Applications of ^{31}P NMR in analytical chemistry
Determination of acidity constants and determination of phosphorus content in beverages and milk

Instrumental analysis practical hand-out

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NMR spectroscopy introduction

Spectroscopic methods study the interaction between the electromagnetic wave and the matter. Nuclear Magnetic Resonance (NMR) spectroscopy takes advantage of the properties of atomic nuclei. It is well known that nuclei have positive charges and behave as though they are spinning. Anything that is charged and moves has a magnetic moment and produces a magnetic field. Therefore, a spinning nucleus acts as a tiny bar magnet oriented along the spin rotation axis. And as the tiny magnet, the spin will orient in the magnetic field (Figure 1).

a) *b)*

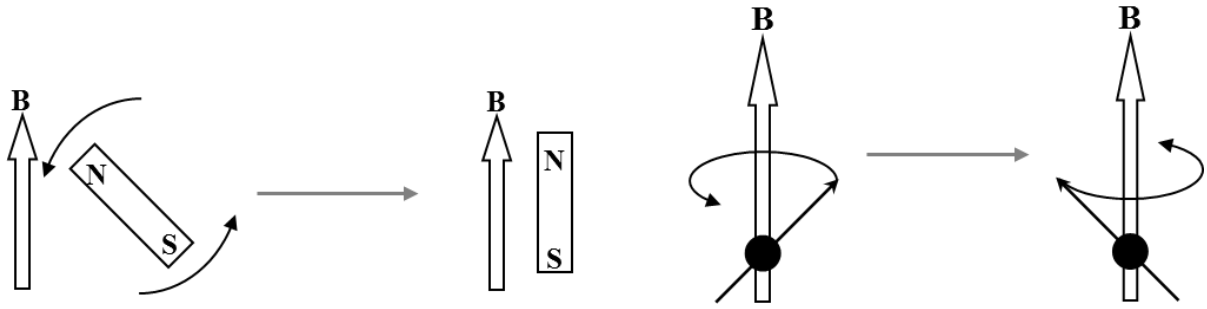

Figure 1. Orientation of a bar magnet (a) and of a nuclear spin (b) in the presence of magnetic field B

In the presence of a much larger magnetic field the orientation will no longer be random. All those nuclei which have nuclear spin quantum number I = l/2 will possess two orientations. The most favorable orientation would be the low-energy state and the less favorable orientation the high-energy state. When a radiofrequency (RF) pulse with energy equal to the difference in energies of the two levels is applied, transitions between the two energy levels will be induced. The nuclear spin system will "resonate"; the spin system absorbs the energy.

The energy required to induce flipping is the energy difference between the two nuclear orientations and is shown in Figure 2 to depend on the strength of the magnetic field Bo in which the nucleus is placed.

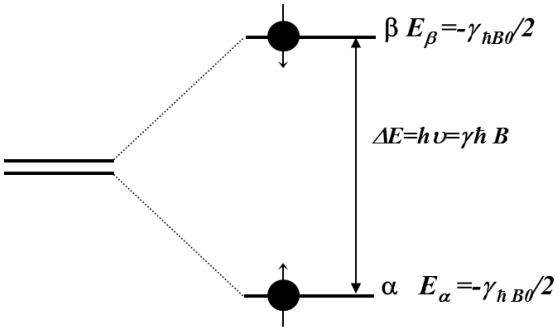

Figure 2. Splitting of energy levels for I=1/2 nuclei

Thus the absorbed energy is:

$\Delta E = \gamma\, h\, B_0/2\pi$ (1)

where h is Planck's constant , γ is *the gyromagnetic ratio* characteristic for each nucleus.

On the other hand

$\Delta E = h\upsilon_0$ (2)

The combination of equations (1) and (2) enables the frequency of nuclear transition to be:

$\upsilon_0 = \gamma\, B_0/2\pi$ (3)

$\omega_0 = 2\pi\, \upsilon_0$ (4)

Equation 4 is referred to as the Larmor equation and ω_0 is the angular *Larmor resonance frequency*.

Following the RF pulse, a signal termed free induction decay or FID can be detected as a result of the voltage induced in the sample by the energy absorption. During relaxation the nuclear spin system relaxes to the thermal equilibrium situation which occurs in the absence of any further perturbing RF pulses. This signal-time function is subject to Fourier transformation and the resulting signal-frequency curve is the *spectrum* (Figure 3).

a) *b)*

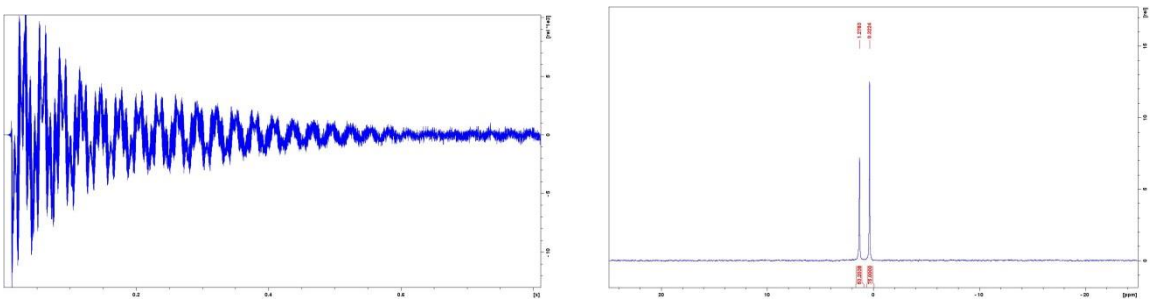

Figure 3. a) ^{31}P FID recorded at 101.25 MHz (250 MHz spectrometer) and b) the resulting spectrum after Fourier transformation. The sample consisted of 75 mM DHPC (dihexanoyl-phosphatidil-choline) in 50mM phosphate buffer at pH=5.5. The resulting two peaks in the spectrum belong to the phosphate-head group of the detergent and to free phosphate, respectively.

The properties for most important I=1/2 nuclei which are frequently measured are:

Nucleus	$\gamma\ 10^7$ /$T^{-1}s^{-1}$	υ_0 (B_0=11.74T) /MHz	natural abundance (%)
^{1}H	26.7	500.00	99.98
^{13}C	6.73	125.72	1.108
^{15}N	-2.71	50.66	0.37
^{19}F	25.18	470.30	100
^{31}P	10.84	202.40	100

Phosphorus-31 NMR spectroscopy

Solution ^{31}P-NMR is a routine NMR technique, as ^{31}P is a ½ nucleus with an isotopic abundance of 100% possessing a relatively high magnetogyric ratio (see the above table). All these properties make spectra acquisition quick and easy for even low, mili-molar concentrations. Moreover, due to the fact that molecules contain low number of phosphorus atoms acquisition of one-dimensional spectra is often enough, and their interpretation is relatively easy. Chemical shifts are referenced to 85% phosphoric acid as an external standard assigned to 0.00 ppm. The ^{31}P chemical shift range is very large, the typical widths is the -250 – 250 ppm domain. The chemical shift is very sensitive to changes in phosphorus environment, a property which can be widely exploited. Often ^{1}H decoupled spectra are acquired with different pulse-sequences. For quantitative purposes due to the inconsistent nuclear Overhauser effect the so-called inverse-gated decoupling scheme should be applied. One-bond ^{31}P-^{1}H coupling constants $^{1}J_{PH}$ characteristic for PH moieties are cca 190 Hz, two-bond $^{2}J_{PH}$ couplings are one magnitude lower.

Applications

Phosphorus is commonly found in organic compounds and in coordination complexes (phosphines). Purity determination and assignment of phosphorus-containing compounds is the most frequent application, which is done routinely.

If phosphorus compounds are used as catalysts, and the reagents/products in the reaction do not contain this nucleus, then ^{31}P NMR can be a brilliant choice for following changes (also under *in situ* conditions) in the catalyst behavior that lead to valuable information regarding mechanism elucidation.

^{31}P NMR is widely used in phospholipid bilayer and native biological membrane characterization studies. These investigations provide a wide range of information about bilayers packing, phase transitions, lipid head group orientation and dynamics, changes in the lipid environment upon protein binding.

Enzimatic reactions can be monitored following for example ATPase, dUTPase, etc. hydrolysis reactions. Determination of structure and dynamic behavior of nucleic acids is an important ^{31}P NMR field for biological applications.

Analytical applications include the quality control of food, beverages and environmental samples.

The **goal of this lab** is to show and prove how efficiently and under which limitations can this technique be applied for analytical purposes. It is also desired to give an overview how should a chemists´ approach be when finding the proper method and developing it for purity determination. There are multiple choice tasks in order to develop skills for precise quantitative measurement elaboration.

Instrument

The 250 MHz Bruker Avance spectrometer equipped with a 5 mm QNP $^{1}H/^{13}C/^{19}F/^{31}P$ probe-head will be used and all parts of the instrument will be presented in detail at the beginning of the lab. The resonance frequency of ^{31}P is 101.2 MHz at this field. Spectrometer handling and measurements will be done with the supervision of spectroscopist.

The sample tube - after corresponding positioning in the rotor - is introduced with the help of compressed air into the spectrometer. The main steps and *commands* (used software version is TopSpin 2.1) are as follows:

Before acquisition, preparation steps:

- open a ^{1}H measurement
- lock to the deuterium signal – *lock*
- wobble to the resonance frequency (present case ^{1}H or ^{31}P) – *wobb*
- correct field inhomogeneities – *shim*

Next step is the acquisition of a ^{1}H spectrum:

eda – edit aquisition parameters – define parameters, set the pulse program: **zg** or **zgig**

zg – zero go – start measurement

Check the measurement in the FID acquisition window, once measurement is over, processing and analysis steps are followed. The same is valid also for $^{31}P\{^{1}H\}$ measurement, there **zgig** pulse program is introduced.

lb – line broadening – the value for ^{1}H spectra is usually 0.3Hz, but strictly < 1Hz, for ^{31}P can be 2-3Hz

em – exponential multiplication, smoothing with the preset LB value

ft – Fourier transformation, resulting the spectrum

pk – phase correction, preferably mostly 0 order

apk – automatic phase correction

abs – automatic baseline – correction

bas – baseline, manual settings

pp – peak picking, choose automatic, just set the minimum height

int – manual integration, define the given interval values.

dconv – determination of line width. Note, the LB value needs to be substracted.

When measurement is over, sample is taken out by pressing the Lift Off button.

As we intend to have the same experimental setup throughout the investigation and we do not want to overwrite the result with the help of the *edc* command a new measurement file in the same directory is saved, by changing only the experiment number.

I. Determination of pK_a values for H_3PO_4

The classical and most precise method for the determination of acidic and stability constants is by potentiometric titrations. For more complex systems the combined NMR/potentiometry technique can be applied, where data are evaluated and constant values are calculated by computer programs (LAKE, PSEQUAD). There are cases, when this approach is not applicable (when need to deal with too small substance amounts, or no ion-selective electrode is available, or when working at extreme pH values, etc). In such circumstances NMR spectroscopy can be the method of choice. Protonation/deprotonation or coordination affects the environment of a nucleus which and as a consequence this is mirrored in the change of chemical shift values. Successful studies were conducted for determination of amino acid residue pK_a values in proteins, or for *in situ* pH measurement in the CF_3COOH system by monitoring the ^{19}F resonance signal.

Task: Determination of pKa1 and pKa2. In the pH=1.5-3.5 (I) and pH=5-8 (II) regions the following two species coexist: H_3PO_4 and $H_2PO_4^-$ (I) and $H_2PO_4^-$ and HPO_4^{2-} (II), respectively. Even though in solution two distinct phosphorus environments exist, due to the rapid proton exchange between them only one signal will be detected. As a consequence, the chemical shift of this resonance is pH dependent and the line width varies as well. At every pH value the following equation is valid:

$$\delta = \delta_1 x_1 + \delta_2 x_2 \qquad (5)$$

where δ is the measured chemical shift, numbers 1 and 2 denote the existing species, δ_1 and δ_2 are the chemical shift values for solutions containing only the corresponding species, $x_1=c_1/(c_1+c_2)$ and $x_2=c_2/(c_1+c_2)$ are the molar fractions.

Considering for example the protonation equilibria:

$$HPO_4^{2-} + H^+ = H_2PO_4^- \qquad (6)$$

characterized with the acidity constant K_{a2}, on the basis of equation (5) and (6) the $\delta = f(H^+)$ relation can be deduced.

Question 1: Deduce the $\delta = f(H^+)$ dependency for K_{a1} and K_{a2}.

Stock solutions: 0.02 M HPO_4^{2-} and 0.02M $H_2PO_4^-$, HCl, NaOH, D_2O

Preparation: In 10 ml beakers six-eight solutions with different pH values are prepared in the desired pH range from the 0.02M stock solutions. In 5mm NMR tubes 600µl is introduced and 60 µl D_2O is added to each solution.

Data evaluation: The measured values are presented for each sample in the following table:

pH	*δ (ppm)*	*LW (Hz)*

Draw the *δ-pH* curve, and determine the inflexion point from the fitted 3rd order polinomial curve. For this the second order derivative is used.

Discuss the discrepancy between literature data, pH-potentiometric and NMR spectroscopic determined pK_a values. Which are the possible error sources?

Check the validity of the deduced $\delta = f(H^+)$ dependency (Question 1) for at least one measured point!

II. Determination of phosphorus content in beverages

If the species to be determined is H_3PO_4 - in different protonation states - based on your previous studies which are the possible classical and instrumental analytical techniques that can be applied for quantitative determination?

Once the method is chosen, there are two ways to conduct such determination: the successive dilution (a) and standard addition (b) methods, both will be tested.

Question 2: Discuss the advantages and disadvantages of all enumerated methods.

Question 3: Discuss the advantages and disadvantages of (a) and (b), how do we get the concentration from the conc – signal intensity curves? Are we allowed to do extrapolation, which is the starting point? What is the meaning of the negative crossing of the x axis for method (b)?

Stock solutions: 80 mM $H_2PO_4^-$, 10 mM $H_2PO_4^-$, 20 mM $H_2PO_4^-$, 1M $H_2PO_4^-$, D_2O

Preparation: For (a) six solutions in the 2-10 mM concentration range needs to be prepared in NMR tubes, with a final volume of 600 µl containing 10% D_2O. Taking into account the available pipettes, calculate and argue which stock solutions would you use and why? Note one has to minimize error sources. The separate beverage solution contains only 10% D_2O, and this dilution needs to be taken into account at calculations!

For (b) argue which stock solution and which pipette will be used in order to avoid sample dilution and to increase at each addition the phosphorus concentration with cca 2 mM.

Data evaluation: (a) The measured values are presented for each sample in the following table:

c (mM)	*Integrated intensity*	*Peak height*	*δ (ppm)*

Determine the calibration curve from both the integrated intensities and peak height values. Under the given experimental conditions which method is more precise?

Give the phosphorus content of the beverage

(b) Data are grouped in the following table:

Added c_p (mM)	*Integrated intensity*	*Peak height*	*δ (ppm)*

Determine the calibration curve from both the integrated intensities and peak height values and give the phosphorus content.

Compare the two experimentally obtained values, also make a comparison with the literature value. Which of the two methods has fewer errors and gives better result? What are the possible error sources? Give an estimate of the average daily phosphorus uptake, and discuss how much beverages need to be drunk in order to fully cover this amount? What makes these beverages unhealthy?

III. Determination of phosphorus content in milk

Phosphorus is an important element for many essential processes in the body. In combination with calcium it is necessary for the formation of bones and teeth. Phosphorus is also involved in the metabolism of fat, carbohydrate and protein, in the effective utilization of many of the B-group vitamins, and in energy metabolism. Phosphorus is widely distributed in both plant and animal foods.

There are several compounds in cow milk that contain phosphorus, amongst which phosphate and phospholipids. Some peaks are narrow others are broad, depending on the investigated milk. The work steps are the same as for beverage determination, with the exception of sample preparation. Neat sample; boiled, centrifuged and filtered sample; and sample containing EDTA can all be tested.

Solution preparation and evaluation is the same as for II.

References:

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