Determination of the phosphorus content of cola drinks by ^{31}P NMR spectroscopy: an introduction to using the external calibration and standard addition approaches

Student handout

I. Background, topics and questions to be discussed

How to determine phosphoric acid in any kind of matrix?
(A) Relying on the ***acid-base properties***. Phosphoric acid is a triprotic acid that dissociates in three steps:

$$H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^- \qquad pK_{a1} = 2.19$$
$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-} \qquad pK_{a2} = 7.21$$
$$HPO_4^{2-} \rightleftharpoons H^+ + PO_4^{3-} \qquad pK_{a3} = 12.44$$

- pH in the equivalence point (pH of the ampholite): $Eq_n = (pK_{a,n} + pK_{a,n+1})/2$
  - First endpoint: pH = 4.7
  - Second endpoint: pH = 9.8
  - Third endpoint: pH = 11.5 (not visible in water)
- titrimetry: using strong base, eg. NaOH as a standard solution (titrant) ↔ bad example: weak base, eg. NH_3
- determining the endpoint:
  - *Classical (chemical) method – with an indicator*:
    - Methyl orange: colour change in pH-range 3.2 – 4.4 (from red to yellow) → first dissociation is detected, H_3PO_4 behaves as a monoprotic acid
    - Thymolphtalein: colour change in pH-range 9.3 – 10.5 (from colourless to blue) → second dissociation is detected, H_3PO_4 behaves as diprotic acid (phenolphthalein is not good: changes colour too soon: 8.2 – 10.0)
  - *Instrumental – electrochemical methods:*
    - Potentiometric titration: Measuring the voltage between the ion selective electrode and a reference electrode upon addition of titrant (sigmoid curve, endpoint at the inflection) → H^+ ion selective: glass electrode ↔ there's no phosphate selective electrode
    - Conductometric titration: Measuring the conductivity of the solution upon addition of titrant → H^+ and OH^- ions have extremely high molar conductivity, there's a minimum at the endpoint (V-shaped curve)

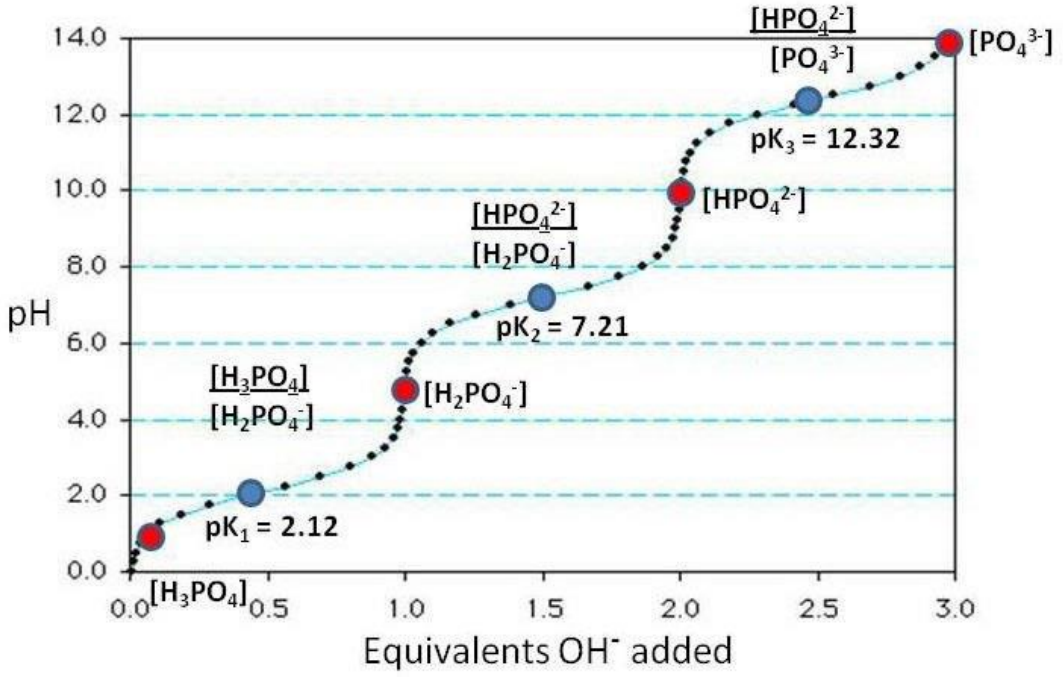

Figure 1: Speciation distribution curve of the phosphate species.

- advantages:
  - fast, inexpensive, easy to carry out
  - electrochemical methods: automation is possible, more accurate than chemical
- drawbacks:
  - interference from matrix
    - colour: endpoint detection of chemical indicator is impossible; not a problem in electrochemical methods
    - other acidic compounds (eg. carbonic acid, ascorbic acid, citric acid): interferes in both cases → not selective!
  - large concentration is needed, trace amounts cannot be measured.

(B) Relying on the *presence of the phosphate ion*.

1) Gravimetry:
   Precipitate the species of interest and measuring the mass of the precipitate. For phosphate magnesium is used in ammonia solution.

$$PO_4^{3-} + Mg^{2+} + NH_3 + 7H_2O \rightarrow \mathbf{MgNH_4PO_4 \cdot 6H_2O} + OH^-$$

   Advantages: inexpensive
   Drawbacks:
   - takes long time, needs a lot of manual work, difficult to carry out, very large concentration is needed
   - interferences: co-precipitation
   - rarely used nowadays.

2) Spectrophotometry**:**

For determination of phosphate the *molybdenum blue* reaction is used. Ammonium-molybdate is added to the phosphate-containing sample, the resulting colourless heteropolyanion is reduced with hydrazine to molybdenum blue (mixed-valence complex).

$$PO_4^{3-} + 12MoO_4^{2-} + 24H^+ \rightarrow [PO_4(MoO_3)_{12}]^{3-} + 12H_2O$$

$$[PO_4(MoO_3)_{12}]^{3-} + N_2H_4 \rightarrow \text{Mo(V,VI)-complex}$$

Advantages:

- very sensitive, small amount of phosphate can be detected
- fast, automation is possible

Drawbacks:

- coloured solutions cannot be investigated
- interference: silicate, arsenate
- more expensive than classical methods (quite cheap and routine nowadays).

3) NMR-spectroscopy

Advantages:

- very selective: no interference (if we are curious about the phosphorus content)
- very sensitive: 100 % natural abundance, large magnetogyric ration, spin half (unlike ^{13}C-NMR)
- fast (1 measurement – approximately minute, easy sample preparation)
- sample usually has a few (usually 1) phosphorus environments, spectrum is simple (unlike proton-NMR)
- ppm scale for ^{31}P is quite wide: signal overlap is rare

Drawbacks: expensive (instrument - hightech: superconducting magnet; maintenance: liquid He and N_2. However, it is a non-invasive method, experimental time is short and combining with ^{1}H NMR measurements a multitude of information is obtained from one experiment about several components in the system.

Table 1 contains the summary of the above discussed methods. In the light of all these the method of choice for the present determination is ^{31}P-NMR spectroscopy.

Table 1. Possible methods for phosphoric acid determination

	H^+ sensitive	PO_4^{3-} sensitive
Classical	titrimetry with chemical indicator	gravimetry
Instrumental	potentiometric titration conductometric titration	spectrophotometry NMR-spectroscopy

II. ^{31}P-NMR spectroscopy

^{31}P is a spin ½ NMR active nucleus (consists of 15 protons and 16 neutrons). As a nucleus with spin I splits into $2I+1$ energy levels, ^{31}P will have two energy states. The energy difference is in the radiofrequency regime of the electromagnetic spectrum. The ω_0 is the angular frequency of the absorbed photon depends on the magnetic field strength (B_0) and the magnetogyric ratio (γ) of the examined nucleus:

$$\omega_0 = \gamma B_0$$

The higher B_0 and the γ are, the better the sensitivity. The magnetogyric ratio is specific to a certain isotope. In the table below the characteristics of the most frequently measured nuclei with ½ spin are collected:

Nucleus	**γ / 10^7 rad/(T·s)**	**Natural abundance**
^{1}H	26.7	99 %
^{13}C	6.7	1 %
^{15}N	-2.7	0.1 %
^{31}P	10.8	100 %

The advantages of ^{31}P-NMR are:

- ^{31}P has relatively high magnetogyric ratio → sensitive measurement, measurement times are short.
- ^{31}P has 100 % natural abundance → further emphasizes the sensitivity of the measurement, and the short experimental time, at the same time low concentration samples can be measured,
- ^{31}P is a building block of many organic compounds → offers complementary information to the ^{1}H and ^{13}C NMR characterisation.
- the number of ^{31}P atoms in one molecule is typically low; → few signal overlap, easy spectral interpretation (unlike in a ^{1}H spectrum). Moreover, the chemical shift range is broad, covers the -250 – 250 ppm spectral range.

Hazards associated with NMR spectroscopy. All hazards associated with strong magnetic fields are common to all NMR laboratories. Students with pacemakers and/ or metallic protheses are not allowed to enter the laboratory in which the instrument is located. Students are required to leave all their magnetizable metal objects (e.g. keys, watches, steel jewellery) and magnetic devices (eg. cell phones, pendrives, RFID cards) outside the laboratory. Both KH_2PO_4 and NaH_2PO_4 are irritants and thus their ingestion, contact with the skin or the eye should be prevented. The ingestion of D_2O should similarly be prevented. Sample preparation must be performed wearing proper shoes, clothing, gloves and goggles.

III. Sample preparation

Stock solutions:

- 0.08 M NaH_2PO_4: for the standards in external calibration method
- 1 M KH_2PO_4: for spiking the solution in standard addition method

Calibration standards:

- mix the necessary amount of 0,08 M stock, water and heavy water in an NMR tube and homogenize it
- the final volume has to be 600 µl which contains 10 % D_2O
- the concentration range is 2 mM – 10 mM → calculate the necessary volume of stock according to the dilution rule: $c_1V_1 = c_2V_2$

Preparing the sample: it contains only coke and 10 % D_2O → 540 µl coke + 60 µl D_2O
Checking the precision: height of liquid in each NMR tube.

III. The ^{31}P and ^{1}H spectra of a phosphate-containing sample.

Instrumentation. A Bruker Avance 250 MHz (101.2 MHz for ^{31}P) instrument equipped with a QNP $^{1}H/^{13}C/^{19}F/^{31}P$ probe-head or a Bruker Avance Neo 400 MHz instrument (161.9 MHz for ^{31}P) equipped with a BBO probe-head, operating in automation mode is used. Automatic operation means sample insertion from the rack into the probe-head, tuning, matching, locking to the D_2O content of the sample and shimming are done without user-intervention. The measurement itself and basic processing of the FID (exponential multiplication, Fourier-transformation and phase-correction) are also performed automatically by the software. This also provides students an example of analytical process automation. Measurement steps, spectrometer setup and parameters as well as spectral processing steps can be discussed during the run-time of the measurements.

If the exercise is adapted for a spectrometer not in automation mode, then the students might need to perform tuning, matching, locking, shimming and processing of the FID manually. This might be very educational from a spectroscopic and technical point of view. However, the instructor needs to make sure that there is enough laboratory time for carrying out all these steps and explaining the underlying concepts sufficiently.

The NMR measurement, technical details.
Locking: The magnetic field and the radiofrequency are not stable enough in a long time → field/frequency stabilization is required. A separate NMR experiment in the "lock" channel runs parallel to the one in the observed channel. The lock signal is held in resonance so the field/frequency relationship is defined also for the observe channel. Usually, the ^{2}H resonance of the deuterated solvent is used to provide the lock signal.
Shimming: In order to gain sharp peaks, the magnetic field has to be very homogenous around the sample. The so-called shim coils surround the magnet generate a weak magnetic field to

cancel out the inhomogeneities of the main magnetic field. The coils are labelled according to the filed profiles they generate, *eg*. x, y or z shims varies linearly in the respective direction, z^2 varies quadratically along the z direction etc. In practice we typically set the z, z^2, z^3, x and y shims. The efficiency of shimming is indicated by the large peak height of the lock signal.

Data acquisition: In the simplest NMR experiment, the radiofrequency pulse excites the nuclei in the sample. Following this, relaxation occurs, *ie.* the initial equilibrium is re-established. According to the vector model the excitation can be explained by the rotation of the magnetization from the *z* direction (parallel to $\boldsymbol{B}_0$ field) to the *xy* plane. The magnetization precesses in the *xy* plane with ω_0 angular frequency, which results in a sinusoidal signal that decays in time due to relaxation. This time-dependent signal is called the free induction decay (FID). The Fourier-transformation of the FID gives the spectrum**,** the time dimension is transformed into frequency dimension. So, the Fourier-transformation sorts out the frequencies present in the sinusoidal time-dependent signal.

Pulse programs used. First, the 1D ^{1}H spectrum of the 2 mM calibrant is recorded. One has to comment the appearance on one huge signal. For the cola sample, water suppression is applied using the noesygppr1d pulse sequence. This gives a nice overview of all ^{1}H-containing components of the beverage.

For ^{31}P measurements several basic pulse sequences can be used: zg, zg30 – these do not include ^{1}H decoupling –, zgpg, zgpg30 – with power gated ^{1}H decoupling and zgig using the inverse gated ^{1}H decoupling. Though only remote, dissociable protons are present, ^{1}H decoupling is still favoured as it makes peaks slightly narrower. It is crucial to emphasize that for analytical purposes it is of utmost importance that spectra are recorded with the same parameters. An example of experimental setup is given in Table 2. Typical experiment time is 1 min.

Table 2. Experimental parameter set for ^{31}P, ^{1}H measurements. Curly brackets around a nucleus indicates decoupling of the given nucleus during the measurement.

Parameter	*Abbreviation/ unit*	*Spectrum*		
		$^{31}P\{^1H\}$, zgpg30	*^1H, zg30*	*^1H wsupp, noesygppr1d*
Spectral width	SW/ ppm	50	14	14
Transmitter offset	O1P/ ppm	-5	6	4.7
Number of transients	NS	16	16	16
Number of dummy scans	DS	4	2	2
Number of points in the time domain	TD	16k	32k	32k
Inter-scan delay	D1/ s	2	1	2
Exponential line broadening	LB/ Hz	1	0.3	0.3
Size of real spectrum	SI	16k	32k	32k

FID processing: The main steps to be discussed and perform are: Fourier-transformation, phase correction and baseline correction. All these are done within the TopSpin software. Chemical shift referencing manual by setting the ^{31}P chemical shift of external 85% phosphoric acid to 0 ppm. Four spectral parameters need to be collected: chemical shift, peak height, peak integral and peak width. Peak picking and integration are done manually. Integration is performed in a 1 ppm wide window around the chemical shift of the single peaks in each spectrum to insure reproducibility of integration. Peak width is determined by Lorentzian deconvolution.

Further questions to be discussed: What do we see in the ^{31}P spectrum of the phosphate containing sample under acidic conditions?

- only one, usually narrow peak (line width can vary with pH)
- pH of the cola drink typically around ~2.5 → equilibrium between H_3PO_4 and $H_2PO_4^-$ species (see the speciation distribution curve on Figure 1)
- regardless that these two species are present, only one peak is detected → reason: fast chemical exchange between the two phosphate species (faster than an NMR-measurement is performed)

IV. Quantification of the NMR spectrum

The detected ^{31}P resonance peak of the phosphate containing sample has an intensity (height) and an integrated intensity. The integral (area under curve) is proportional to the concentration of the sample. If peak widths are the same within experimental uncertainty, then peak height is also proportional to the concentration. Figure 2 depicts the main characteristics:

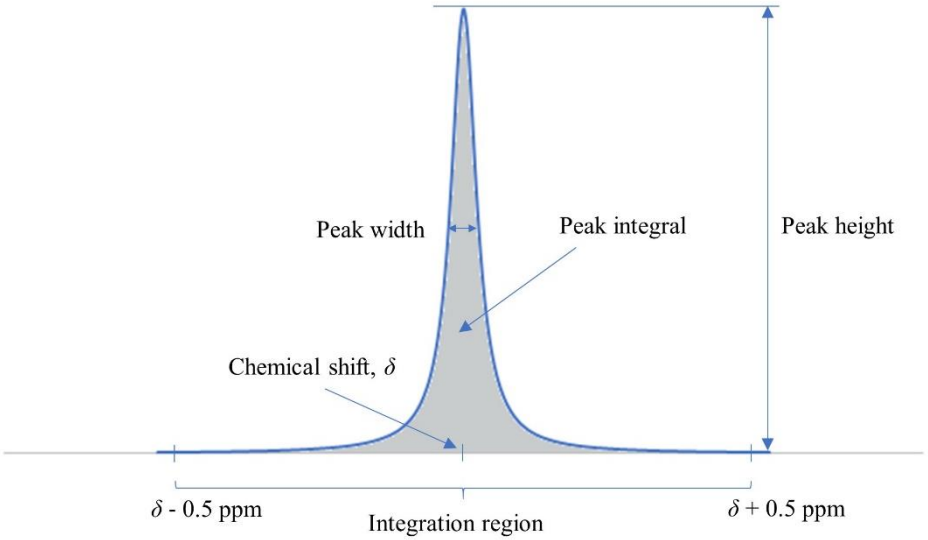

Figure 2. The Lorentzian NMR peak – similar to the experimental phosphorus resonance – and the main parameters to be determined: chemical shift denoted δ, peak height and peak width. Peak integral is the grey area under the curve inside the integration region.

However, the absolute peak height/ integral itself cannot be directly related to concentration (I_{abs} = 562342 → c = ?) → calculating concentration from measured data.

(A) External calibration

A series of calibration standards incremented linearly are prepared. The sample concentration must fall between the smallest and largest concentration of the calibration standards (in the calibration region) and the calibration curve should be linear.
The concentration of the sample can be calculated from the fitted line's equation: $I = Bc + A$. Intercept (A) should be zero theoretically, but in practice it is just expected to be much smaller than the slope (B).
Advantages:

- suitable for "high throughput" analysis: one calibration curve is enough for numerous similar samples

Drawbacks:

- extrapolation is not allowed, only interpolation
- **matrix effects** are neglected.

For external calibration, signal intensity is plotted against calibrant concentration. A linear function is fitted to the obtained experimental points as shown in Fig. 3a. At the same time, the meaning of the parameters A and B in the general linear equation $y = A + B \cdot x$ is discussed. Special attention is paid to the fact that the detected intensity for 0 sample concentration should be 0. Therefore, parameter A should be small, comparable to the integral of spectral noise. The concentration of the unknown sample is determined by interpolation according to Eq. 1:

$$c_{\text{sample}} = \frac{I_{\text{sample}} - A}{B} \qquad (1)$$

where c_{sample} is the total phosphorus concentration of the sample solution, I_{sample} is the intensity (either peak height or integral) for the sample solution, while A and B are the y-intercept and the slope of the corresponding fitted line, respectively.

(B) Standard addition

The sample is spiked with a known amount of the analyte. It is preferable if the added amount is so small that dilution can be neglected (best: addition of solid analyte to the solution ↔ if that is not possible: addition of concentrated solution, dilution is in the % range)
The added concentration is plotted versus the analytical signal. The sample's concentration is put to $c = 0$ (as it contains no added analyte). If we reduce the sample's concentration until it reaches $I = 0$ (we move into the negative region of the concentration axis), we get the exact concentration of the sample (removing analyte from the sample is technically impossible though). So, the absolute value of the intercept of the x axis is the concentration of the sample.
Advantages:

- matrix effects are not a problem: matrix is the same for every spiked samples as we can neglect dilution

Drawbacks:

- we need some *a priori* knowledge about the concentration of the sample so that the spiked concentrations are commensurable with it
- spiking has to be done for each and every sample → slower in case of more samples, not high throughput.

Quantitation via the sequential standard addition method is evaluated by plotting the measured intensities against the increase of phosphorus concentration in the sample solution upon consecutive additions. The measured intensity of the original sample solution belongs to the value zero on the *x*-axis. The 3 standard additions yield 4 points for linear fitting with *y*-intercept *A*' and slope *B*'. Check whether volume increase and the resulting dilution of phosphorus content can be neglected during calculations. As a total of 9 µl standard solution is pipetted to a 600 µl sample, corresponding to an only 1.5% volume increase, the neglection is justified.
The concentration of the sample solution in sequential standard addition is calculated by Eq. 2.

$$c_{\text{sample}} = \left|\Delta c_{\text{sample}}\right| = \left|-A'/B'\right| \qquad (2)$$

Eq. 2 yields the absolute value of the *x*-intercept of the fitted line, that is, the sample solution concentration. The *x*-intercept itself is always negative and corresponds to the concentration change that would have to be introduced to the original sample in order to get zero signal intensity, i.e. remove all the analyte.

Graphical representation of the two different approaches is shown in Figure 3.

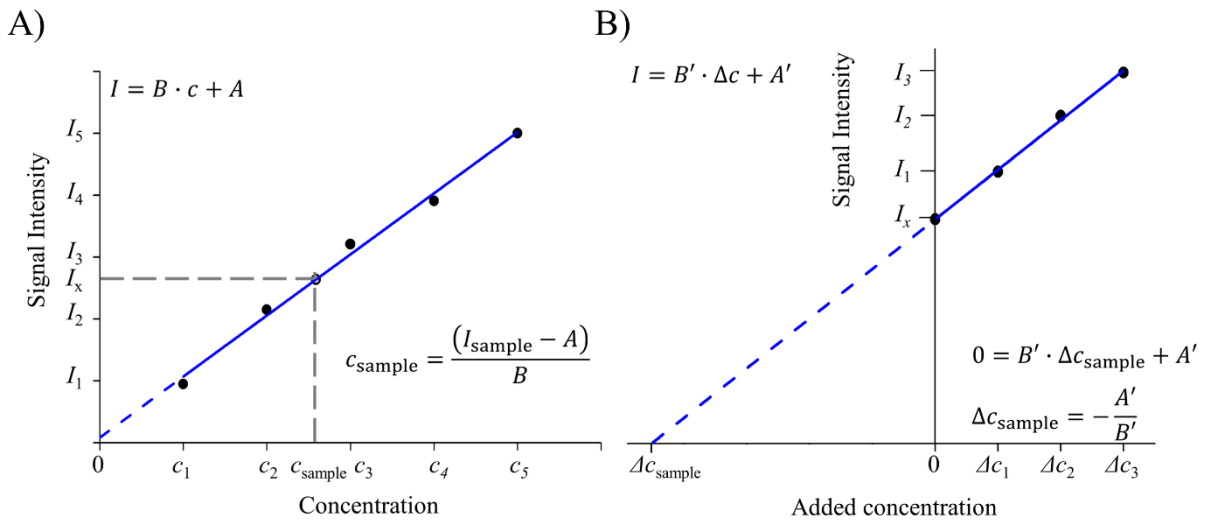

Figure 3. Scheme of linear fitting and calculating the resulting concentration in A) external calibration and B) sequential standard addition.

V. Results and discussion

All processed spectra are subjected to quantitative evaluation. The total phosphorus content of the cola drink sample is determined according to both external calibration and sequential standard addition. Evaluation has to be done separately by analyzing signal intensities from peak height and integral.

The obtained two values have to be compared, special attention has to be given to the possible errors, which are/were the error sources during the practical. These errors can originate from all steps: stock solution preparation, sample preparation, calculation, measurement, ignoration of the 10% D_2O content.
And finally, a literature search has to be done to find out the phosphorus content determined by other methods. Comparison of the own results to the literature data.

The overview of the practical: