{B} \exp\left(i \frac{1}{4} \omega_Q^{(1)} \tau\right) * \exp\left(-i \frac{1}{4} \omega_Q^{(1)} \tau\right) = -i \frac{1}{3\sqrt{2}} \quad (37)$$

which are now notably independent of $\omega_Q^{(1)}$ due to having been refocused. While the second delay must be at least as long as the first, it is normally optimized to a longer duration to let signal acquisition start at the point where the echoes, i.e. the refocused coherences, reach their maximum intensity. ⁴⁰

2.4 Phase identification using relative signal intensity enhancements of ¹³C-NMR techniques

The aforementioned CP and INEPT pulse sequences provide different signal enhancements compared to direct excitation of nuclei (i.e. the DP-sequence). The enhancements are known to be dependent on two properties related to the ¹³C nuclei: relaxation in the form of the rotational correlation time τ_C and molecular segment mobility in the form of the order parameter S , seen in eq.(1). A common interpretation of the degree of order for C-atoms in lipids is how restricted the C-H bonds are. Thus, In this equation for the order parameter (Eq.(1), pp. 10), θ is the angle between the C-H bond vector and the normal of the structure surface in the relevant phase, for instance, the lamellar bilayer phase.

For soft organic materials known to adopt a number of phases, this dependence can be used to assign NMR-spectra containing a type of fingerprint distribution of relative signal enhancements per carbon atom to particular lipid phases seen in Figure 21 below. In particular, CH₂-groups exhibit a range of states of segment mobility that gives rise to a number of characteristic relative signal enhancements. Meanwhile, high CH₃-group mobility (i.e. INEPT signal enhancement) is interpreted in tandem as a general indicator of transition from a solid crystal state to a liquid crystal state. This method of comparing signal enhancements has previously been penned “dynamics based spectral editing”.^{43 44}

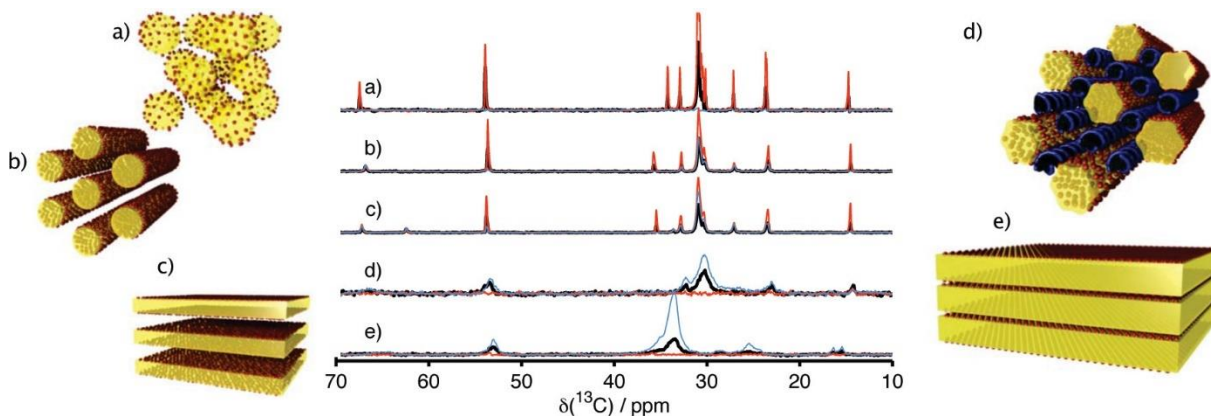


Figure 21: Typical ¹³C-NMR spectra for various phases of a system of the lipid cetyltrimethylammonium and succinate. The phases are a) micellar/cubic or Qm, b) hexagonal or H1, c) lamellar or Lalpha, d) amorphous solid and e) crystalline solid. In the NMR-spectra, black lines are DP signal, blue lines are CP signal and red lines are INEPT signal. Reprinted with permission from Nowacka A., C. Mohr P., Norrman J., W. Martin R., Topgaard D. (2010). Polarization Transfer Solid-State NMR for Studying Surfactant Phase Behavior. *Langmuir* 26, 22, pp. 16848–16856, Copyright 2010 American Chemical Society

Moreover, changes in ppm for CH₂-groups upon melting is expected for lipids containing saturated fatty acid chains. This is due to transition from a state of all-trans conformation (in the solid state) to a mixture of trans- and gauche conformations (in the liquid state).⁴⁵ This change in ppm is shown for a set of triglycerides in Figure 22 to the right.⁴³

For the CP signal enhancement, the approximation (eq. (38)) can be used provided that the relaxation delay used during experiments is adequately long to permit complete T₁ relaxation. Other aspects that follow, pertaining to the observability of the signal enhancement described by the equation, are magnetic field homogeneity (effectively an issue of sufficient sample packing and spectrometer field quality), ¹H-decoupling efficiency and MAS-rate.^{46 47}

$$\frac{I_{CP}}{I_{DP}} = \frac{\gamma_H}{\gamma_C} \frac{\exp\left(-\frac{\tau_{CP}}{T_{1\rho}^H}\right) - \exp\left(-\frac{\tau_{CP}}{T_{CH}}\right)}{1 - \frac{T_{CH}}{T_{1\rho}^H}} \quad (38)$$

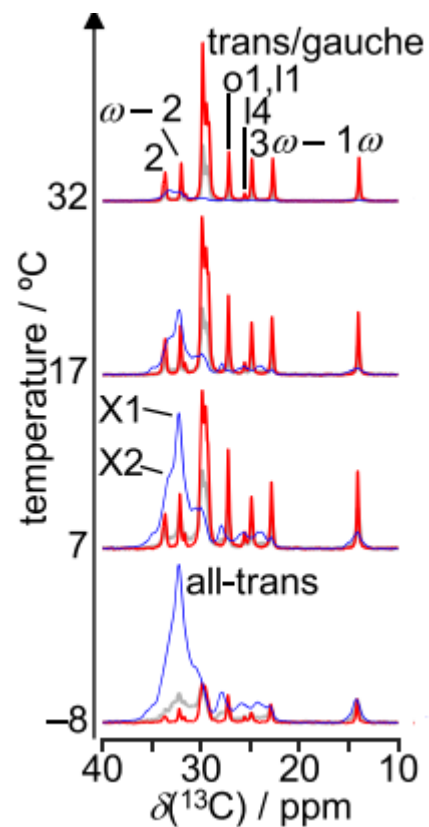


Figure 22: A change in chemical shift due to partial trans-to-gauche conformation change caused by melting. The lipids (triglycerides) were sourced from animal food products. Reprinted with permission from Nowacka A., C. Mohr P., Norrman J., W. Martin R., Topgaard D. (2010). Polarization Transfer Solid-State NMR for Studying Surfactant Phase Behavior. *Langmuir* 26, 22, pp. 16848–16856, Copyright 2010 American Chemical Society

In eq.(38), τ_{CP} is the cross-polarization contact time, T_{CH} is the cross-polarization time-constant, $T_{1\rho}^H$ is the 1H longitudinal relaxation in the rotating frame and γ_H and γ_C are the respective gyromagnetic ratios of 1H and ^{13}C . Rigid sample conditions facilitate direct spatial dipolar polarization transfer, which is expressed as a shorter (lower) T_{CH} and a greater signal enhancement. Mobile sample conditions, by contrast, lead to a longer (higher) T_{CH} and causes the signal enhancement to decay with $T_{1\rho}^H$.⁴³

For INEPT signal enhancement, the signal enhancement can be written as

$$\frac{I_{INEPT}}{I_{DP}} = \frac{\gamma_H}{\gamma_C} \sin(2\pi J_{CH}\tau) \sin(2\pi J_{CH}\tau') \cos(2\pi J_{CH}\tau') \exp\left(-\frac{2\tau}{T_2^H} - \frac{2\tau'}{T_2^C}\right) \quad (39)$$

The enhancement is at its most effective use in liquid samples. In eq.(39), γ_H and γ_C are once again the respective gyromagnetic ratios of 1H and ^{13}C , J_{CH} is the C-H scalar coupling constant (i.e. the frequency of polarization transfer events through covalent bonds), τ and τ' respectively are the two coherence transfer delays in the INEPT pulse sequence and T_2^H and T_2^C respectively are time constants for dephasing events due to transverse relaxation for the two nuclei. The transverse dephasing events are counteracted by adjusting the values of τ and τ' . Long (high) time constants, as is the case in highly liquid sample conditions, gives well-defined values of τ and τ' , namely $1/4 J_{CH}$ and $1/8 J_{CH}$ respectively. By contrast, short (low) time constants, as is the case under rigid sample conditions, causes faster INEPT signal decay and thus reduced signal enhancement.⁴³

Quantitative use of these equations for estimation of expected signal enhancements hinges on the ability to estimate the relevant time constants mentioned throughout the above paragraphs. Those time constants depend on fluctuations in local magnetic fields of the investigated nuclei. For ^{13}C -nuclei in CH_2 -segments, the two bonded H-atoms are the main influence on the local magnetic field. For the time-dependent influence of 1H -atoms on the local magnetic field of a heteronucleus, two-step models sensitive largely to a single order parameter have long been in use.⁴⁸ These models estimate the time-averaged dependence of the local magnetic field via two terms; one for fast, isotropic reorientations and one for slow, anisotropic reorientations as shown in

$$g(\tau) = (1 - S^2)e^{\frac{\tau}{\tau_c}} + S^2e^{\frac{\tau}{\tau_s}} \quad (40)$$

where the latter term depends on a slow, system-representative reorientation time τ_s as opposed to τ_c . The system-representative reorientation may be, for instance, molecular movement between anisotropic sample regions with different orientations. Obviously, describing the whole sample system with only two reorientation motions is not fully representative of reality. It does, however, serve well to illustrate how the utilized techniques rely on different sample dynamic properties for their respective signal enhancements. Under MAS, the time-averaged fluctuation is better described by eq.(41), which introduces a frequency modulation due to the spinning.⁴⁹ In eq.(41), ω_R is the magic angle spinning rate.

$$g(\tau) = \left[(1 - S^2)e^{\frac{\tau}{\tau_c}} + S^2e^{\frac{\tau}{\tau_s}} \right] \left[\frac{2}{3} \cos(\omega_R\tau) + \frac{1}{3} \cos(2\omega_R\tau) \right] \quad (41)$$

Finally, fourier transformation of eq.(41) yields a reduced spectral density $j(\omega)$ in eq.(42) below⁴³:

$$j(\omega) = \left[(1 - S^2) \frac{2\tau_C}{1 + \omega^2\tau_C^2} + S^2 \frac{2\tau_C}{1 + \omega^2\tau_C^2} \right] \otimes \left[\frac{1}{3} \delta(\omega \pm \omega_R) + \frac{1}{6} \delta(\omega \pm 2\omega_R) \right] \quad (42)$$

where " $\otimes$ " denotes a convolution of the function around a central value, similar to NMR-peak splitting around a central value due to J-coupling, otherwise known as convolution. δ denotes the Dirac delta function. The Dirac delta function is defined to have an amplitude only at the quantity inside its parenthesis and zero at all other values. It is to be interpreted as specifying centering frequencies for the convolution peaks.

Eq.(42) allows for estimations of the aforementioned relevant time constant via insertion of frequencies at which the various dipolar fluctuation events in the local magnetic fields occur. For homonuclear dipolar relaxation, static fluctuations (i.e. interaction at $j(0)$) and fluctuations at the Larmor frequency of the nucleus contribute, giving rise to eq.(43) below. Conversely, heteronuclear dipolar relaxation occurs via fluctuations at the Larmor frequency and at the nutation frequency, resulting in eq.(44) below.⁵⁰ Finally, for cross polarization interactions, relevant fluctuations occur at the difference between the nutation frequencies of the involved nuclei leading to eq.(45) below.⁵¹

$$\frac{1}{T_2^X} = \frac{\gamma_X^2 b^2}{2} [j(0) + j(\omega_0^X)] \quad (43)$$

$$\frac{1}{T_{1\rho}^X} = \frac{\gamma_X^2 b^2}{2} [j(\omega_1^X) + j(\omega_0^X)] \quad (44)$$

$$\frac{1}{T_{CH}} = \frac{\gamma_C^2 b^2}{2} j(\omega_1^H - \omega_1^C) \quad (45)$$

In the above equations, b denotes the local magnetic field at the ^{13}C nucleus, X is an index denoting some nucleus in question (in this case ^{13}C or ^1H), ω_1^X denotes the nutation frequency of said nucleus and ω_0^X denotes the Larmor frequency of said nucleus.

The implications of this model for the signal enhancements in question are as follows. The maximum spectral density and thus the maximum signal intensity can be found at $\omega=0$ for static experiments and $\omega=\omega_R$ as well as $\omega=2\omega_R$ for MAS experiments. For INEPT, which merely relies on coherence transfer for its signal enhancement and only has that signal enhancement reduced by homonuclear dipolar relaxation, optimization occurs by finding the correct transfer delays τ and τ' . For cross polarization, signal enhancement is optimized by matching the MAS rate and the difference in nutation frequency between the ^1H and ^{13}C nuclei.

Armed with equations for estimating the relevant time constants, a map of theoretical signal enhancements as a function of the aforementioned order parameter S and rotational correlation time τ_C can be calculated for some assumed experimental values of b , τ , τ' , J_{CH} , τ_{CP} , ω_1^H , ω_1^C , ω_0^H and ω_0^C .

2.5 Phase identification using the width and pattern of D₂O quadrupolar splitting patterns in static ²H-NMR

²H is a quadrupolar nucleus and may exhibit quadrupolar splitting patterns in NMR spectra. The width in kHz of the quadrupolar splitting (interpreted as the distance in kHz between the characteristic "horns" of the spectral pattern) depends on how restricted the movement of the molecule containing the ²H nucleus is, as well as the orientational freedom of the molecule (i.e. its anisotropy), here expressed as the order parameter based on the angle between the D-O bond vector and the normal of the surface of the fatty acid chain (the model is explained further over the course of this section). The dependence leads to recurring splitting parameters (the aforementioned widths) and splitting patterns. When studying D₂O as it is incorporated into to phase structures, the dependence is due to hydrophobic interactions with the non-polar segments of the surfactants around it. The patterns are indicative of particular phases and are shown in Figure 23 below. For instance, a total width of about 4 kHz (corresponding to a 2 kHz distance between the "horns") is indicative of a lamellar phase at maximum bond ordering (i.e. a small angle θ) for nonionic surfactants.^{52 53} Phospholipids could exhibit different values.

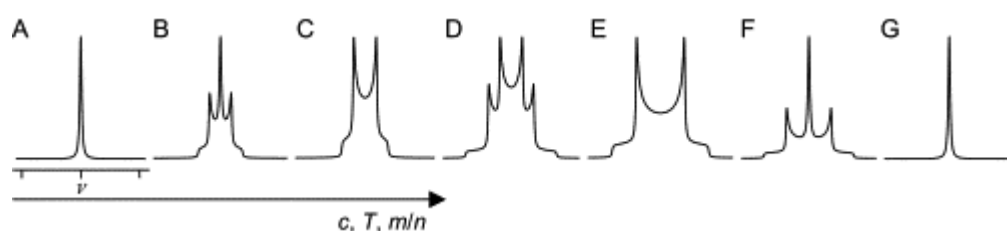


Figure 23: Quadrupolar splitting patterns representative of nonionic surfactant phases. Changes in concentration (c) and temperature (T) or elapsed time (min) typically cause a pattern to transition to its neighboring pattern. The phases from left-to-right are one-phase micellar/cubic, two-phase micellar/cubic and hexagonal, one phase hexagonal, two-phase hexagonal and lamellar, one-phase lamellar, two-phase lamellar and reverse micellar/cubic and finally one-phase reverse micellar/cubic. Reprinted with permission from Ferreira T.M., Bernin D., Topgaard D. (2013). Chapter Three - NMR Studies of Nonionic Surfactants. *Annual Reports on NMR Spectroscopy* 79, pp. 73-127. Copyright 2013 Elsevier.

Similar to the utilization of the C-H bond vector angle order parameter pertaining to signal enhancement for the ^{13}C -NMR techniques, describing the environment that governs the order of the D_2O -molecule in terms of a set of angles is convenient. The relevant directions in this framework are **I**, the direction of the main magnetic field of the instrument (also referred to as the laboratory direction), **m**, the ^2H -O bond direction, **n**, the direction that is normal to the hydrophilic-hydrophobic interface of the phase structure and **d**, the main symmetry axis direction of the phase as a whole. The directions are illustrated in Figure 24 below.⁵³

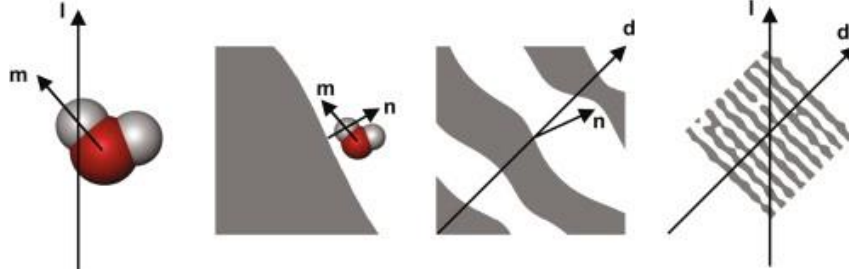


Figure 24: Directions (represented by arrows) used to define angles featured in order parameter definitions for the binding of D_2O in lipid phases. **I** is the main system direction, **m** is the D-O bond direction, **n** is the direction normal to the fatty acid chains and **d** is the direction of propagation in the observed crystallite. Index letters next to each arrows are used for indexing of angles and order parameters pertaining to those angles throughout this section. Reprinted with permission from Ferreira T.M., Bernin D., Topgaard D. (2013). Chapter Three - NMR Studies of Nonionic Surfactants. *Annual Reports on NMR Spectroscopy* 79, pp. 73-127. Copyright 2013 Elsevier.

Quadrupolar splitting pattern widths in kHz ($\Delta\nu_Q$) in anisotropic liquid crystals are determined by

$$\Delta\nu_Q = \frac{3}{2} \chi \overline{P_2(\cos \theta_{\mathbf{ml}})} \quad (46).$$

The overbar denotes a time-averaging and χ is the frequency of quadrupolar interactions in D_2O . For the contrasting case of an isotropic liquid, the order parameter $S_{\mathbf{mn}}$ is time-averaged to zero, leading to the absence of quadrupolar splitting. For anisotropic liquid crystals, $\theta_{\mathbf{ml}}$ time-averages to a non-zero, unclear value since the molecules maintain extensive yet restricted movement.

In order to make the model quantitatively useful, one can extend the expression of the quadrupolar splitting from dependence solely on $\theta_{\mathbf{ml}}$ to dependence on $\theta_{\mathbf{mn}}$, $\theta_{\mathbf{nd}}$ and $\theta_{\mathbf{dl}}$ and thus the order parameters related to them via

$$\Delta\nu_Q(\theta_{\mathbf{dl}}) = \frac{3}{2} \chi \frac{n_b c_s}{c_w} S_{\mathbf{mn}} S_{\mathbf{nd}} P_2(\cos \theta_{\mathbf{dl}}) \quad (47)$$

The order parameter $S_{\mathbf{dl}}$ is expressed in terms of the second order Legendre polynomial, as in eq.(1).

In eq.(47), n_b is the molar amount of water per fatty acid chain, c_s is the concentration of surfactant (in this case lipid) and c_w is the concentration of water. In this extended model, one introduces the assumption that no exchange takes place between differently oriented microcrystallites in the material, meaning that $\theta_{\mathbf{dl}}$ only takes a single value for each observed D_2O -molecule, fixing the value of the order parameter $S_{\mathbf{dl}}$. One more order parameter needs to have a fixed value in order to permit quantitative estimations. This order parameter is $S_{\mathbf{nd}}$, which inherently is equal to 1 for the lamellar phase, -1/2 for the two hexagonal phases (normal and inverted) and 0 for isotropic phases (including micelles and cubic structures) and thus does not change for any one D_2O molecule, since it is only present in one phase at a timescale longer than the frequency of the quadrupolar interaction.

Eq.(47) expresses the quadrupolar splitting for any one angle θ_{dl} . The most likely θ_{dl} for a molecule to experience is $\pi/2$, owing both to the fact that a microcrystallite oriented in a plane perpendicular to the axis l maintains the same θ_{dl} when rotated about the l axis and that a microcrystallite oriented in any other plane with respect to l will have an angle θ_{dl} of $\pi/2$ at two points when rotated about the l axis. This leads to the occurrence of two "horns" of higher intensity at the quadrupolar splitting corresponding to $\theta_{dl} = \pi/2$. Taking this into account along with previous assumptions gives the more compact equation in

$$\Delta v_{Q,\pi/2} = \left| -\frac{3}{4} \chi \frac{n_b c_s}{c_w} S_{mn} S_{nd} \right| \quad (48)$$

for estimation of expected "horn-to-horn distance" in quadrupolar splitting patterns for D₂O. Estimation using eq.(48) with some low⁵⁴ (but importantly nonzero) assumed value of S_{mn} , corresponding to water bound at the hydrophilic-hydrophobic interface, is what yields values for the $\theta_{dl} = \pi/2$ quadrupolar splitting of approximately 2 kHz mentioned previously.

It is important to stress that S_{mn} is difficult to quantify even theoretically for many materials, making it difficult to predict a specific expected value of quadrupolar splitting before experiments. Thus, to reliably determine the phase behavior of a material, especially with respect to possible transitions between lamellar and hexagonal phases, several experiments at different temperatures and water concentrations are needed. The aforementioned transition can then be observed as a sudden, as opposed to gradual, change in $\Delta v_{Q,\pi/2}$ owing to a change in S_{nd} from 1 to $1/2$ or vice versa (this transition may still occur via a two-phase pattern). Gradual change merely indicates slight increases or decreases in ²H-O bond orientation order within the present phase(s) due to the aforementioned changes in temperature and/or water concentration. Common phase transitions and their theoretical resulting ²H NMR spectra are shown in Figure 23.

As an alternative to D₂O, deuterated species of phospholipids can be used to similar effect, yielding the same types of spectral patterns but with considerably different values of $\Delta v_{Q,\pi/2}$, thus requiring adjusted models.⁵³

2.6 Phase identification using chemical shift anisotropy in static ^{31}P NMR

In many materials, ^{31}P exhibits strong chemical shift anisotropy in the solid state, owing to its high number of electrons and thus have its chemical shielding tensor asymmetrized. Clearly, in molecules with the ability to aggregate in various phases (such as phospholipids), different molecular segments may position themselves in a variety of ways and experience a range of molecular mobilities.³³ In such an environment, the axial dependence of the chemical shift of ^{31}P becomes highly apparent in NMR spectra.⁵⁵ For typical phospholipid phases, this results in characteristically broad and asymmetrical peak shapes. Specific shapes are recurring for each phase, since they represent different axial restrictions of freedom of movement.⁵⁶⁻⁵⁷ These shapes are shown in Figure 25 above. Most notably, lamellar and hexagonal phases can be distinguished by

the ppm-direction and width of the "shoulder" of the pattern in conjunction with the ppm position of the high-intensity peak within the pattern. The simultaneous occurrence of two phases result in spectra of mixed character. These often effectively appear as a shoulder in either direction depending on which phase is the most prevalent, together with the existence of two high-intensity peaks. Though the technique is well-established, a lingering limitation is that for mixtures of similar molecules, molecules present in lower quantities may have their spectral patterns obscured by the main sample constituent. I.e., superpositioned patterns may not be resolved.⁴⁰

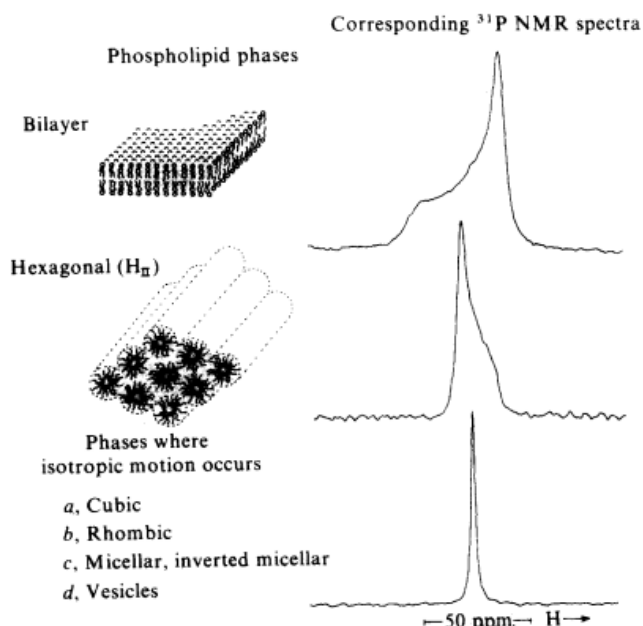


Figure 25: ^{31}P NMR spectra representative of the L_α (top), H_{II} (middle) and the L_1 , L_2 and I_1 phases (bottom). Reprinted with permission from P.R. Cullis, De Kruijff B. (1979). Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 559, 4, pp. 399-420. Copyright 1979 Elsevier

3 Materials and methods

3.1 Sample preparation

Powdered Polar Brain Lipid extract (pPBLe) was purchased from Avanti Polar Lipids, Inc, Birmingham AL. Samples were prepared by transferring pPBLe from the sealed glass vials from the manufacturer into Eppendorf tubes. Each Eppendorf tube was weighed before and after transfer on a Precisa-type scale to determine the mass amount of pPBLe that had been transferred. A 50/50 amount of D_2O and H_2O by mass was then added to meet the desired water content per dry weight of each sample based on the previously transferred mass amount of pPBLe (which was approximated as dry weight). The goal of sample A9 was to achieve water saturation of the sample at room temperature. To that end, water was added in a controlled fashion until the characteristic pale yellow color of the hydrated extract changed to a pale translucent appearance. The samples were stirred in the Eppendorf tubes using a spatula to facilitate

equilibration. Then, each sample was passively equilibrated in its Eppendorf tube at 4°C (277 K) for a minimum of two weeks. Sample preparation was carried out at room temperature in ambient air.

The samples were transferred to plastic rotor inserts for 4mm Bruker ZrO₂ rotors using Sterican-type needles as picking tools. Between 20 and 30 mg of material was able to be transferred into inserts for all the samples. Samples were packed inside of the rotors using a flat-head hexagonal key to combat the formation of air bubbles, though the elastic and sticky nature (attributes that increased with increasing water content) of the hydrated pPBL_e caused new air pockets to form upon the retraction motion of each compaction. The packing process for each sample was halted upon perception of a state of minimum bubble formation.

Additionally, a sample of pPBL_e with no added water was prepared for the purpose of checking for possible degradation occurring in the other samples during their equilibration time. The sample could also provide information on material behavior at very low water content and an estimate of how much moisture is absorbed during preparation of a sample at ambient conditions. Lipids were transferred directly from the sealed shipping vials into 4 mm Bruker plastic rotor inserts using the same procedure and conditions as above. The transfer and packing of the sample and the sealing of the rotor insert was completed within 23 minutes and 13 seconds.

3.2 NMR measurements

Spectra were recorded on a 500 MHz Bruker Ultrashield spectrometer operating at the Larmor frequencies $\omega_0/2\pi = 500.13$ MHz for ¹H, $\omega_0/2\pi = 202.45$ MHz for ³¹P, $\omega_0/2\pi = 125.77$ MHz for ¹³C and $\omega_0/2\pi = 76.77$ MHz for ²H.

The spectrometer was calibrated for MAS-experiments using KBr and adamantane standards. First, the instrument angle is varied while rotating a KBr standard at 5 kHz. KBr was used due the resonance frequency of ⁷⁹Br being close to that of ¹³C and the absence of quadrupolar coupling due to the FCC structure of the material. The latter helps yielding narrow lines in the frequency domain. The angle resulting in observation of the highest number of rotational echoes of the highest amplitude was selected for MAS. Next, pulse-acquire experiments on an adamantane sample were used to find resonance frequencies and 90° pulse lengths for ¹H and ¹³C. Finally, a CP/MAS experiment was performed on the adamantane standard to confirm that CP signal could be acquired. The choice of adamantane as the standard is due to facile averaging of ¹H homonuclear dipolar interactions at moderate spinning rates and a tendency for strong CP signal with a low number of scans.⁵⁸

For MAS-experiments, ¹³C spectra were recorded on a 4 mm HX CP-MAS probe of type Efree using the DP, CP and INEPT techniques (pulse sequences shown in Figure 20) described in the preceeding section at 5 kHz MAS with a 100 ms acquisition time, a 5 s relaxation delay and 256 scans. The spectral width was 248 ppm. Also on the Efree probe under 5 kHz MAS, ³¹P DP- and CP spectra were recorded using a 100 ms acquisition time, a 5 s relaxation delay and 128 scans. The spectral width was 494 ppm. Finally, ¹H spectra using a pulse-acquire sequence with 0.4 s acquisition time, a single scan and a spectral width of 500 ppm were recorded, also under 5 kHz MAS.

For the ^1H -decoupling used in all the DP, CP and INEPT measurements, a 62 kHz SPINAL-64 sequence⁵⁹ was used. For CP experiments, a ramped CP sequence ramping down from 72 kHz to 40 kHz was used with a 1 ms contact time along with a nutation frequency of 80 kHz for ^{13}C and ^{31}P . For INEPT measurements, a 1.8 ms delay was used before and after the first 180 degree pulse on the ^1H channel and a 1.2 ms delay before- and after the second one. For hard RF pulses, a nutation frequency of 80 kHz was used for ^{31}P , ^{13}C and ^1H .

For static ^2H and ^{31}P spectra, a broadband (BBO) probe with separate ^1H channel was used. The broadband channel was tuned to ^{31}P or ^2H depending on the experiment. A pulse-acquire sequence without high-power ^1H -decoupling was used for ^{31}P experiments and a quadrupolar echo sequence was used for ^2H experiments. Due to time constraints, no static experiments were performed for the sample with 150 wt% water by dry weight lipid.

All experiments were carried out at variable temperature starting at instrument temperatures 263 K through 343 K in 5 K increments. The temperatures of 263 through 273 K were omitted for the sample of 150 wt% water by dry weight of lipid due to concerns of rotor cap ejection during MAS due to freezing. Upon reaching a target temperature the samples were allowed 10 minutes of equilibration time before measurements proceeded. Due to technical difficulties regarding cooling, 263 K and 268 K spectra were recorded last under supervision when required. 298 K spectra were recorded before and after each experiment series to check for changes in the sample and spectrometer drift due to the temperature ramping. For each temperature, the ^{13}C and ^{31}P spectra were recorded in a sequence preceded by- and followed by a ^1H spectrum as a means of monitoring for chemical degradation. Including the temperature ramping and all experiments described above, a typical MAS experiment series for one sample lasted about 28 hours.

For static experiments, actual temperatures were calibrated using water-free reference samples of methanol for the temperature range 263-300 K and glycol for the temperature range of 300-343 K. In lieu of water-free methanol- and glycol samples for MAS-experiments, samples in 4 mm rotor inserts were prepared and a separate calibration curve was established for those samples via experiments on the BBO probe. The difference in calibrated temperature compared to the water free sample turned out to be linear. This difference was thus assumed to originate from a constant displacement in chemical shift of the OH-peak in the respective samples, brought on by the influence of sample water content. Under this assumption, temperatures were calibrated on the Efree probe using the water-containing samples and the calibrated temperature difference from the BBO calibration experiments was subtracted from the MAS-calibrated temperatures. Due to technical circumstances related to cooling, an elevated cooling gas flow rate was used to achieve low temperatures. Over the course of the temperature series, gas flow rate was then variably ramped back down to normal flow rate, resulting in an additional source of error. To account for this, temperature calibration was carried out at multiple flowrates for every instrument temperature, spanning the lowest and highest flow rates recorded at each temperature during experiments on PL samples. The maximum difference in calibrated temperature due to flow rate recorded for any one set temperature was 2.68 K. The precision of the temperature calibration method varied across the temperature range with the poorest precision recorded being 0.26 K. Finally, evaluation of the temperature equilibration over time added another potential 0.4 K of error. Summarizing these errors gives an effective temperature error margin of +/- 3.4 K for both the Efree and BBO probe. For both the Efree- and BBO probe, temperatures were typically calibrated as soon as a stable instrument temperature was reached. Temperature calibration data and figures are presented in Appendix A1.

For the sample with no added water, the full MAS experiment series as described above was completed within 34 hours of the rotor insert being sealed. Static experiments were carried out after two weeks.

Relative molar contents of fatty acid chains and water for each sample were estimated by peak integration of terminal methyl- and water peaks respectively in ^1H spectra. For the relative molar content estimation, equation (49) below was used⁴⁴ where A_w is the area of the water peak and A_m is the area of the terminal methyl peak. Normally, the area of the water peak would be divided by two since H_2O contains two hydrogen atoms. However, since half of the added liquid by weight was in the form of D_2O (which has a molar mass of about 20.03 g/mol compared to 18.02 g/mol for water) and the signal, the ^1H peak corresponding to water only represents half of the added liquid. Thus, a factor of 0.95 is used instead to account for the presence of a nearly equal amount (by mole) of undetected heavy water. The 343 K post-series ^1H spectra of each sample were used for the estimation. Via comparison with spectra from other samples and resolution enhancement (usage of various negative line broadening values) it was determined that no water peak could be resolved for the sample with no added water. An integration of the plateau area of about 0.1 ppm width around the central ppm-value of the water peak in higher water content samples was used instead to give an estimate of the highest possible water content in the sample. Using the same method, molar content of cholesterol per mol PL was estimated by assuming that each PL contains two terminal CH_3 -groups on average, enabling calculation of a peak area ratio between the PL terminal CH_3 -peak and the chol 19-peak. The content was estimated using the 343 K spectrum for extract with no added water.

$$\frac{\eta_w}{\eta_m} = \frac{A_w * 0.95}{A_m/3} \quad (49)$$

$$\frac{\eta_{chol}}{\eta_m} = \frac{A_{chol}}{A_m/2} \quad (50)$$

where A denotes area and w, m and $chol$ indexes water, methyl (terminal CH_3) and cholesterol respectively. For processing, zero filling was performed to 8x the original size of the FID for all experiments. The FID was then multiplied by an exponential window function according to the matched filter principle, yielding line broadening of 10 Hz for ^{13}C and ^{31}P and 2.5 Hz for ^1H for data from MAS-experiments. For static experiments, line broadening of 19.53 Hz for ^{31}P and 32.55 Hz for ^2H was used according to the same principle. Automatic phase correction and baseline correction was performed on all spectra except static ^{31}P spectra, which were corrected manually. For spectra where automatic correction was visibly insufficient, processing was re-done with manual correction. All spectral processing was performed in Bruker Topspin 4.3.0.

Peak assignment was done based on previous NMR work on the known constituent phospholipids^{25 33 38 60 61 62 63 64}. ^{13}C and ^1H spectra were able to be assigned using pertinent work on similar substances while ^{31}P was inferred based on reported relative phospholipid contents from the manufacturer and reported relative chemical shifts in a solvent different to the one used in this study^{65 66}. For ^{13}C , the 343 K DP-, CP- and INEPT spectra of the 30 wt% sample was used as basis for assignment. For ^{31}P , the MAS-spectrum at 298 K of the 50 wt% sample was used. Finally, for ^1H , MAS-spectrum at 343 K of the 20 wt% sample was used. These spectra showed the best resolved peaks and, in the case of ^{13}C , the highest signal-to-noise ratio.

Peak referencing was performed internally by setting the CH₃ peak to 14 ppm for ¹³C and 0.9 ppm for ¹H. For ³¹P, the largest peak (PE) was set to 0 ppm.

4 Results and Discussion

4.1 Peak assignment

Images in this section were produced using MATLAB⁶⁷. For images containing NMR spectra, RBNMR⁶⁸ was used to import data from NMR files to MATLAB.

Labeled ¹³C-, ³¹P- and ¹H-spectra based on previous work are shown in Figure 26 below. Detailed labeling of the ¹³C spectrum is presented in Appendix A2.

The ¹³C results featured peak distributions congruent with previously documented ¹³C NMR experiments on PLs (sections 2.2.5 pp.10 and section 3.2 pp.40) except for two cases. The unidentified peak cluster at 146 ppm features chemical shift values comparable to that of carbon bond configurations of an OH-group bonded to an aromatic carbon seen in molecules such as rosmarinic acid⁶⁹. Such a configuration could potentially be seen on an unknown cholesterol derivative contained in the sample. The non-negligible DP signal in the range of 100-110 ppm is presumed to belong to anomeric carbons on sugar moieties in ganglioside species.⁷⁰

Notably, the resolved ³¹P-MAS peaks at positive ppm values do not match either of the PLs declared in the table of contents from the producer. These peaks (designated by asterisks) may belong to lysophosphatidylethanolamine in its two conformations (LPE and 2-LPE) as well as cardiolipin (CA) (section 3.2 pp.40). LPE is PE with only one fatty acid group on the glycerol backbone. CA is a GPL with no head groups, instead having two phosphate groups on the 1- and 3-positions of its glycerol backbone, each connected to a phosphatidyl group.

For ¹H, most peaks were able to be labeled barring ambiguity within clusters known to correspond to certain bond configurations. The head groups expected from the composition summary were able to be identified. One notable unidentified peak was found. It is presumed to belong to the same configuration as the unidentified ¹³C peak (section 3.2 pp.40).

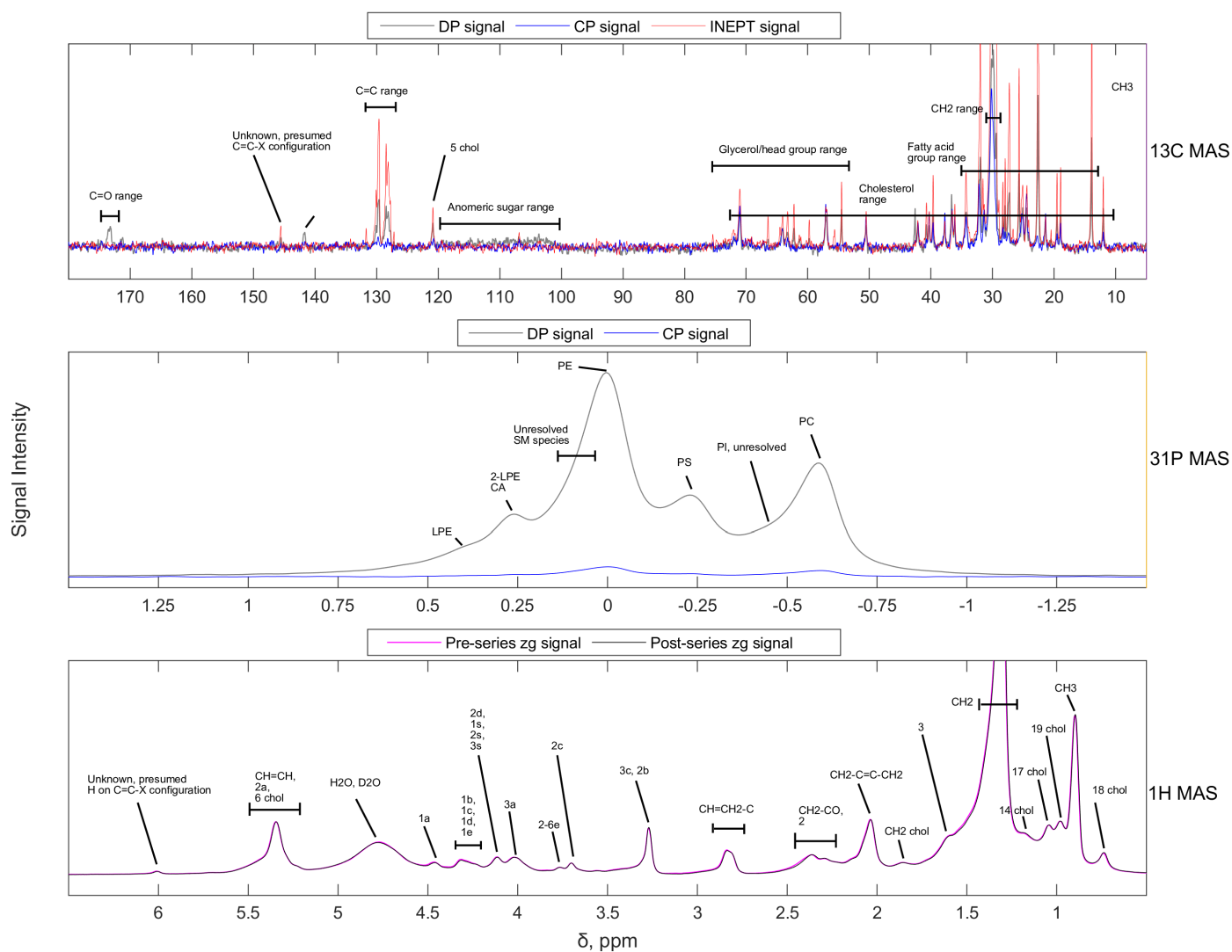


Figure 26: Peak assignments for the pPBL. Aside from previously annotated PLs, LPE and 2-LPE denotes 1- and 2-conformations of lysophosphatidylethanolamine while CA denotes cardiolipin. Spectra were recorded at 343, 298 and 343 K respectively from top to bottom. The water contents were 30%, 50% and 20 wt% water per dry weight lipid respectively from top to bottom. The intensity axis of the ^{13}C and ^1H spectra are scaled for visibility.

4.2 ^1H -NMR measurements

^1H -MAS spectra for a series of key temperatures are shown in Figure 27 below while a zoomed-in series of spectra featuring resolved peaks are shown in Figure 28. All zoomed-in ^1H -measurements are shown in Appendix B1 for quality-control purposes. The overlap of black lines (pre-series measurement) and red lines (post-series measurement) both throughout the temperature series and the series of water contents indicates consistent sample conditions and absence of chemical degradation during experiments. Spinning sidebands occur even at 343 K for all water contents, indicating that neither sample reached a liquid state (as opposed to liquid crystalline) across the temperature range (section 2.3.7, pp.18). At 298 K, only the sample free from added water shows a considerable band of peaks while at 308 K, only line broadening due to solid conditions (i.e. homonuclear dipolar interactions) remains for all water contents. The absence of sidebands in favor of a solid peak band (with a total width of about 160 ppm at the lowest water content and temperature) for all water contents at 263 K indicates that all samples were solid at this temperature, presumably simply due to subzero conditions. Meanwhile, the distinct occurrence of sidebands at temperatures of 278 K and higher for water contents of 20 wt% and higher indicates that a water content between 10 wt% and 20 wt% sees an onset of formation of water-binding liquid crystals. There is some change in the central ppm-value for the water-peak across the temperature range. Since the change evident at maximum temperature happens both for samples that will be shown to adopt a H_{II} and L_{α} phase respectively, this is thought to be due to slight temperature dependence of the hydrophobic interactions in the liquid crystal structures. Finally, the absence of a sharp peak corresponding to isotropic water even at the highest water content and temperature investigated (the entire peak structure at 4.7 ppm exhibits line broadening) suggests that no separate phase of pure water was observed in this study.

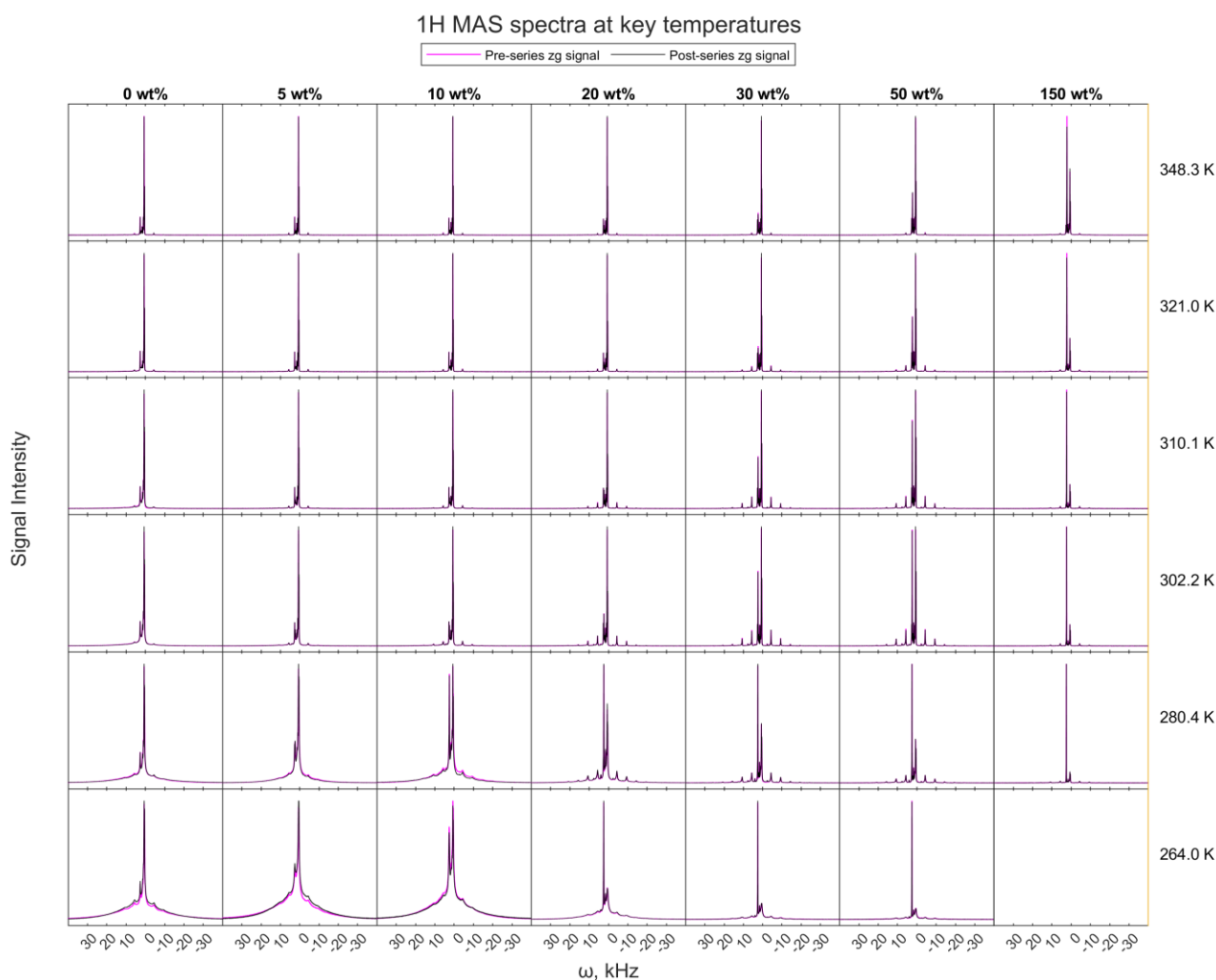


Figure 27: ¹H MAS-NMR spectra for a series of key temperatures for all water contents with water contents above each column and temperatures displayed to the right. Black lines represent the spectrum recorded before each experiment series and red lines represent the spectrum recorded after each experiment series. Overlap of black and red lines indicate consistent sample conditions throughout the experiment series. Disappearance of peak bands indicate that the transition from solid to liquid crystalline phases occurs at about 298 K for water contents of 10 wt% by dry weight lipid and lower while it may occur as low as 278 K for water contents of 50 wt% by dry weight lipid. For 150 wt% water by dry weight lipid, scaling to the highly intense water peak makes the sidebands very small compared to the main peak cluster and thus invisible in full intensity view. The existence of sidebands even at 343 K for all water contents means that in the investigated temperature range, the PLs in the extract only melt to an anisotropic liquid crystalline state as opposed to any isotropic liquid state.

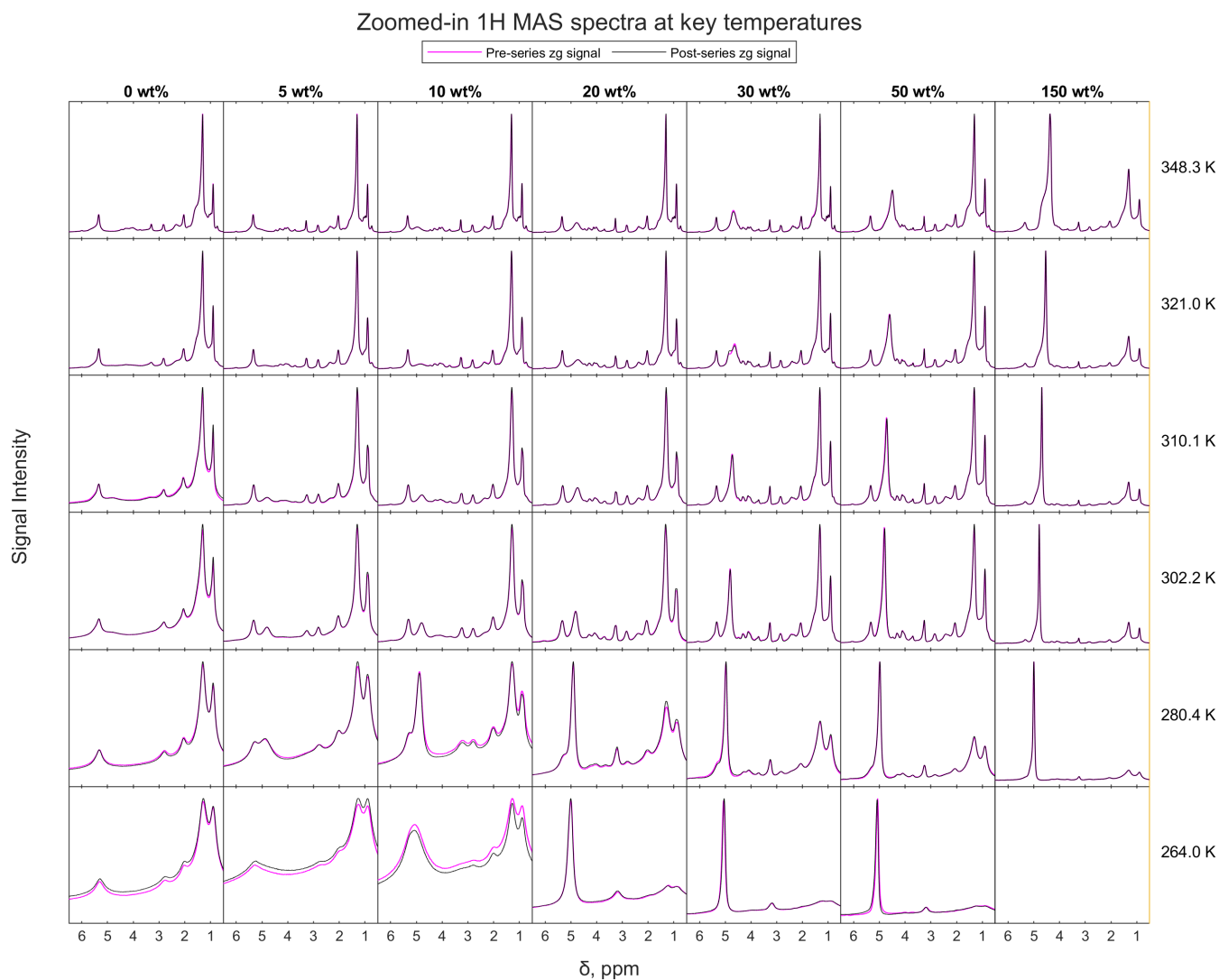


Figure 28: Zoomed-in ^1H MAS-NMR spectra for a series of key temperatures for all water contents with water contents above each column and temperatures displayed to the right. The intensity axis of each spectrum is individually scaled to 110% of the intensity of the tallest peak in the spectrum. Black lines represent the spectrum recorded before each experiment series and red lines represent the spectrum recorded after each experiment series. Overlap of black and magenta lines (seen as visibility of the black graph only) indicates consistent sample conditions throughout each experiment series. The presence of line broadening at all temperatures means that in the investigated temperature range, the PLs in the extract only melt to an anisotropic liquid crystalline state as opposed to any isotropic liquid state.

4.3 ^{13}C -NMR measurements

^{13}C -MAS spectra for the same series of key temperatures as for the ^1H -measurements are shown in Figure 29 below. All ^{13}C spectra are shown in Appendix B2 for reference.

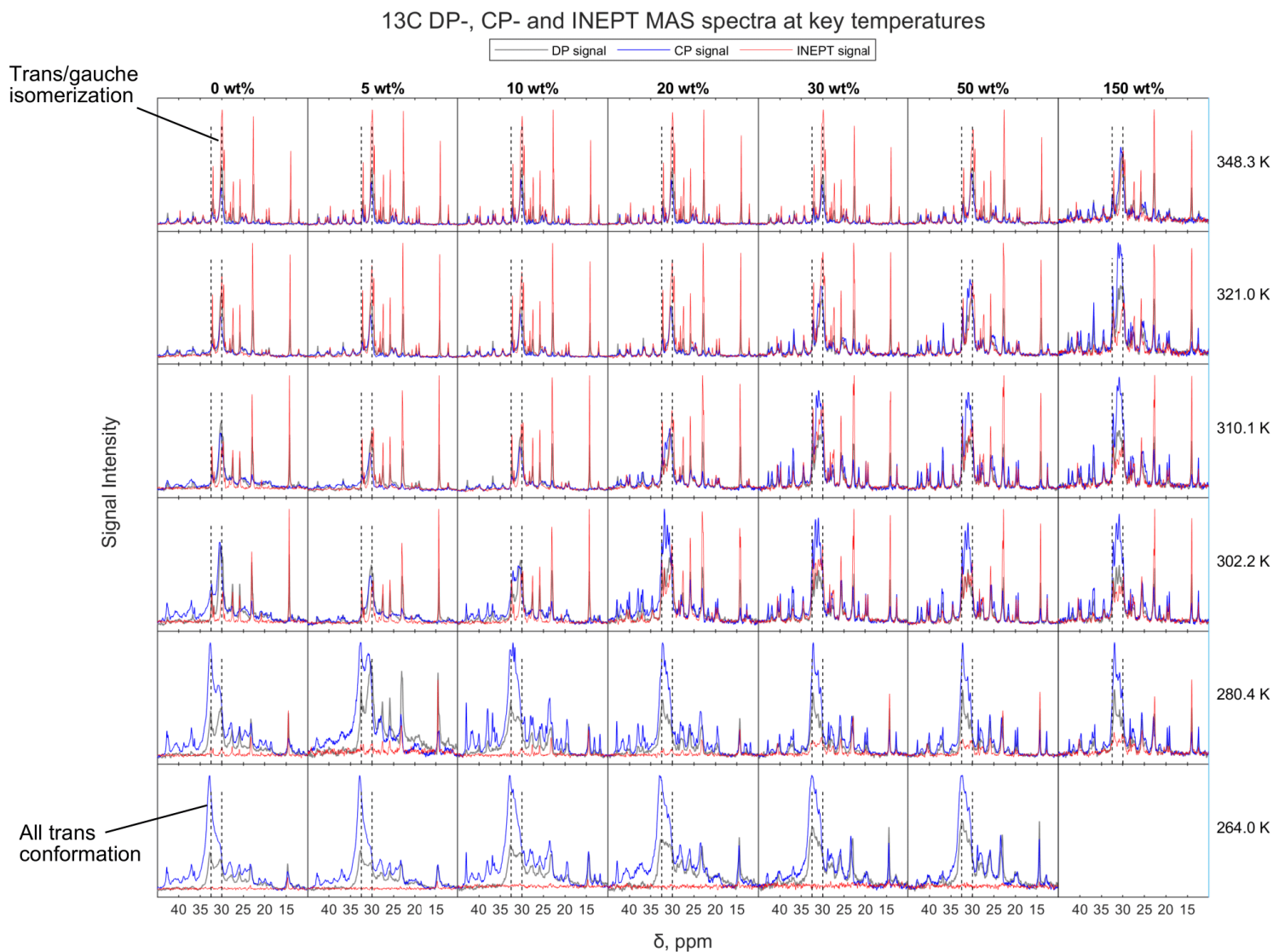


Figure 29: ^{13}C MAS-NMR spectra for a series of key temperatures for all water contents with water contents above each column and temperatures displayed to the right. The spectra show the 10-45 ppm range which is the one mainly used to obtain phase information. The intensity axes of all spectra are internally scaled to 110% of their respective highest intensity peaks. This is the cause the character of the 5 wt% 280.4 K spectrum: the 32 ppm peak cluster is in the progress of transitioning away from all-trans conformation, resulting in a lower highest-intensity and thus a more compact scaling. L_c -phase shows no trans/gauche shift with predominantly CP signal across the full ppm range. L_α phase is distinguished by spectra featuring INEPT as the highest signal at 14 ppm while simultaneously featuring higher-or-similar CP signal compared to DP and INEPT in the 30-35 ppm range (this trait required supporting evidence in the form of results from experiments on other nuclei). L_β features spectra with some trans/gauche shift and predominantly CP signal except for at 14 ppm where it may be superseded by DP. H_{II} is distinguished by spectra featuring INEPT as the highest signal across the ppm range while retaining notable CP signal only in the 30-35 ppm range.

Keeping in mind that cholesterol contributes considerable CP signal compared to reference literature samples (sections 2.2 pp.8-11 and 2.4 pp.30), results suggest that L_C phase is predominant at subzero temperatures for water contents of 10 wt% and lower due to CP being the predominant type of signal and limited trans/gauche shift taking place (section 2.4 pp.31). For water contents of 20 wt% and higher, an L_β phase appears to occur since trans/gauche shift is in motion and the CP signal is slightly superseded by DP signal at 14 ppm. Above the melting point of water, water contents of 5 wt% and lower appears to result in a transition to liquid crystal states largely influenced by the lipid itself, seen as considerable trans/gauche shift while the INEPT signal at 14 ppm is consistently higher than the other signals. For water contents of 30 wt% and higher, the dominance of the INEPT-signal at 14 ppm with retained higher CP signal in the 30-35 ppm cluster suggest the onset of L_α phase formation. Notably, this appears to coincide with the onset of L_β formation at lower temperatures, suggesting a general onset of lamellar liquid crystal formation somewhere between 10 wt% and 20 wt%. At moderate temperatures, L_α phase appears to occur for water contents of 20 wt% and higher. Conversely, for lower water contents, the samples have completed a shift from all-trans to trans/gauche isomerization by 302.2 K, suggesting that chain melting occurs near this temperature. Retention of noticeable CP-signal enhancement and high CH_3 INEPT-signal suggests a H_{II} -phase, considering the low water content. At 321.0 K, water contents of 20 wt% and lower have transitioned to a H_{II} -phase while 30 wt% possibly retains some L_α phase behavior, here seen as a retainment of CP signal enhancement comparable to the INEPT signal enhancement in the 30-35 ppm cluster. Complimentary evidence from experiments on other nuclei is required to gain distinction between the phases.

4.4 ^{31}P -NMR measurements

^{31}P -MAS NMR spectra for the same series of key temperatures as above are shown in Figure 30 below while a close-up of the resolved peak region for high water contents and temperatures is shown in Figure 31. All ^{31}P spectra are shown in Appendix B3 for reference. Results agree with the onset temperatures of chain melting indicated by the ^{13}C results (see the above section). A decrease in peak resolution at 150 wt% by dry weight lipid and high temperature further indicates, in conjunction with ^{13}C -results, that comparatively less mobile liquid crystalline phases (such as L_α as opposed to H_{II}) are present to a greater extent at considerably higher temperatures for highly hydrated extract. (section 2.3.7, pp.18)

31P DP- and CP-MAS spectra at key temperatures

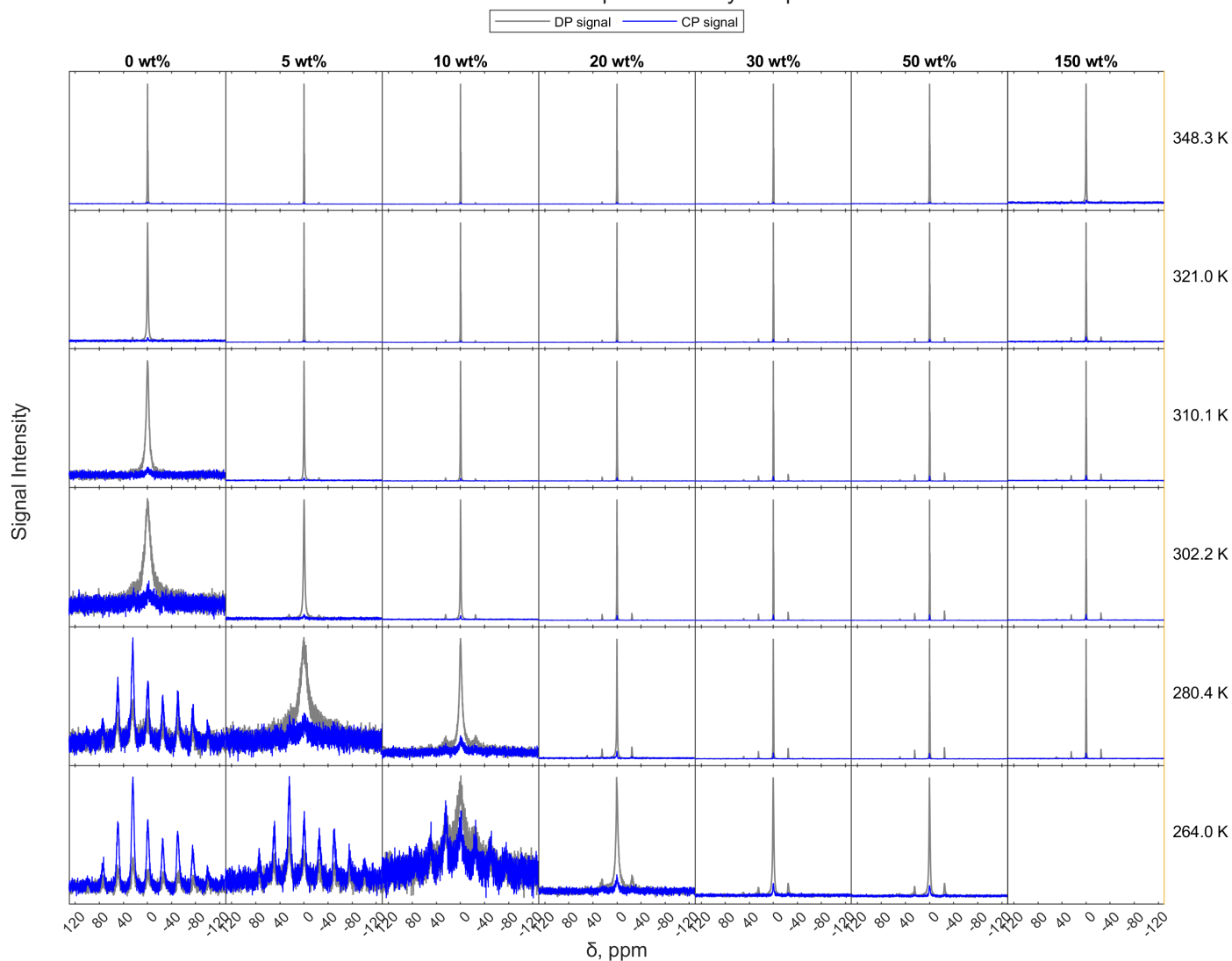


Figure 30: ^{31}P -MAS-NMR spectra for a series of key temperatures with water contents above each column and temperatures displayed to the right. Grey lines are DP-signal and blue lines are CP-signal. All spectra are scaled to 110% of their respective highest intensity peaks. The apparent feature of smaller peaks in the 321 and 348.3 K spectra for 5 wt% water is due to overlap of increasing and decreasing curve segments shading each other. Occurrence of minor sidebands and/or peak band widths as opposed to a major sideband pattern indicate complete transition to a liquid or liquid crystalline state by 318 K for hydrated samples and 343 K for the non-hydrated sample.

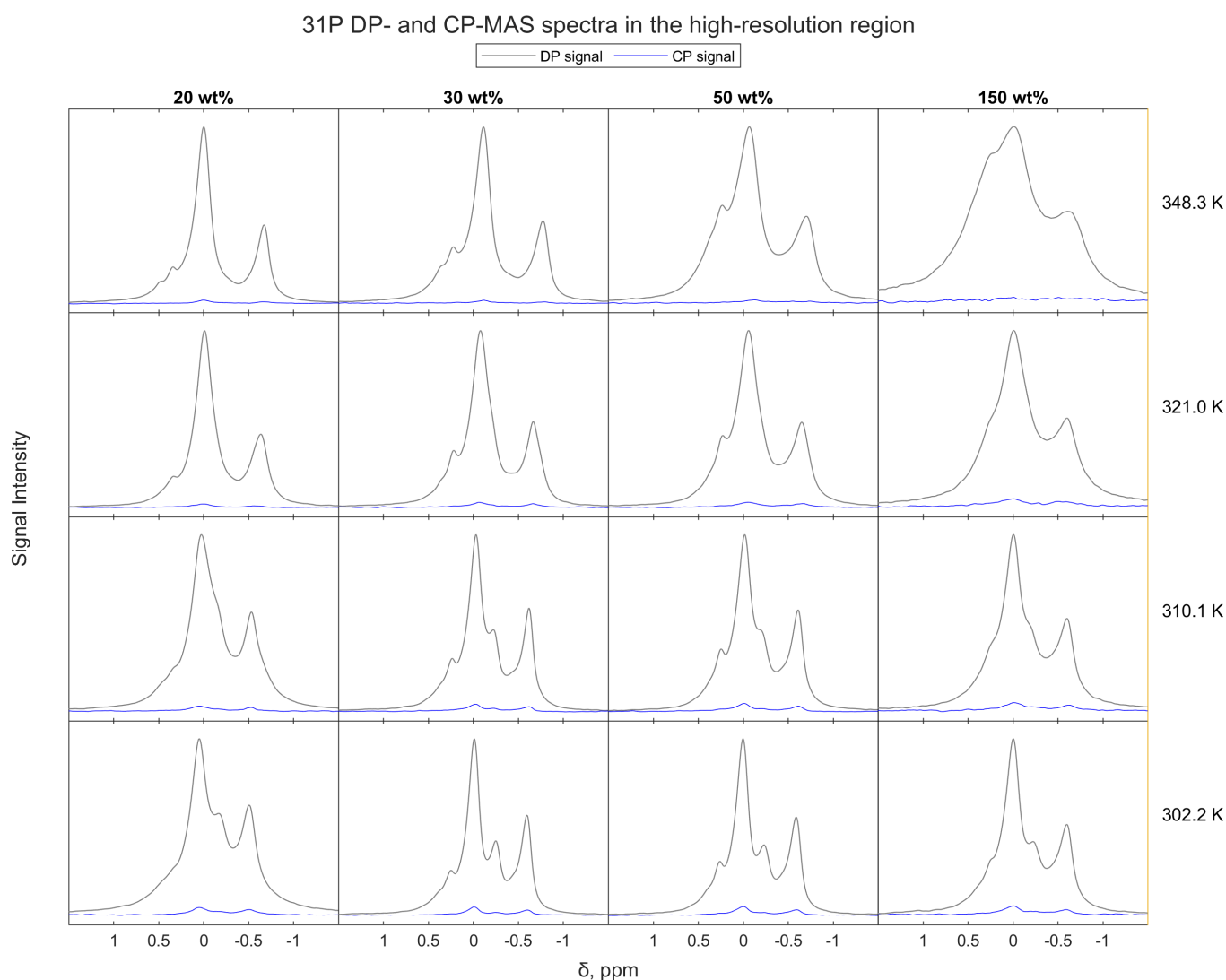


Figure 31: Zoomed-in ^{31}P spectra in the resolved peak range of temperature and water content. A decrease in peak resolution at higher temperatures for very high water content indicates a decreased level of mobility at higher temperatures for 150 wt% water by dry weight lipid compared to lower water contents.

Figure 32 below shows static ^{31}P NMR spectra for the same series of key temperatures as above. The results are congruent with previous results and notably suggests two-phase regions for water contents of 20 wt% and above (section 2.6, pp.17). Furthermore, peak patterns at moderate temperatures for water contents of 30 wt% and higher suggest that the presence of multiple phases. Considering the various phase behaviors previously reported for the constituent phospholipids, this is proposed to be due to phase separation among the individual phospholipid species. For lower temperatures, the phase seen for water contents of 30 wt% and higher that was thought to be L_c may also be a mixture of liquid crystal phases, a notion supported by the prevalence of the ^{13}C DP- and INEPT signals at 263 K and 278 K respectively. In summary, the ^{31}P results suggest that higher water contents promote the retention of L_α -phase with increasing temperature for the PL species in this extract.

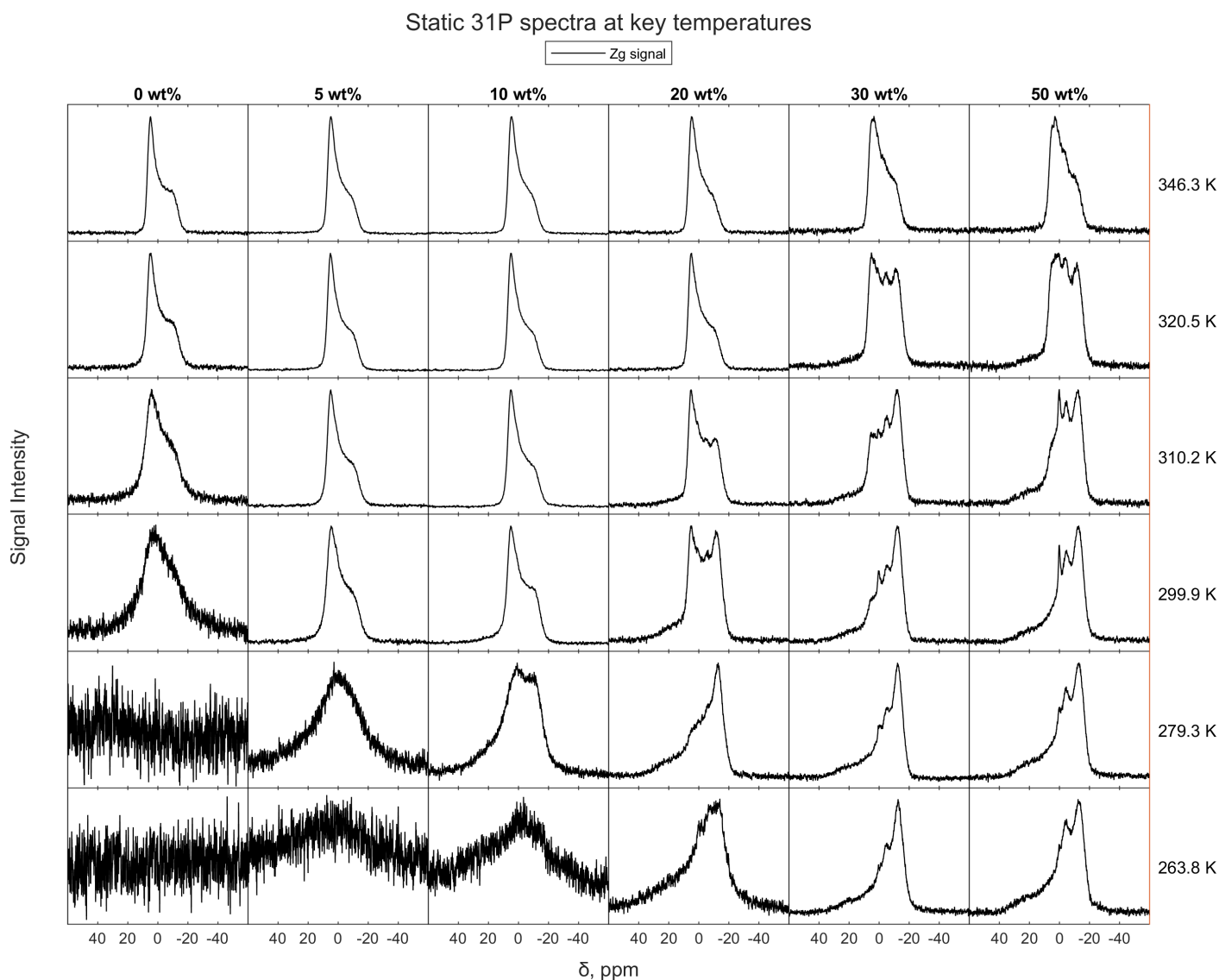


Figure 32: Static ^{31}P -NMR spectra for a series of key temperatures with water contents above each column and temperatures displayed to the right. All spectra are scaled to 110% of their respective highest intensity peaks. Results indicate existence of H2 as the sole liquid crystalline phase for water contents of 10 wt% by dry weight lipid and lower with an onset of formation at about 298 K. Further indications are of more extensive liquid crystal formation for water contents of 20 wt% by dry weight lipid and higher and particularly, L_α phase formation to various extents for these water contents at temperatures of 308 K and lower. The presence of small, jagged peaks which protrude from the shoulder patterns at different chemical shifts at these water contents indicate unresolved phase behavior patterns of individual PL species.

There is a lack of signal seen in the spectra recorded at low water contents and low temperatures in Figure 32. Figure 33 below shows a side-by-side comparison of the static spectrum at 263 K and 5 wt% water and the MAS-spectrum at 263 K and 0 wt% water. It appears that the CSA pattern widths seen in the MAS-spectrum and static spectrum are comparable and on the order of 240 ppm. It is proposed that the lack of signal is thus due to rapid FID decay due to dipolar coupling (sections 2.3.7.2 pp.20 and 2.3.10.4 pp.29). The line broadening used in the static spectrum has been exaggerated (300 Hz) for ease of comparison.

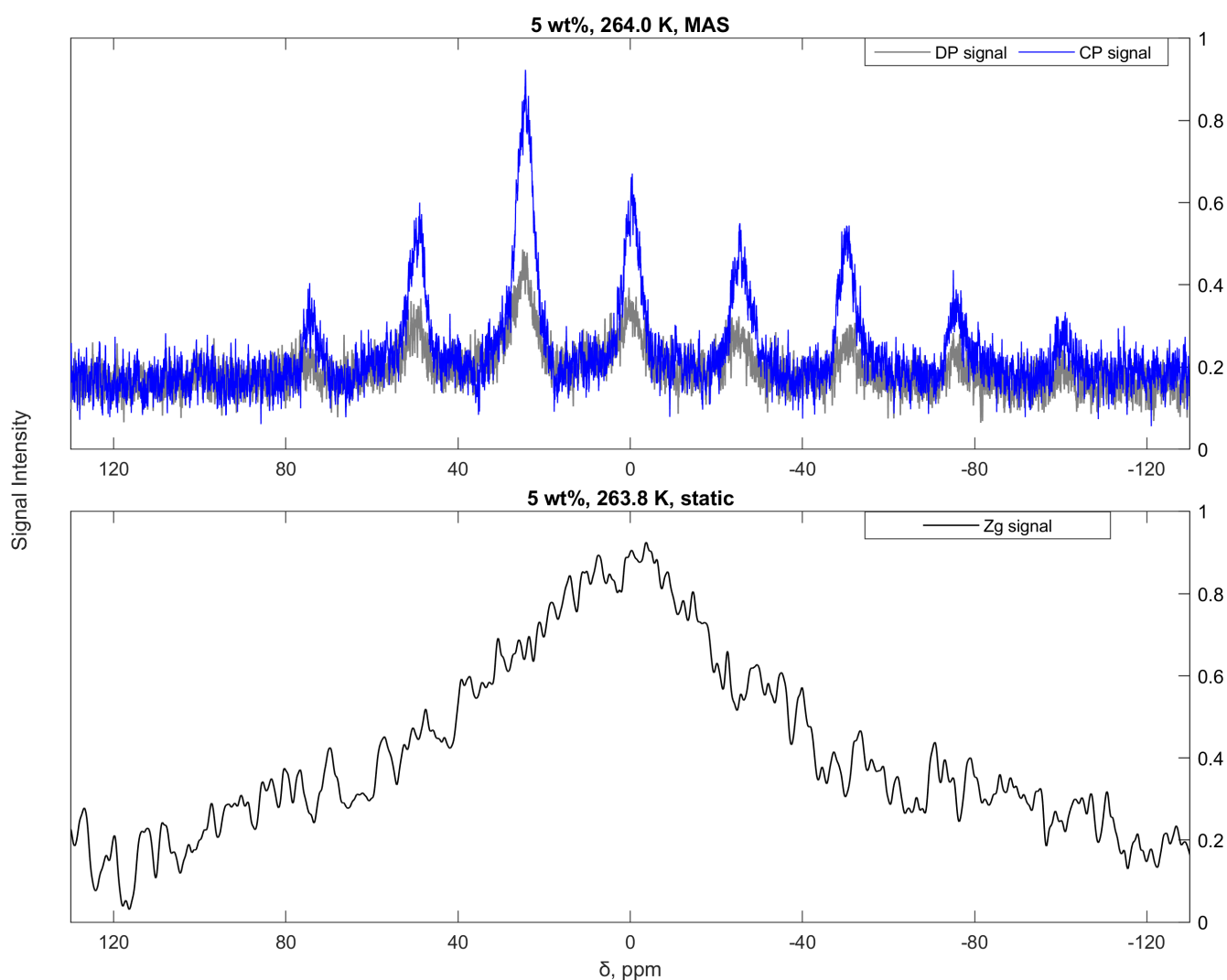


Figure 33: MAS (top) and static (bottom) ^{31}P -spectra with water content and temperature shown in the figure title above each plot. The line broadening in the bottom spectrum was exaggerated to 300 Hz for ease of comparison. The scales of the y-axes in the figure are relative to 110% of the maximum peak intensity. The absolute scales of the y-axis in arbitrary units which are not comparable due to recording on different NMR probes. The similar widths of the sideband pattern and the static peak band suggests that apparent signal loss at low temperatures is due to rapid FID decay.

4.5 ^2H -NMR measurements

Static ^2H NMR spectra for the same series of key temperatures as above are shown in Figure 34 below. All ^2H spectra are shown in Appendix B4 for reference. The implications of the results agree with those of the results of the ^{13}C and ^{31}P measurements. Furthermore, the widths of the splitting patterns (section 2.5 pp.34) suggest that the phase thought to be L_α -phase at low temperatures for 20 wt% water content may be a

different phase, such as L_β . Meanwhile, the width of the patterns from the samples with 30 wt% water and higher are consistent with expected values for L_α -phase. Finally, the absence of distinguished powder patterns in the quadrupolar splittings of water contents of 10 wt% and lower, along with the occurrence of partially resolved two-phase patterns is in agreement with literature, where a water content of 8% of total weight was reported as the onset of formation of a two-phase system of L_α and H_{II} and 16% water of total weight was reported as the optimal water content for L_α phase formation, persisting to 297 K.

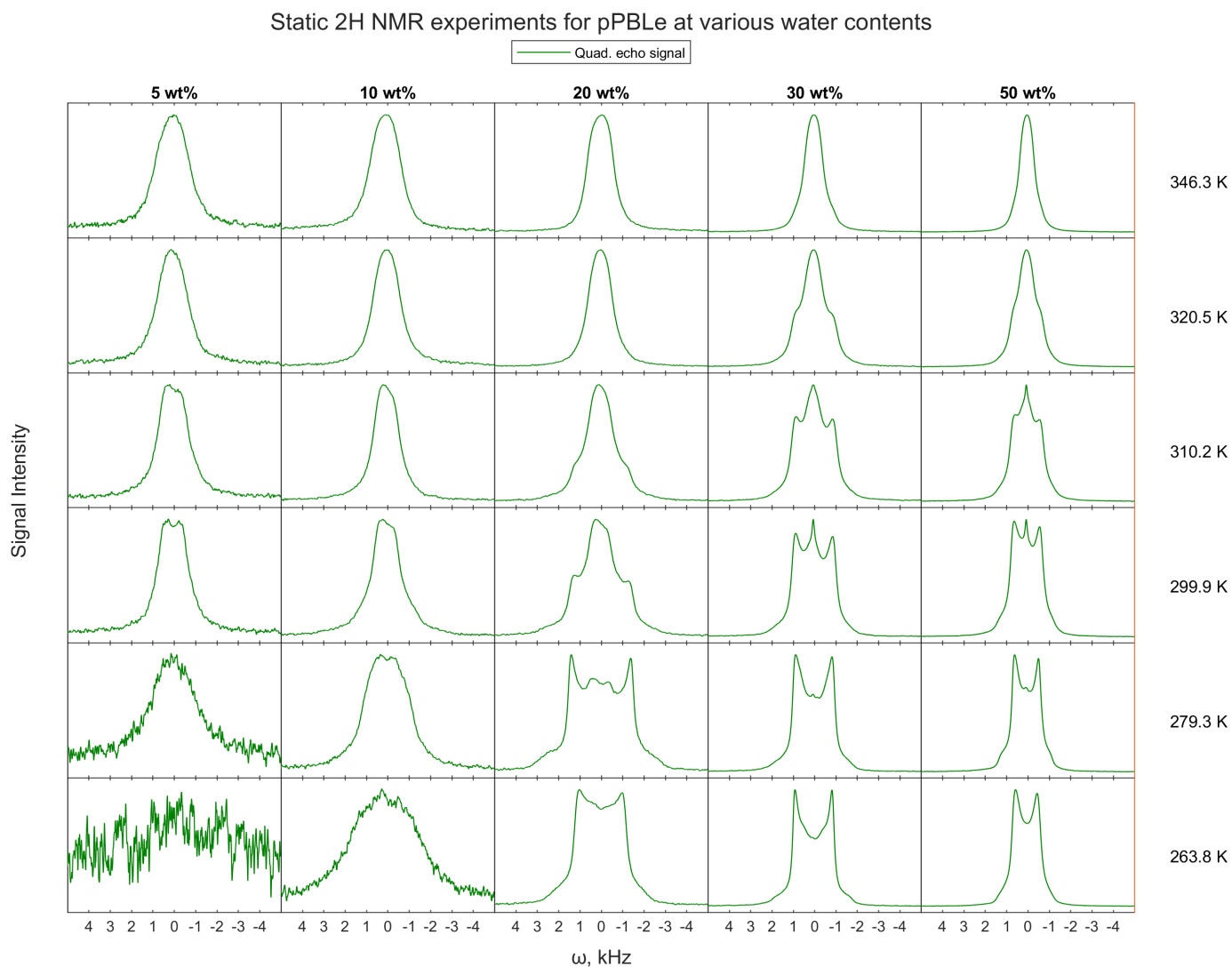


Figure 34: Static 2H-NMR spectra for a series of key temperatures with water contents above each column and temperatures displayed to the right. Results indicate none-or-limited formation of liquid crystals for water contents of 10 wt% by dry weight lipid and lower, liquid crystal formation for water contents of 20 wt% by dry weight lipid and higher and particularly, L_α phase formation to various extents for these water contents at temperatures of 308 K and lower. Pattern widths suggest that lamellar liquid crystal phases other than L_α are present at lower temperatures for water contents of about 20 wt% by dry weight lipid.

4.6 Summary of representative NMR spectra by phase and nucleus

Spectra representative of nearly pure phases (when viewed sample-wide) were identified. These are shown in Figure 35 below. The L_β -phase is distinguished from the L_c -phase via the transition of ^{13}C CH_2 -chemical shifts away from 34 ppm, in this case particularly visible in the DP-signal where a notably higher intensity close to 34 ppm (L_c) is replaced by a cluster of approximately equally intense peaks (L_β). The effect is owed to the onset of notable chain mobility in the fatty acids, enabling some of the energetically less favored gauche configurations to occur while reducing available dipolar interactions for CP (section 2.4, pp.31). Observing other nuclei, distinguishment occurs via loss of CP signal and strong narrowing of the peak band (^{31}P -MAS) (sections 2.3.10.3 pp.28 and 2.3.7 pp.18), narrowing of the solid band to approximately half width (^1H -MAS) and onset of detectable patterns (static ^{31}P and ^2H) (sections 2.6 pp.17 and 2.5 pp.34), all brought on by the formation of a liquid crystal phase with reduced solid-state interactions, giving much longer FIDs.

The L_α -phase is distinguished from the L_β -phase by better peak resolution in the CH_2 -cluster and considerable INEPT signal on the 14 ppm CH_3 -peak (^{13}C), further narrowing of the solid band to reveal clear rotational sidebands (^1H) and narrowing of the quadrupolar splitting to about half of the width of that of L_β . The effects on the MAS-spectra are owed to gradually increasing chain mobility with increasing temperature (^{13}C), starting from the terminal CH_3 (14 ppm) and continuing to the near-terminal CH_2 groups and the lowest-index CH_2 groups that are largely responsible for generating chain angle. For ^2H , increased water mobility gives a higher D-O bond angle relative to the chain angle, a lower order parameter S_{mn} (section 2.5, pp.35-36), and a smaller quadrupolar splitting.

H_{II} is identified by INEPT prevailing as the highest signal across the 10-45 ppm range (^{13}C) owing to high chain mobility, the change to a low-ppm shoulder (^{31}P) due to a change in the symmetry of the crystal structure (no longer lamellar) and a Gaussian peak of approximately the same width as the quadrupolar splitting seen for L_α (^2H). The latter effect is owed to exchange broadening; the water is mobile enough to diffuse out of crystallites at time constants faster than those of the frequencies covered by the quadrupolar splitting (the assumption of a constant θ_{H} no longer holds) (section 2.5, pp.35-36).

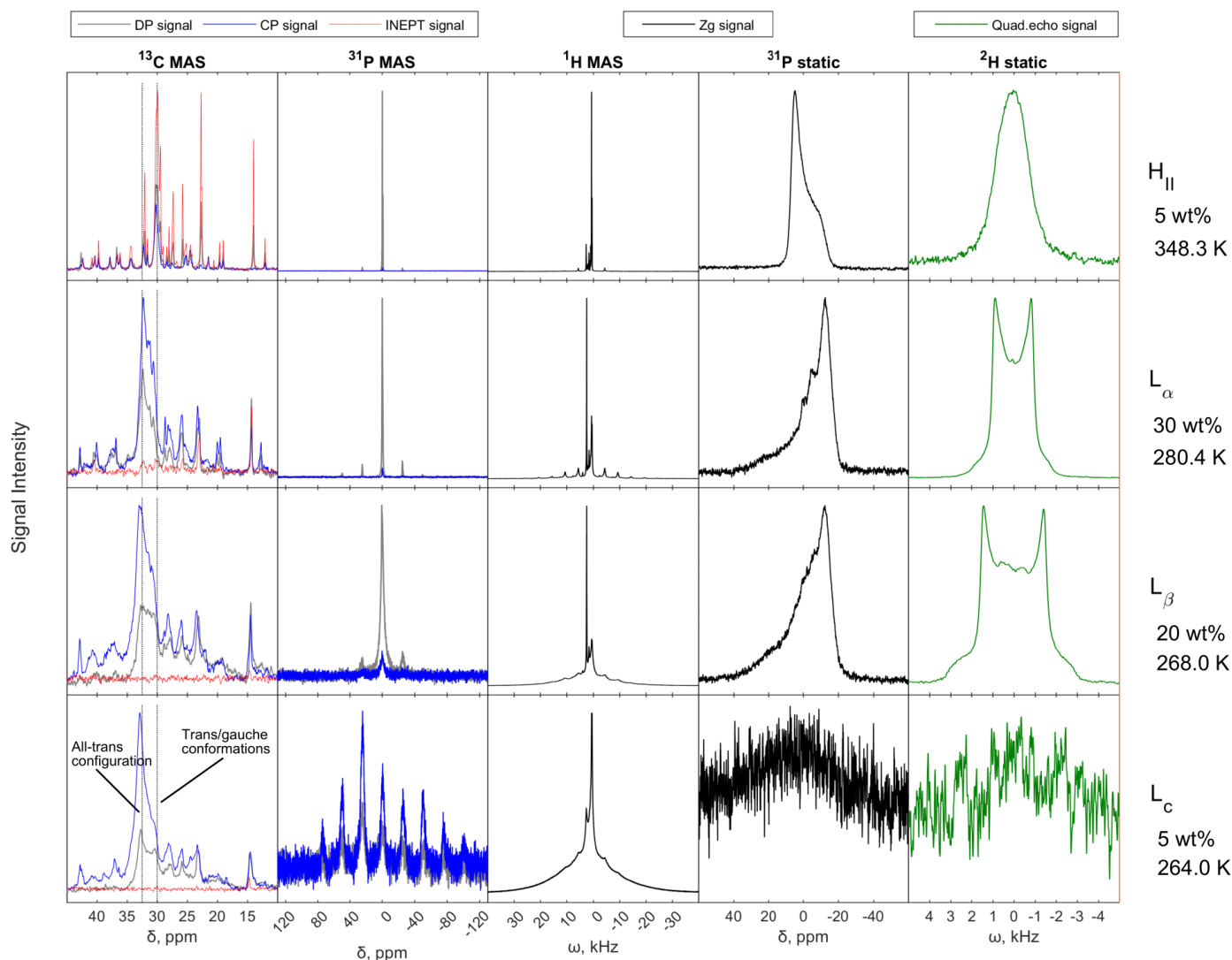


Figure 35: NMR spectra representative of approximately pure PL phases (rows). Columns represent NMR techniques and nuclei. Color coding in diagrams are the same as in previous figures. For ^{13}C , the ratio of INEPT signal (red) to the DP-(grey) and CP-signals (blue) especially for low ppm peaks as well as the break-up of the CH_2 -cluster into peaks of different chemical shifts (trans/gauche shift) are key identifiers. For ^{31}P -MAS, the disappearances of CP-signal (blue), readily visible peak broadening and sidebands identify the phases. For ^1H , the identifiers are solid band widths of about 60 kHz and 30 kHz for L_c and L_β respectively and sidebands at ± 10 kHz and ± 5 kHz. For static ^{31}P NMR, the criteria are onset of strong signal (heteronuclear dipolar interactions with ^1H shortens the FID at low temperatures in the absence of decoupling), adherence to patterns predicted by literature (wide, high-ppm should for L_α and smaller, low-ppm shoulder for H_{II}) (see section 2.6 and Figure 25) and the resolution of lower intensity peaks (L_α). For ^2H , the identifiers are onset of strong signal and quadrupolar splittings: L_β has a “horn-to-horn distance” of about 4 kHz and L_α features lower distances (a discrete distinction does not appear). At higher temperatures, small crystallite size relative to D_2O mobility leads to exchange broadening of the peak. Thus, H_{II} is identified not by its predicted pattern but as a Gaussian peak of a width close to that of the L_α -phase pattern. The utilized example pairs of water contents and temperatures by ascending rows are 5 wt% at 264 K, 20 wt% at 268 K, 30 wt% at 280.4 K and 5 wt% at 348.3 K. The temperatures are valid for MAS experiments.

4.7 Phase map for the polar brain lipid extract

The molar amount of water per molar amount of fatty acid groups for the samples are shown in Table 3 below.

Table 5: Conversion between weight percent added water by dry weight lipid, molar amount of water per molar amount of fatty acid group and molar amount of water per total molar amount of water and phospholipids.

Weight percent added water by dry weight of lipid	Molar amount of water per molar amount of fatty acid group	Molar amount of water per total molar amount of water and fatty acid chains
0	0.76	0.14
5	1.43	0.55
10	2.31	0.70
20	4.09	0.80
30	7.46	0.88
50	10.89	0.92
150	28.19	0.97

Converted to molar ratios of water per total molar amounts of fatty acid chains and water, the proposed phase map is shown in Figure 36 below. A phase map differs from a phase diagram in that it concerns the whole sample rather than its constituents.

Comparing the phase map with the previously reported phase diagram for PE by Sholaev et al (section 2.2.1 pp.) and reported phase behaviors of the other PLs (sections 2.2.2-5 pp.) enables verification and explanation of the results.

Starting with water contents of up to 0.70 water content by total moles of water and PLs (henceforth shortened to “water content”), the sample adopts a H_{II} phase above 303 K at all water contents. This mirrors the phase behavior of its main constituent PE (section 2.2.1, pp.8). The L_C phase exists for higher water contents and temperatures than expected when compared to the previously reported phase diagram. This is thought to be due to PLs other than PE requiring higher temperatures and higher hydration to form water-incorporating liquid crystals, particularly PC. Similarly, the continued occurrence of L_β above 268 K at water contents beyond 0.65 molar content (about 10 wt%) can be explained by PC and PS together (sections 2.2.2-3 pp.9). The onset of decrease of L_C -phase at about 0.60 water content (about 6.5 wt% water) coincides with the onset of L_α - and H_{II} formation at 278 K previously reported for PE.

For water contents higher than 0.70, L_α phase formation to various extents starts to become apparent. This coincides roughly with the previously reported onset of consistent L_α formation above 273 K at about 0.78 water content (about 18 wt% water). On a related note, the sample first starts to show general L_α characteristics at the early L_α formation optimum seen for PE at about 0.76 water content (16 wt% water). For higher water contents, assuming that the sample largely behaves like PE, L_α would be expected to decrease with increasing temperature and water content within the previously investigated range ending at about 0.88 water content. While its prevalence continues to decrease with increasing temperature for water contents of 0.75-0.86, the trend is the opposite for increasing water content. This is thought to be because PC and PS which form L_α more stably are sufficiently hydrated to form L_α to an extent that makes

it a prominent phase in the sample. At water contents beyond 0.88 water content the sample is thought to approach full hydration, enabling PE to also form L_α up to some threshold temperature, leading L_α to become the prevailing phase depending on temperature.

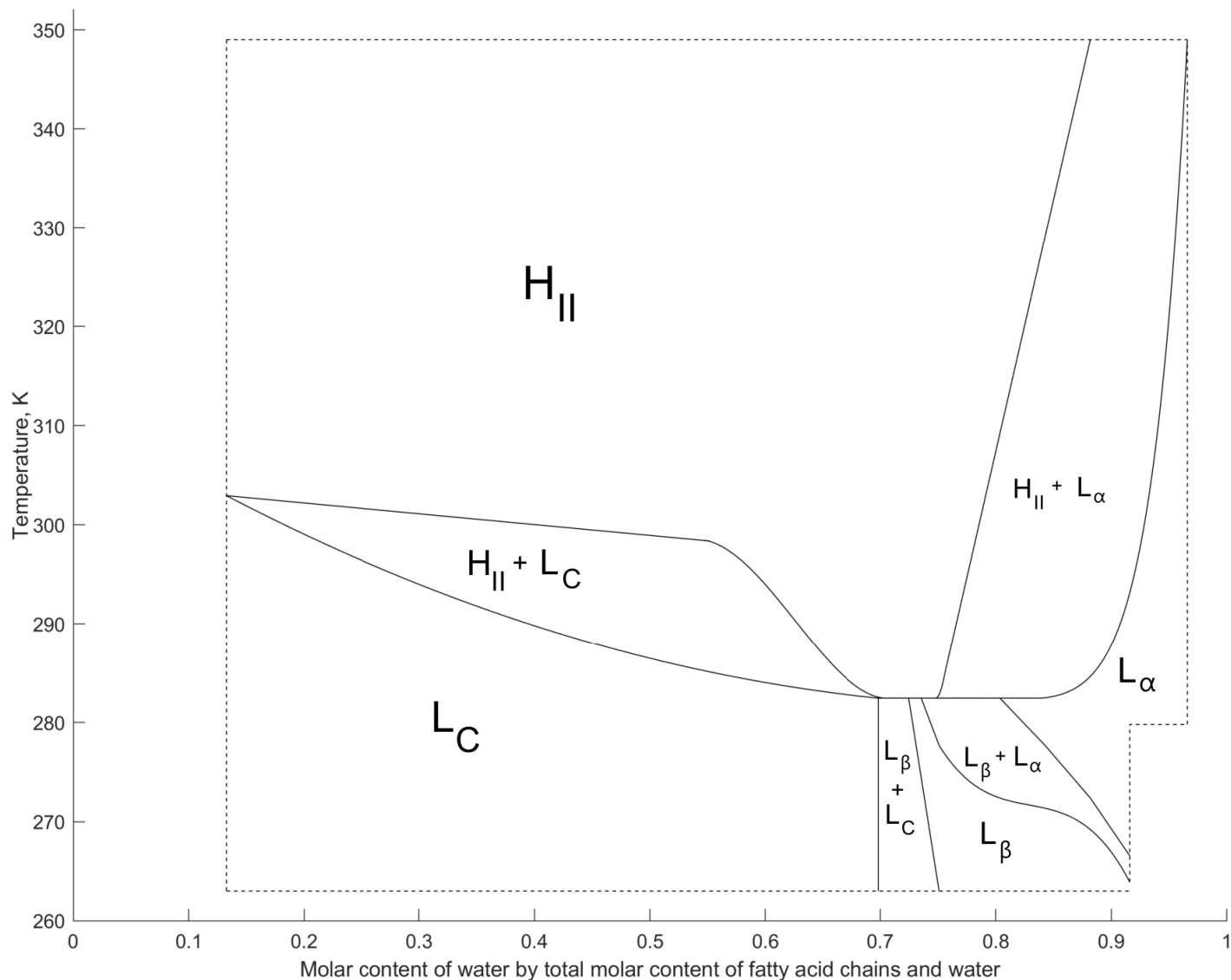


Figure 36: Phase map for the pPBL in the investigated temperature range and with molar ratio of water per molar content of fatty acid chains. Four principal phases were identified in the sample (phase labels are defined in Table 2). Phase boundaries were drawn using temperatures and water contents between quantities corresponding to recorded spectra when a drastic shift was observed between two investigated temperatures and, conversely, drawn using temperatures of recorded spectra when transitions were observed to occur gradually across several recorded spectra. The dashed line indicates the boundary of the investigation in terms of temperature and water contents examined.

Table 6: Conversions between molar content H₂O by total molar content of H₂O and PLs (left column) and weight percent water by weight percent water per dry weight of extract (right column) for each of the prepared samples.

Molar content of water by total molar content of water and PLs	Weight percent water per dry weight of lipid extract (%)
0.05	0
0.30	5
0.45	10
0.59	20
0.72	30
0.79	50
0.91	150

4.8 Cholesterol content and its impact on the phase map

The cholesterol content was estimated to be 32 mol% by total molar amount of PL fatty acid chains and cholesterol.³ Assuming that each PL has two fatty acid chains on average, this corresponds to 16 mol% of cholesterol by total molar amount of PL and cholesterol. In comparing with previous studies, a few expectations of the sample follow.^{30 31 32 33 34 35 36 39}

Some L_O-phase is expected to occur where L _{α} -phase occurs above 314 K. The retained ¹³C CP-signal at high temperatures coinciding with two-phase patterns in both static ³¹P and static ²H, enabling distinguishment of L _{α} from H_{II} in ¹³C, suggests that L_O is indeed contributing fatty acid chains with reduced mobility compared to those of L _{α} , possibly also enhancing the stability of the latter. It is, however, difficult to estimate how much L_O-phase is present since it is mainly associated with PC, a minority constituent in this extract. The detailed fatty acid content of PC is also not known.

A notable decrease in L _{α} -to-H_{II} transition temperature for PE should be present even at full hydration. Since much of the sample remains in the H_{II} phase at higher temperatures even after full hydration sets in (at about 0.9 molar content), the sample behaves as could be expected from the reference literature.

No phase of pure cholesterol should be observed. This claim necessitates an explanation of the presence of many resolved cholesterol peaks in the ¹³C spectra. Since cholesterol assembles into raft regions that are rich in cholesterol when appearing as L_O, such an explanation is at hand.

Finally, assuming a non-negligible fraction of unsaturated fatty acid chains (this is likely the case judging by the considerable signal in the C=C cluster in the ¹³C spectra), the transition from solid to liquid crystalline phases in general should occur at about 293 K or lower, which indeed seems to be the case (see above throughout Results and Discussion section). Barring these overarching effects, detailed analysis of the impact of cholesterol on the phase diagram is difficult without more extensive phase information on the individual PLs in the extract.

5 Conclusions

5.1 Phase map construction and reliability of sample preparation

The phase behavior suggested by these results, barring any unresolved peak patterns, is largely consistent with POPE⁷¹, indicating that the extract behaves much like its main constituent until the molar water content becomes very high. Motivations include the lowest required water concentration to achieve lamellar liquid crystalline structure as well as the apparent melting point from solid to liquid crystalline. Solid crystalline and liquid crystalline structures are adopted throughout the investigated parameter ranges. Four distinguishable phases were identified. For PAMPA working conditions (full hydration and body temperature), the extract largely adopts an L_{α} phase with an unknown amount of L_O regions. For water contents of 20 wt% and higher, two-phase regions were present across many temperatures. In addition, static experiments potentially contained unresolved CSA patterns and quadrupolar splittings respectively. It is unknown if this is due to individual PLs each adopting phases that they would display in isolation under sufficient hydration. Considering that the above information could be obtained and connected to previously reported phase behavior for the constituent PLs, the simple sample preparation methods are deemed sufficient for whole-sample analysis of this kind.

5.2 Efficacy of multinuclear NMR for phase analysis

Dynamics based spectral editing for ^{13}C supported by established methods of phase evaluation for other nuclei is a powerful tool for determining phase behavior for mixtures of PLs, GPLs, triglycerides and sterols. For pPBLe specifically, dynamics based spectral editing alone can distinguish four phases rather than three via the retainment of- or loss of CP signal in the CH_2 -cluster at about 32 ppm at moderate temperatures, a conclusion drawn after verification with supporting information from ssNMR experiments on other nuclei. ^{31}P -MAS, ^1H -MAS and ^{13}C -MAS in conjunction provide extensive possibility for peak labeling, allowing for a degree of chemical analysis without adding much experiment time if there are also concerns regarding sample composition. For analysis of mixtures other than pPBLe, supporting results from dynamics based spectral editing with static NMR results (^{31}P CSA and/or ^2H quadrupolar splitting after addition of D_2O) are still recommended to draw reliable conclusions.

5.3 Suggestions on further research

The ^{31}P and ^2H measurements indicate possible presence of multiple phases but could just as well be unresolved patterns of different PLs adopting the same type of phase individually. 2D recoupling experiments for ^{31}P -NMR such as PROCSA would provide species-specific phase information for the constituent PLs in the pPBLe and illuminate concerns regarding heterogeneity in the hydrated extract.

Considering the notable difference in phase transitions when comparing seemingly similar PLs such as POPE and DOPE, chemical analysis with the aim of determining fatty acid content would clarify which phase behavior to expect among the variety encountered when canvassing prior work, beyond the knowledge that a considerable ratio of unsaturated fatty acids is to expect for brain extract.

Finally, performing the static ^{31}P and ^2H experiments on highly hydrated extract would verify the presence of L_α -phase at higher temperatures and water contents more securely than the ^{13}C MAS-experiments of this study alone. In this study, phase information for high hydration was only established using ^{13}C -NMR and consistently re-encountering relative signal enhancement patterns seen in less hydrated samples for which ^{13}C -derived phase information was supported by static ^{31}P - and ^2H experiments.

Appendix A1

Temperature calibration was performed to account for instrument temperature discrepancy, differences in flow rate, precision issues and temperature equilibration time. In summary, calibrated temperatures were determined successfully and an effective error margin of ± 3.4 K was found to account for both flow rate, precision and equilibration time.

Figure A1-1 shows calibration curves for water-contaminated methanol and glycol respectively together with a water-free reference graphs and a set-temperature graph (i.e. the temperature as indicated by the NMR instrument without calibration). Using this graph, a calibration curve was fitting to the reference graphs. It was, in turn, used to find average calibration errors due to water contamination for glycol and methanol respectively. These errors were then subtracted from the temperature values calibrated for rotor samples (i.e. water-contaminated samples) to estimate true temperatures for those samples.

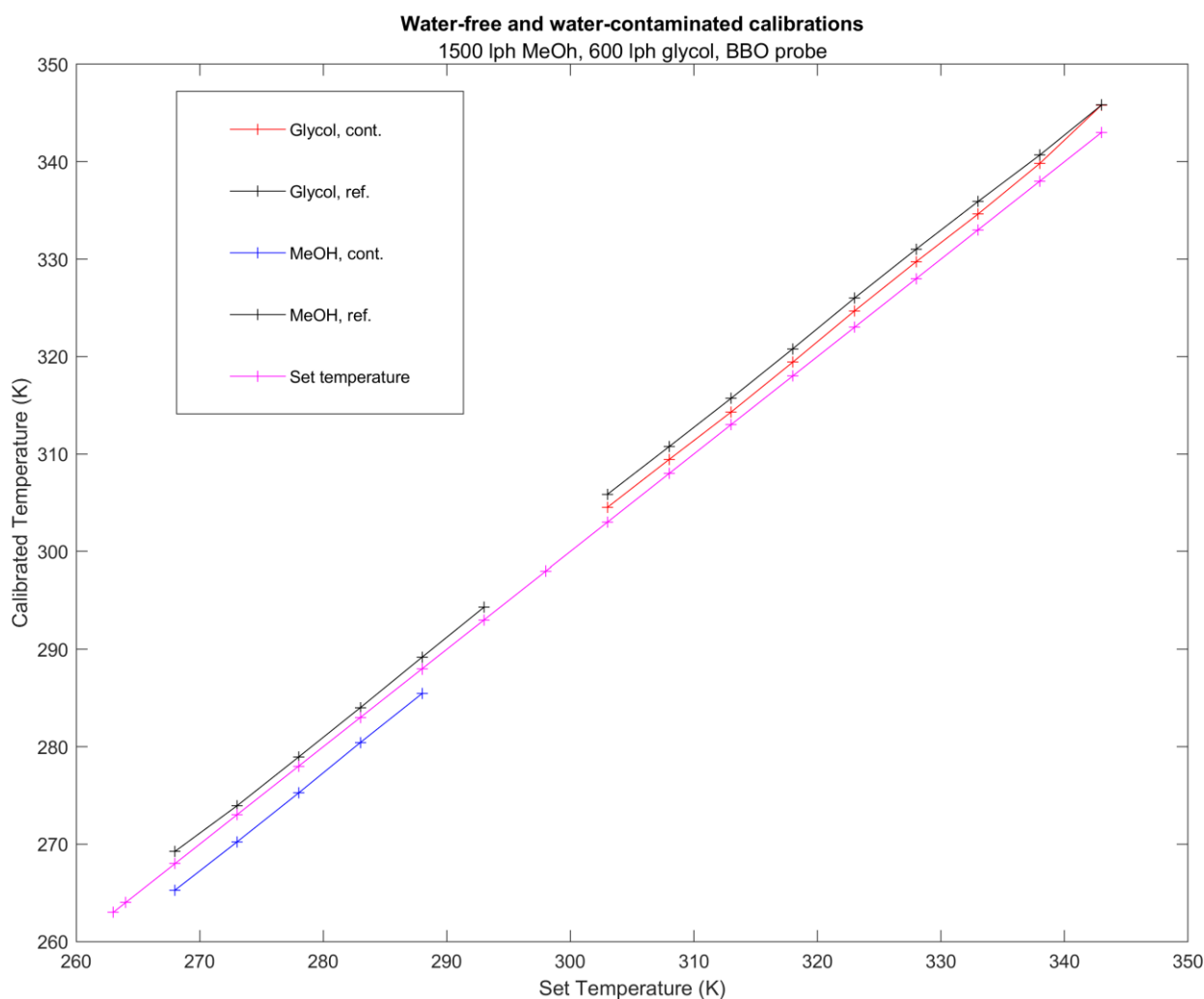


Figure A1-1: Calibration curve for MeOH in a rotor (blue), glycol in a rotor (red) and methanol and glycol in water-free reference tubes (black). Both MeOH and glycol consistently calibrated at lower temperatures compared to the reference line. Flow rates (shown in title) were the same for rotor- and reference samples.

Figure A1-2 shows graphs of calibrations with a rotor sample of glycol performed at three different flow rates while Figure A1-3 shows the same type of graphs for a reference sample at different flow rates. It can be seen that MAS conditions on the Efree probe causes the instrument to raise the sample temperature more at higher instrument temperatures compared to static conditions on the BBO probe. Reasons could include machine overcompensation for higher cooling gas flow rates, higher heat flow from the heater to due the spinning motion and/or heating by the spinning motion itself. The difference between the calibrated temperatures for 400 lph and 900 lph at 343 K instrument temperature under MAS conditions (Figure A1-2) was the highest difference found due to difference in flow rate and was used as basis for the error margin.

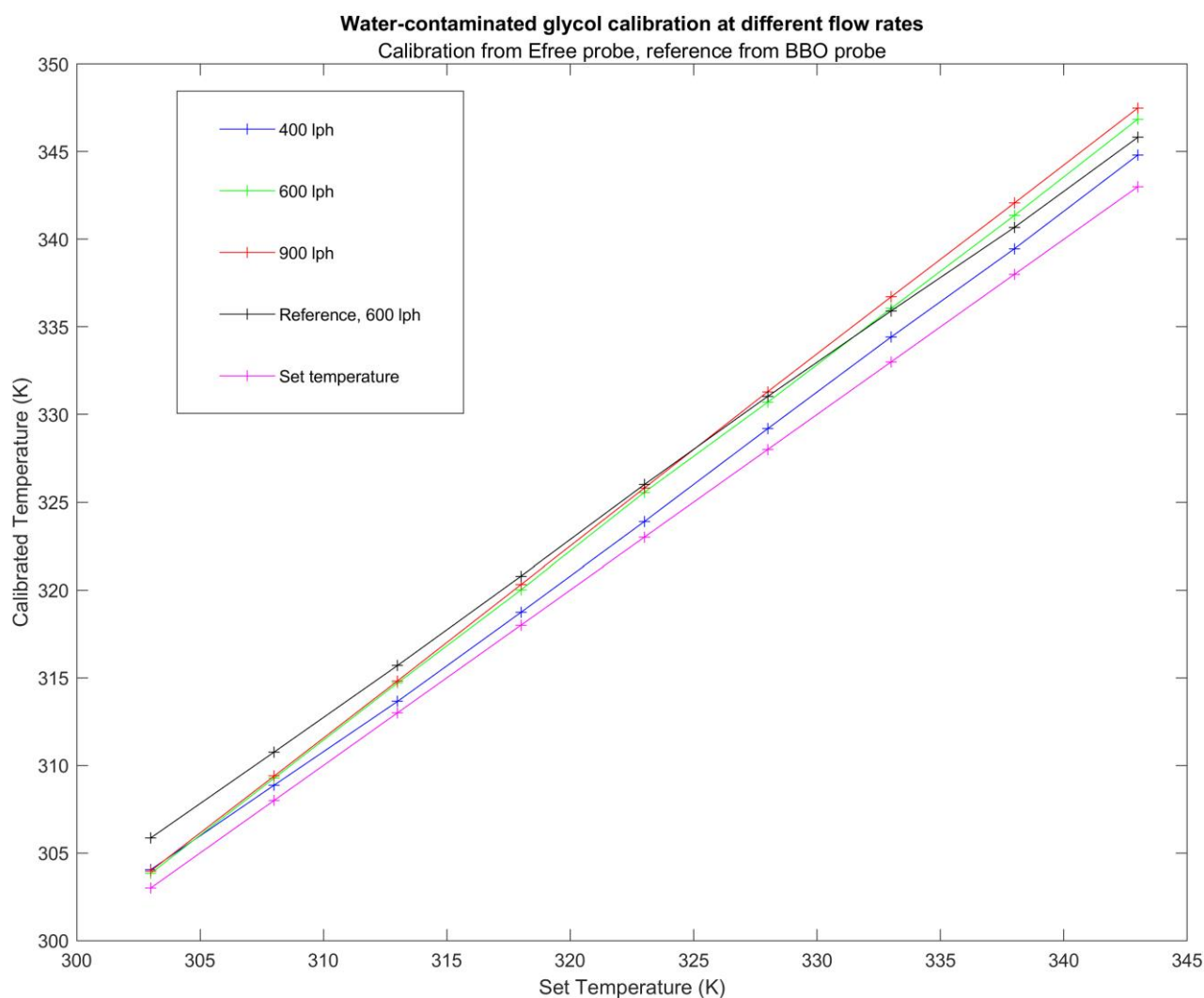


Figure A2-2: Calibration curves at different flow rates (shown in the plot legend) for a rotor sample under MAS conditions on the Efree probe (red, green, blue) plotted against a reference curve for a rotor sample on the BBO probe (black).

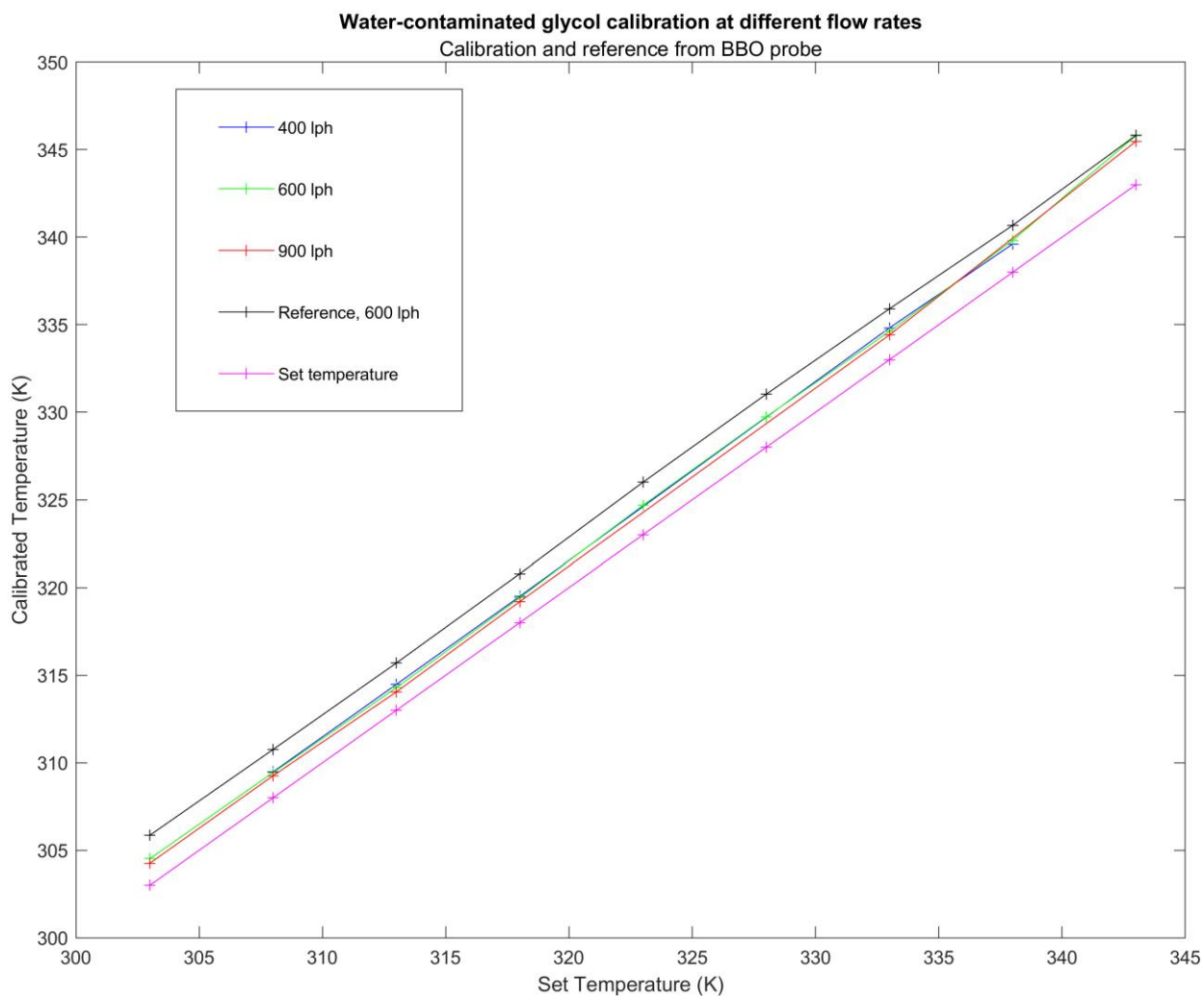


Figure A1-3: Calibration curves at different flow rates (shown in the plot legend) for a rotor sample under static conditions on the BBO probe (red, green, blue) plotted against a reference curve for a rotor sample on the BBO probe (black).

Figure A1-4 shows the evolution of temperature over time for a moderate-high temperature within the study as well as a medium flow rate at MAS conditions (red) plotted against a line representing the recorded reference value under static conditions for the same temperature and flow rate (black, dashed). The difference between the reference line and the plateau starting at about 70 minutes was used as basis for the error margin due to temperature equilibration. It is worth noting that much of each experiment series took place at times at-or-near the plateau. On the other hand, temperature evolution could be more severe at other temperatures, particularly those higher than 323 K. Thus, some measure of error margin is warranted.

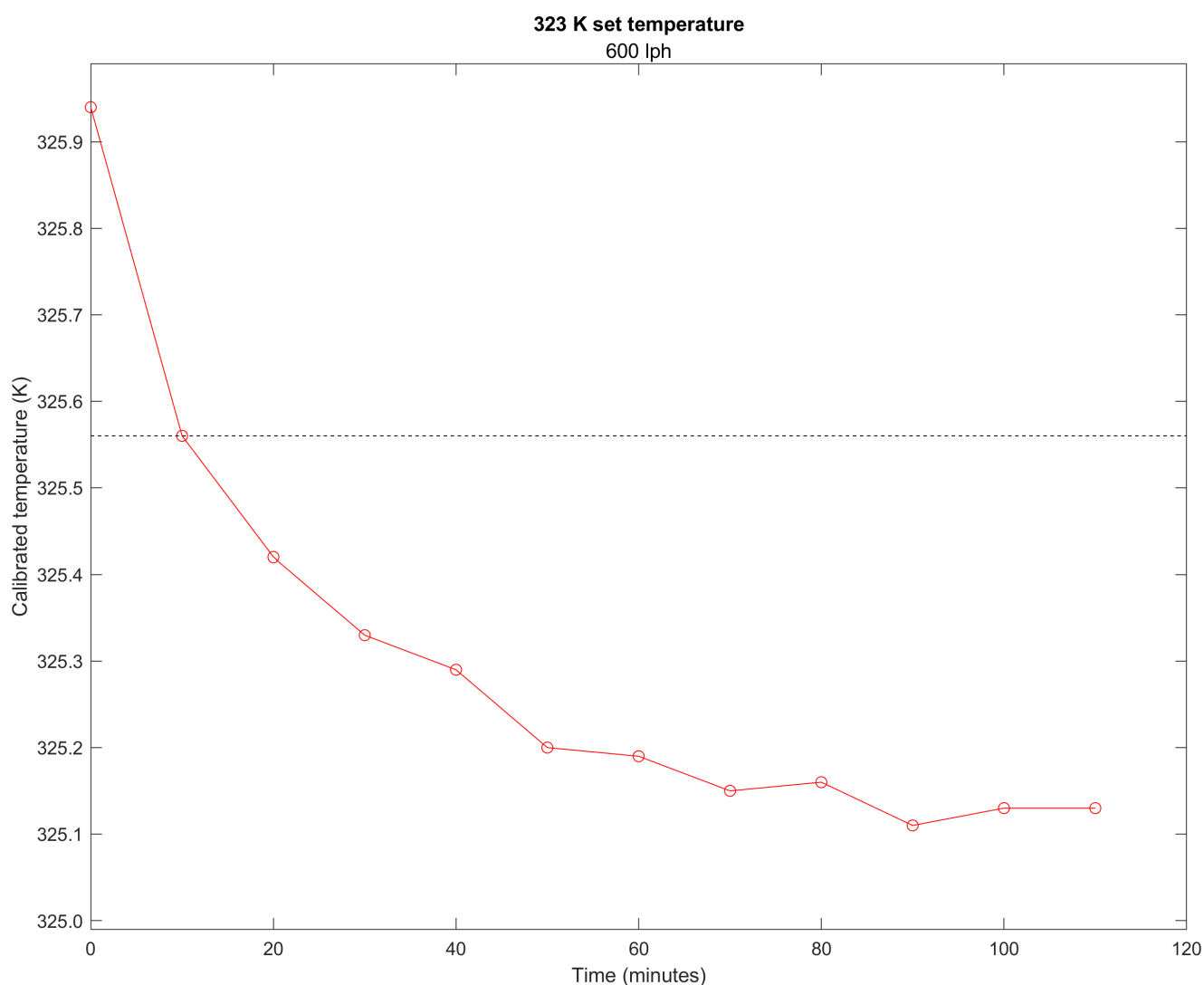


Figure A1-4: Temperature evolution over time for a moderately high temperature and a medium flow rate (shown in the plot title) in the context of the study. An error margin can be seen between the reference line (black, dashed) and the sample graph (red)

An error margin due to difference in flow rate across the full temperature series was established by finding the largest difference in calibrated temperature between different flow rates for each instrument temperature for each sample (reference methanol, rotor methanol, reference glycol, rotor glycol) with rotor methanol and rotor glycol also being calibrated under MAS conditions. All error were then sorted into vectors by sample type and compared. The largest individual error across all samples and temperatures was then selected as error margin for the full temperature series. The maximum errors for each sample type across all of their calibrated temperatures are shown in Table A1-I with the largest error in bold font.

Table A1-I: Errors in calibrated temperature due to varying cooling gas flow rate

Sample type	Largest error due to flow rate (K)
Reference Methanol	1.08
Rotor Methanol (static)	0.24
Rotor Methanol (MAS)	1.60
Reference Glycol	0.76
Rotor Glycol (static)	0.43
Rotor Glycol (MAS)	2.68

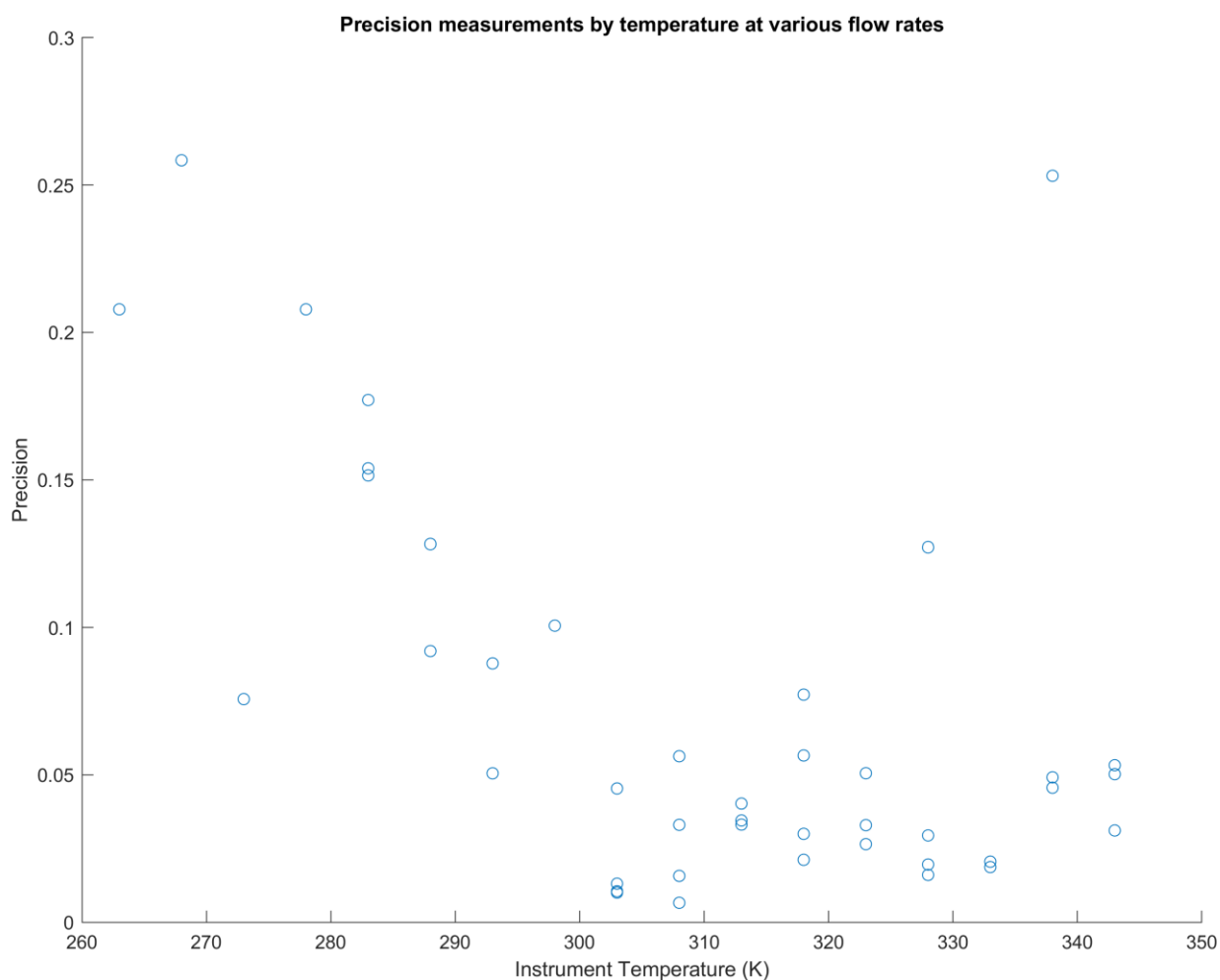


Figure A1-5: Scatter plot of measurement precision across the temperature range for various flow rates and samples.

Figure A1-5 shows a scatter plot of all recorded measurement precisions (three times the standard deviation of a series of repeated measurements at the given temperature and flow rates) across the temperature range in this study for any given combination of temperature, flow rate and sample. It appears that poor precision largely occurs at temperatures far removed from room temperature. I.e., the more cooling or heating that is required, the higher likelihood that precision is affected. Results suggest that precision is correlated with flow rate, considering that a high flow rate of cooling gas was commonly required to achieve low temperature under the technical circumstances of this study.

Appendix A2

Figure A2-1 shows detailed labeling of clustered peak regions in the ^{13}C -MAS spectra of the 30 wt% water sample at 348.3 K. Most notable peaks were able to be labeled. No reference values to distinguish the different PI (index e) peaks could be found. The asterisk denotes unknown primary alcohol moieties likely contributed by undeclared PG (phosphatidylglycerol) as well as saccharide moieties on ganglioside molecules.

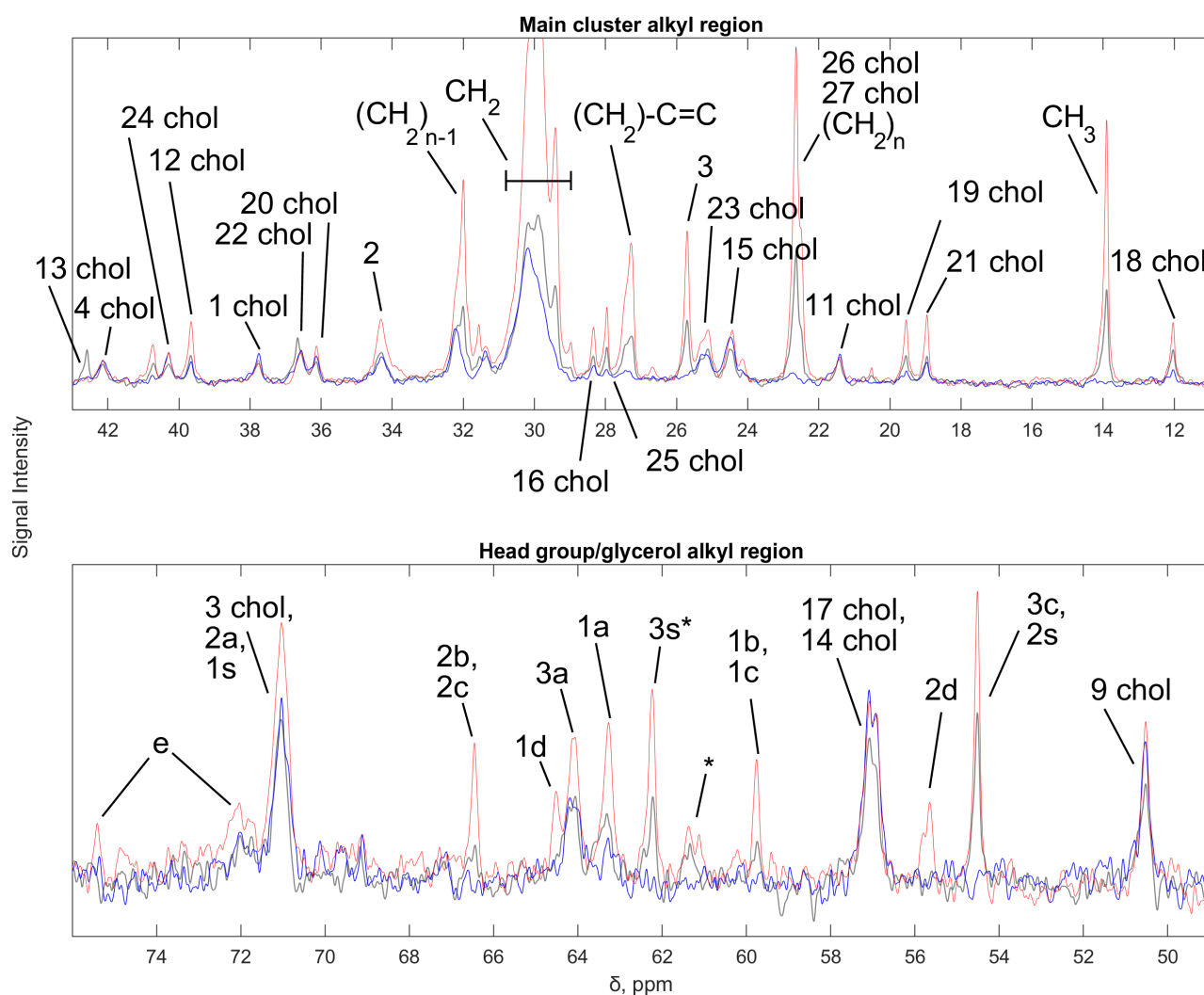


Figure A2-1: Detailed labeling of clusters in the 343 K ^{13}C -MAS spectra for the 30 wt% sample. Indexes are used as declared in the theory section in the main report. The CH_2 number indices, where applied, are labeled counting down from the CH_2 before the terminal CH_3 group. I.e., $(\text{CH}_2)_n$ is the highest index CH_2 -group. PI (index e) peaks were not able to be distinguished by position within their associated head group. The asterisk denotes unknown primary alcohol moieties thought to be contributed by PG (phosphatidylglycerol) and saccharide moieties.

Appendix B1

^1H -MAS spectra for all investigated temperatures are shown below. Figure B1-1 shows full view and Figure B1-2 shows a view of the central peak cluster. Low (ideally no) visibility of the pre-series graph indicates high consistency in sample conditions at the observed temperature. In Figure B1-2, the pre-series graph can be seen to appear in a clearly observable manner only at subzero temperatures where it still largely overlaps the post-series graph. The peak shapes change while the ppm values remain consistent. Since the overlap is high-complete at temperatures above subzero, these two factors indicate that the change between the graphs is due to slower equilibration. In summary, this indicates consistent sample conditions for all temperatures and water contents.

The top line contains a repeat of the 302.2 K experiment performed after the full temperature series for quality control purposes. Similarity with the mid-series experiment (particularly with regards to peak distribution) indicates consistent sample chemistry across temperature changes.

1H MAS spectra at key temperatures

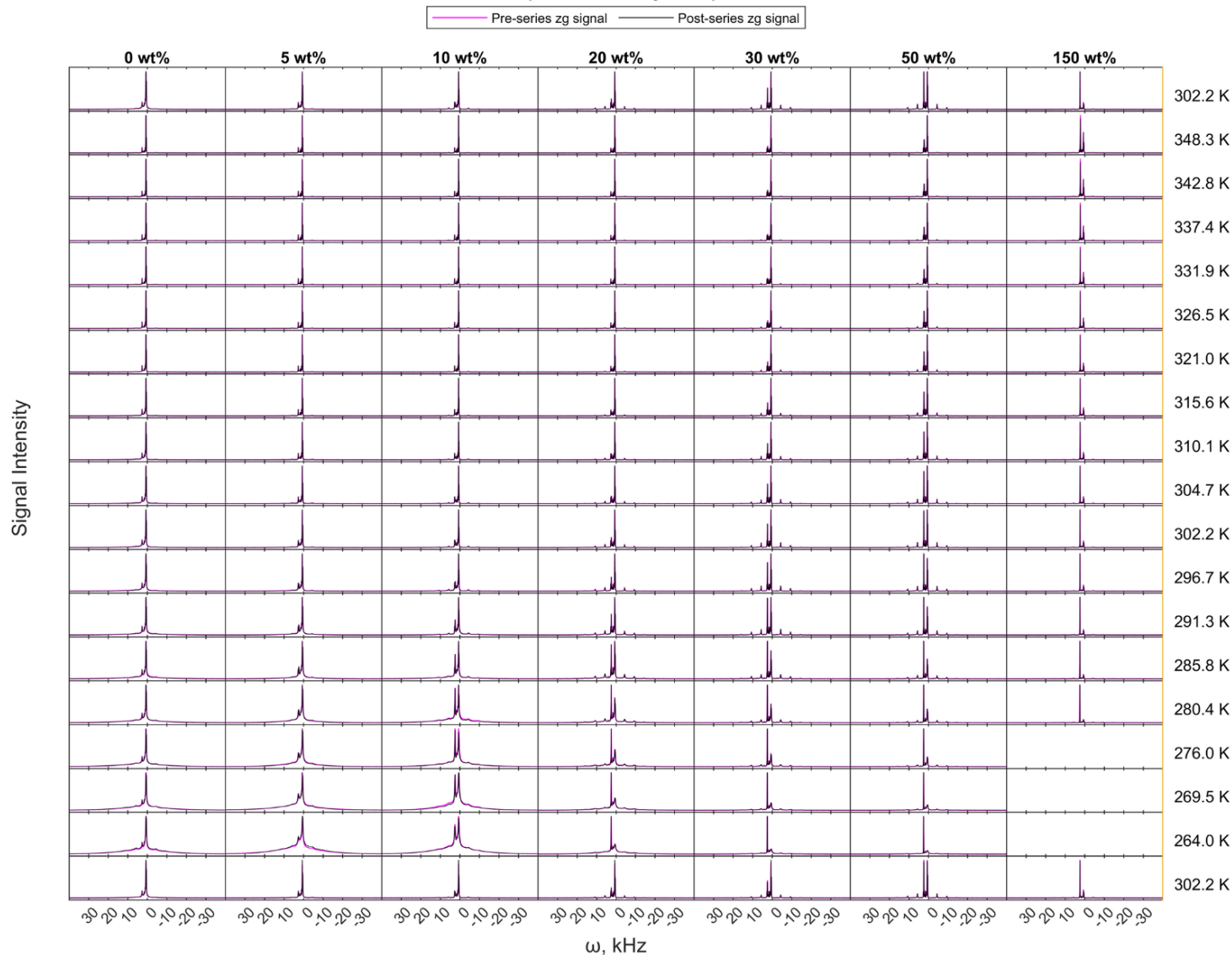


Figure B1-1: ^1H -MAS spectra for all investigated temperatures and water contents. The top row contains the post-series measurement in room temperature. The intensity axis of each spectrum has been individually scaled to 110% of its maximum peak intensity. The top and bottom lines of spectra contain room temperature experiments performed before and after the temperature ramping for the verification of consistent sample conditions. Consistent peak positions and relative signal intensities indicate consistent sample conditions.

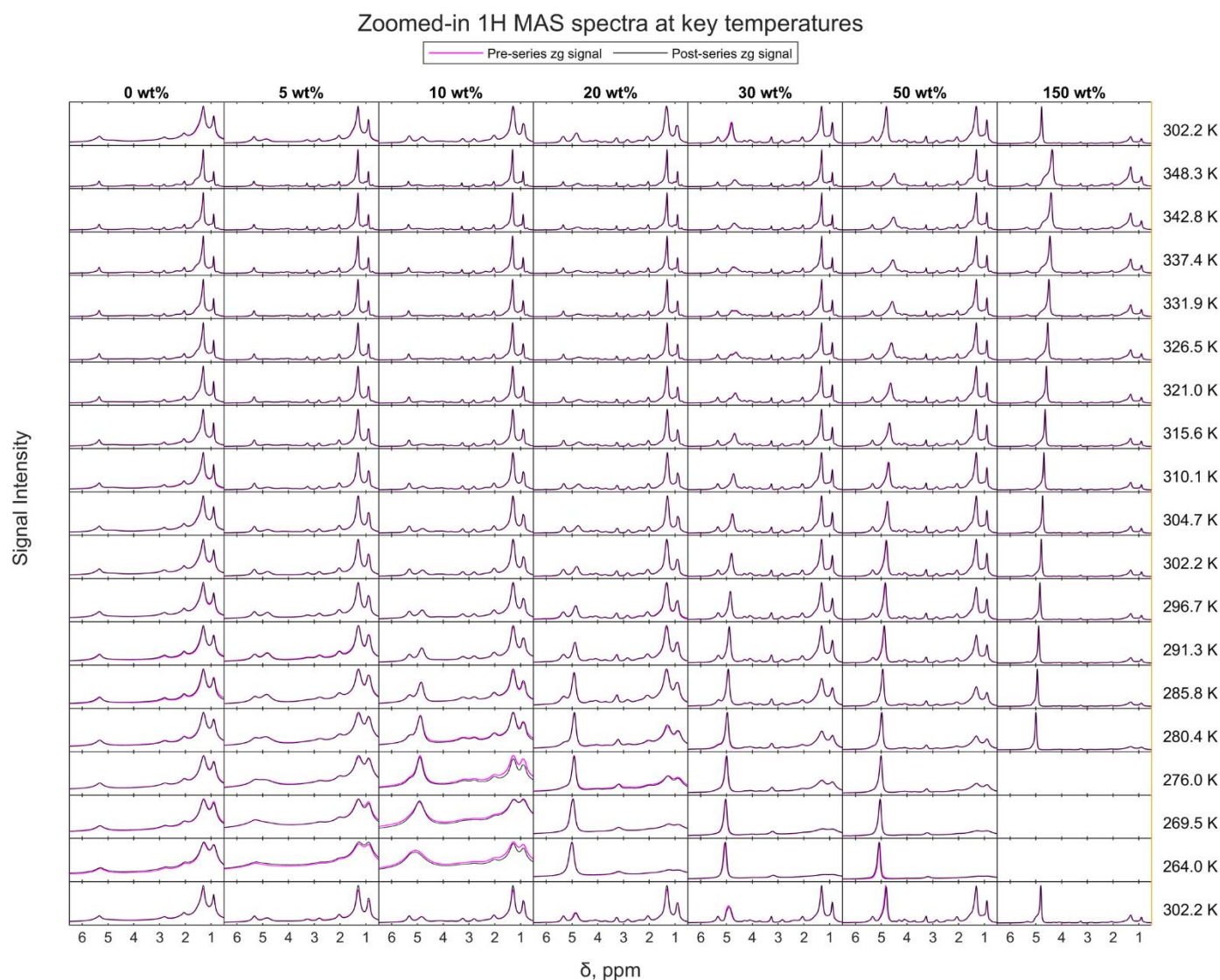


Figure B1-2: View of the central peak cluster of ^1H -MAS spectra for all investigated temperatures and water contents. High visibility of the pre-series graph (magenta) indicates low consistency in sample conditions during the course of the experiment for the temperature in question. Conversely, visibility of the post-series graph only (black, printed after the magenta graph) indicates high consistency. The intensity axis of each spectrum has been individually scaled to 110% of its maximum peak intensity. The top and bottom lines of spectra contain room temperature experiments performed before and after the temperature ramping for the verification of consistent sample conditions. Consistent peak positions and relative signal intensities indicate consistent sample conditions.

Appendix B2

Figure B2-1 shows a grid of ^{13}C -spectra for all investigated temperatures and water contents in this study. The top line contains a repeat of the 302.2 K experiment performed after the full temperature series for quality control purposes. Similarity with the mid-series experiment (particularly with regards to peak distribution) indicates consistent sample chemistry across temperature changes.

^{13}C DP-, CP- and INEPT MAS spectra at key temperatures

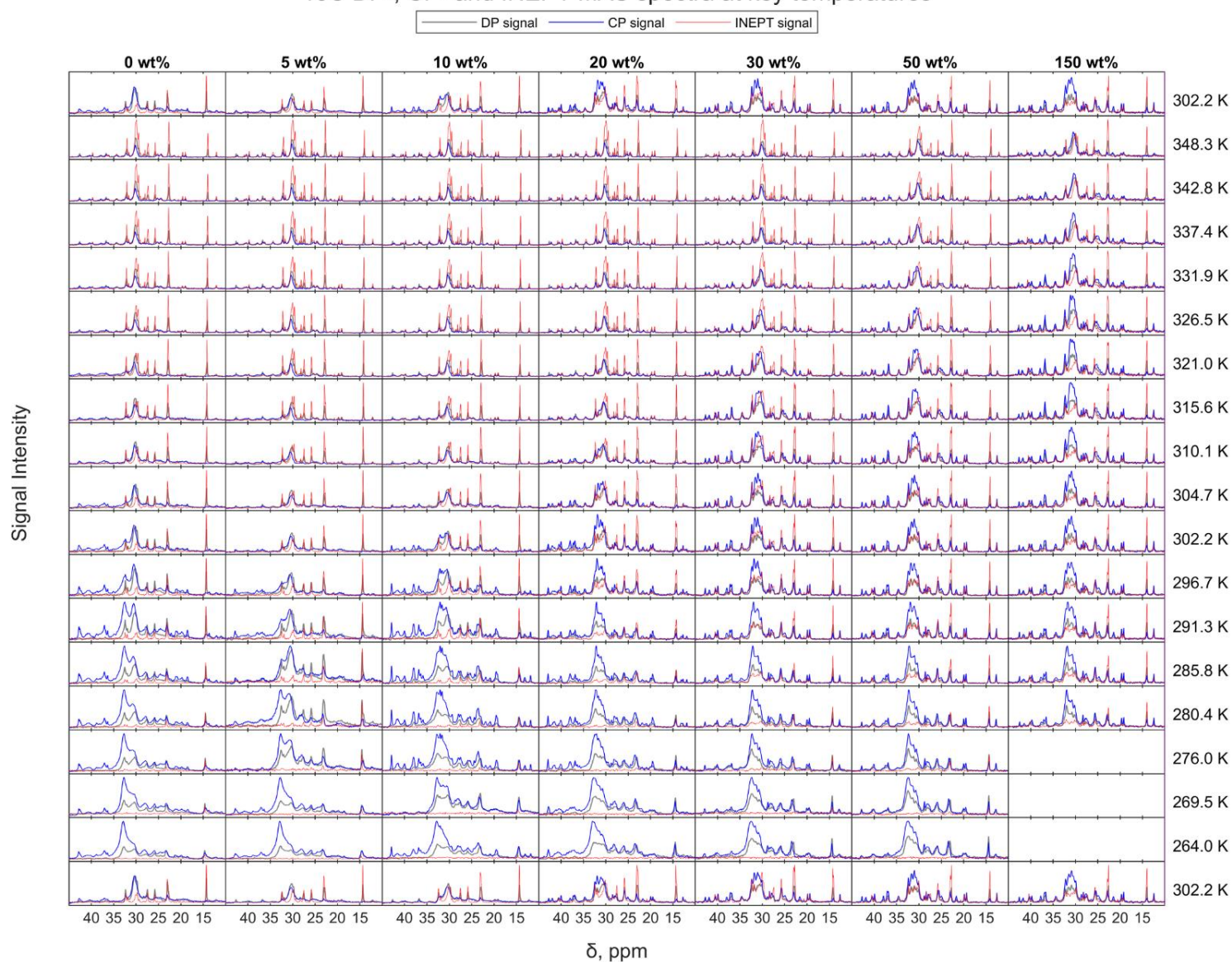


Figure B2-1: ^{13}C -MAS spectra in the 10-45 ppm range for all temperatures and water contents investigated. Grey indicates DP-signal, blue indicates CP-signal and red indicates INEPT-signal. The intensity axis of each spectrum has been individually scaled to 110% of its maximum peak intensity. The top and bottom lines of spectra contain room temperature experiments performed before and after the temperature ramping for the verification of consistent sample conditions. Consistent peak positions and relative signal intensities indicate consistent sample conditions.

Appendix B3

Figure B3-1 shows a grid of ^{31}P -spectra for all investigated temperatures and water contents in this study while figure B3-2 shows the same type of grid for all static ^{31}P -spectra. The top lines contains a repeat of the 302.2 K and 299.9 K experiments respectively performed after the full temperature series for quality control purposes. Similarity with the mid-series experiment (particularly with regards to peak distributions) indicates consistent sample chemistry across temperature changes.

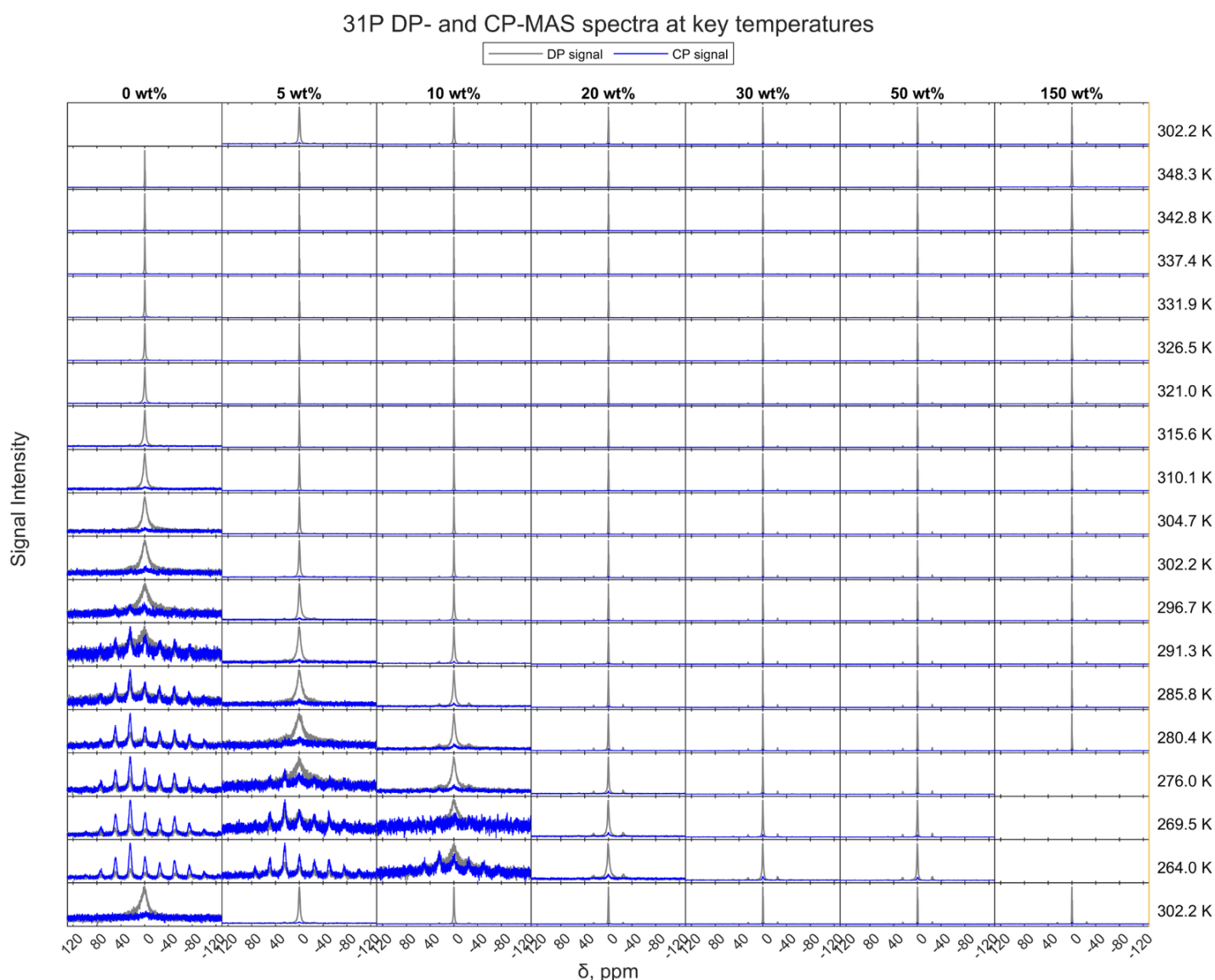


Figure B3-1: ^{31}P -MAS spectra for all temperatures and water contents investigated. Grey indicates DP-signal and blue indicates CP-signal. The intensity axis of each spectrum has been individually scaled to 110% of its maximum peak intensity. The top and bottom lines of spectra contain room temperature experiments performed before and after the temperature ramping for the verification of consistent sample conditions. Consistent peak positions and relative signal intensities indicate consistent sample conditions.

Static ^{31}P spectra at key temperatures

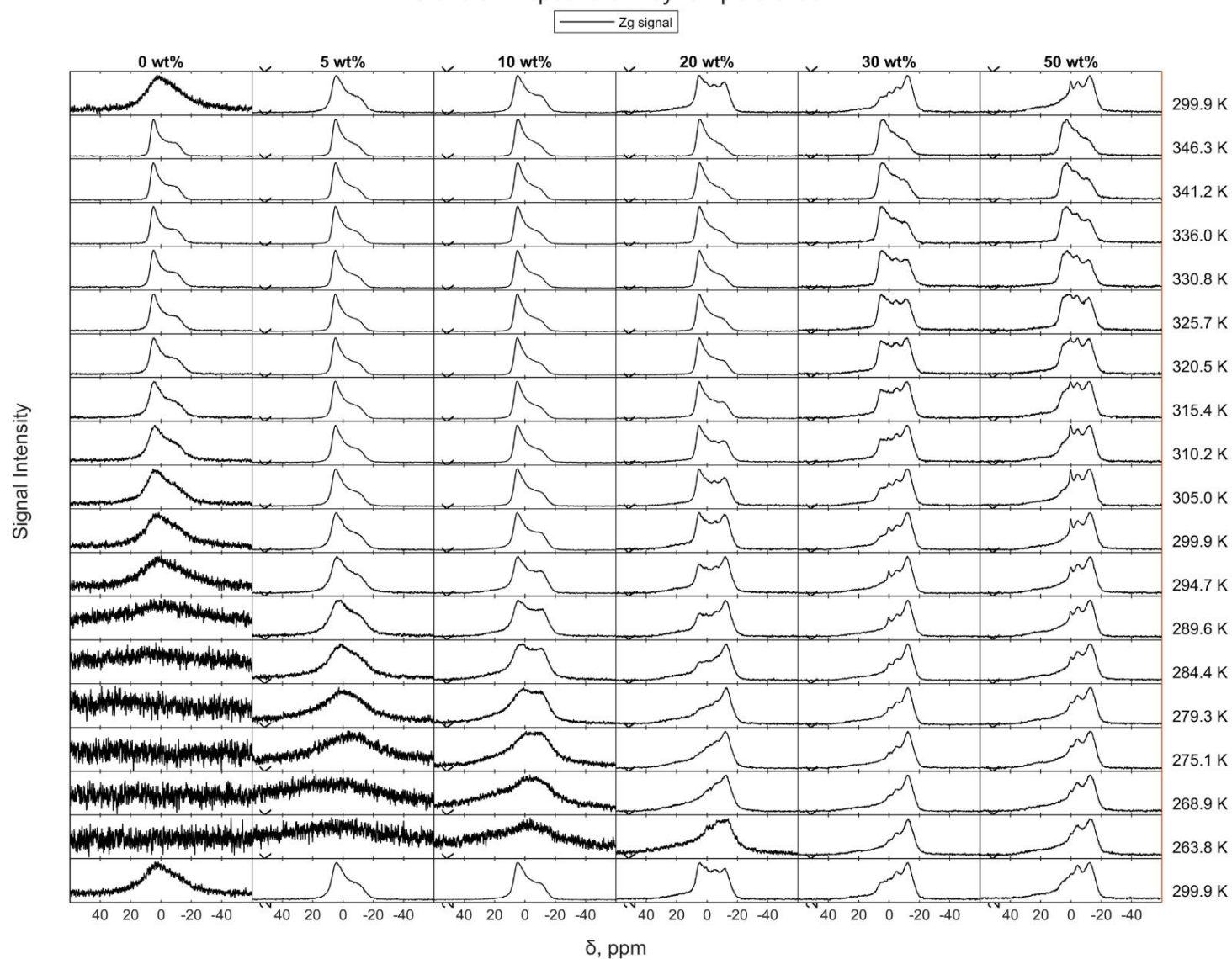


Figure B3-2: Static ^{31}P spectra for all temperatures and water contents investigated in this study. The intensity axis of each spectrum has been individually scaled to 110% of its maximum peak intensity. The top and bottom lines of spectra contain room temperature experiments performed before and after the temperature ramping for the verification of consistent sample conditions. Consistent peak positions and relative signal intensities indicate consistent sample conditions.

Appendix B4

Figure B4-1 shows a grid of ^2H -spectra for all investigated temperatures and water contents in this study. The top line contains a repeat of the 299.9 K experiment performed after the full temperature series for quality control purposes. Similarity with the mid-series 302.2 K experiment (particularly with regards to peak distribution) indicates consistent sample chemistry across temperature changes.

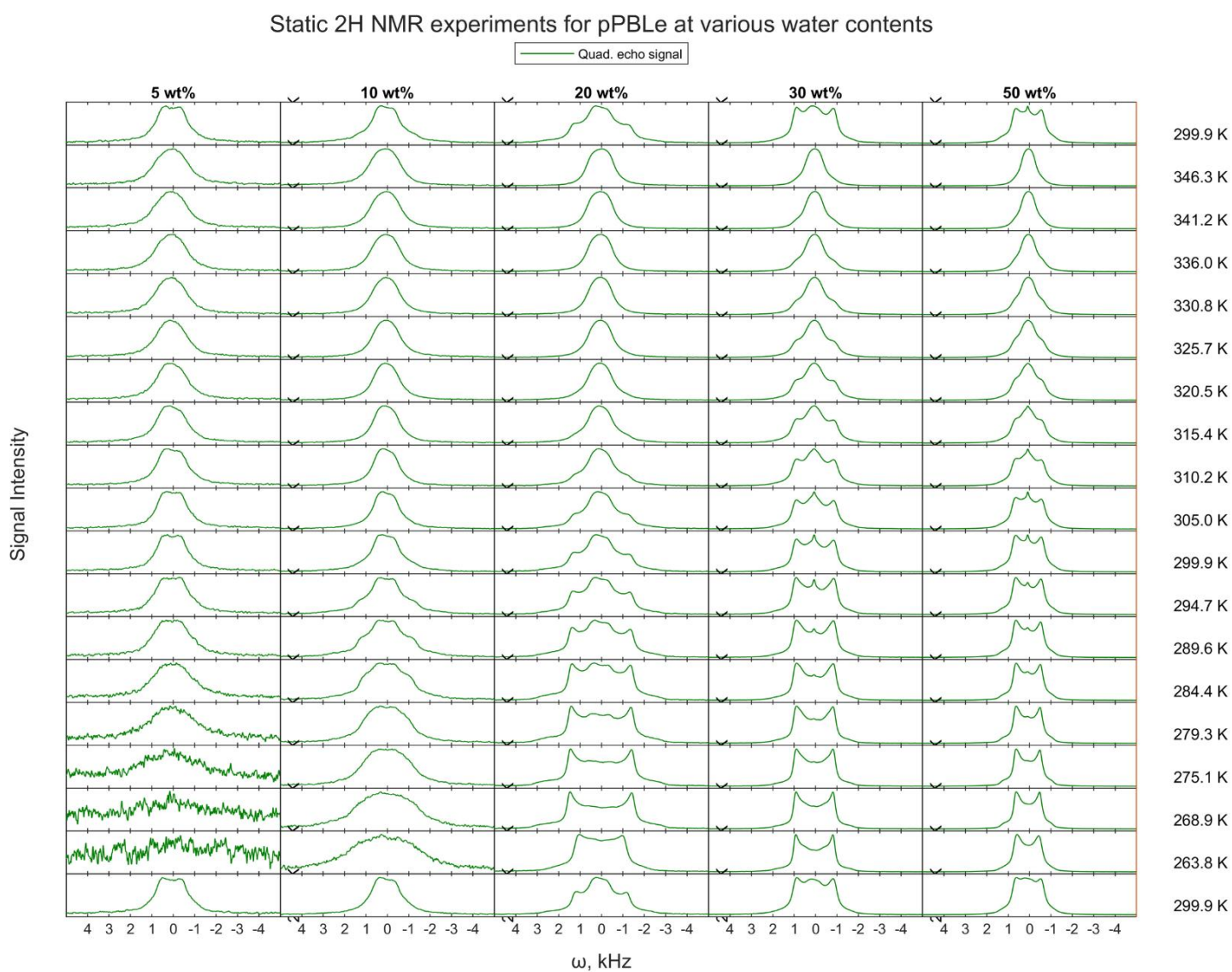


Figure B4-1: Static ^2H spectra for all temperatures and water contents investigated in this study. The intensity axis of each spectrum has been individually scaled to 110% of its maximum peak intensity. The top and bottom lines of spectra contain room temperature experiments performed before and after the temperature ramping for the verification of consistent sample conditions. Consistent peak positions and relative signal intensities indicate consistent sample conditions.

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