Methylphosphonic acid as a $^{31}$P-NMR standard for the quantitative determination of phosphorus in carbonated beverages†

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Methylphosphonic acid (MPA) is introduced as an internal standard for the determination of the phosphorus content of carbonated beverages (cola drinks) by quantitative $^{31}$P-NMR analysis. Phosphorus in cola beverages was analyzed as phosphate in ten commercially available drinks. Phosphate concentration in the studied samples was in the range of 3.5 to 6.1 mM. The preparation of the samples included: degassing of commercially available beverages, addition of MPA as an internal standard, $D_2O$ for signal locking and a solution of NaOH to ensure a basic environment in the sample. The calibration graphs of the MPA standard and analyte showed a linear response in the concentration range of interest. The measured values of the phosphoric acid standard solutions lay within the confidence limits of the MPA, showing that these compounds give a similar response in NMR analysis. The measured concentrations of phosphorus were in good agreement with the values detected by the traditional spectrophotometric method. The presented $^{31}$P-NMR method using MPA as an internal standard is reliable and simple for the determination of the phosphorus content of cola type beverages.

Introduction

Phosphorus is an essential element and makes up about 1% of the adult body. 85% of body phosphorus is in the bones and the remaining is distributed throughout the soft tissues. In blood and extracellular fluid, an inorganic phosphate compartment is located, into which phosphate is inserted upon absorption from the diet. The recommended daily allowance of phosphorus for adults is 700 mg.1

Phosphoric acid and its phosphates are used as food additives for acidity regulation and chelation, and as antioxidants or as enhancers for other antioxidants. Phosphates can be found in carbonated beverages, chocolate, processed meat, cheeses, dressings, fats, oils and bakery products. There are several methods used to quantify phosphate concentration in food. The Association of Official Analytical Chemists (AOAC) recommends a colorimetric method, applying antimony phosphomolybdate.2 International standard ISO 6878 specifies spectrophotometric determination of phosphorus using ammonium molybdate in water.3 The determination of phosphorus in beverages has been described several times by colorimetric analysis using molybdate/vanadate,4 molybdate5,6 color reagents and pH titration.5,7 Among other methods, ion chromatography,8 flow injection methods with IR9 and colorimetric detection applying thyolphthalein10 have been reported.

Nuclear magnetic resonance spectroscopy is a good method to gain simultaneous qualitative and quantitative data in the analysis of food products.11 The most widely applicable analysis method is proton NMR, which was recently well reviewed by Mannina et al.12 The carbon NMR is probably the most attractive method for qualitative analysis of mixtures, however due to low natural abundance of the magnetically active $^{13}$C isotope, simultaneous quantitative information is difficult to collect.13–18

Quantitative $^{31}$P-NMR analysis accuracy has been shown to be comparable to chromatographic methods.19 $^{31}$P-NMR has also been proven to be a good method for food analysis and has been covered in a review by Spyros et al.20 Phosphates have been analyzed by $^{31}$P-NMR in milk,21,22 meat23,24 and olive oil.25,26 Soft drinks have been analyzed only by $^1$H-NMR27,28 but, somewhat surprisingly, we were unable to find any literature data on the determination of phosphates in beverages by $^{31}$P-NMR. While $^1$H-NMR has been shown to be a suitable quality control method for soft drinks,27 an additional $^{31}$P-NMR measurement from the samples under study would clearly enrich the available data. Also methylphosphonic acid has not been evaluated as a standard for the quantitative $^{31}$P-NMR analysis.

In this paper, we describe a new method for the quantitative determination of phosphate in soft drinks by $^{31}$P-NMR, applying methylphosphonic acid (MPA) as an internal standard.
Materials and methods

Samples, chemicals and stock solutions

The samples (Coca-Cola®, Coca-Cola Light®, Coca-Cola Zero®, Cola®, Pepsi®, Angry Birds Tropic Cola®, RC Cola® and Dr Pepper®) were purchased from supermarkets in Tallinn, Estonia. Internal standard methylphosphonic acid (MPA) (purum, ≥98.0%) was purchased from Sigma Aldrich, Na₂HPO₄ (puriss. p.a., 99%) from Honeywell Riedel-de Haén and NaOH solution (10.05 M) from Fisher Chemical.

53.2 mM methylphosphonic acid or 52.8 mM MPA and 51.3 mM Na₂HPO₄ stock solutions were prepared from pure substances in distilled water. A 3 M or 6 M NaOH solution was prepared by dilution with distilled water from the concentrated solution.

NMR sample preparation and measurements

The soft drinks were degassed by ultrasound at 640 W and 25 °C for 15 min on a Bandelin Sonorex Digital 10 P. Analyte samples were prepared in the following way: to a 0.5 cm NMR tube were added 500 µL of the degassed soft drink, 100 µL of D₂O, 100 µL of MPA stock solution (52.8 mM to samples 1–5 resulting in the concentration of 7.0 mM in the NMR tube and 53.2 mM to samples 6–10 resulting in the concentration of 7.1 mM in the NMR tube) and 50 µL of 6 M NaOH (>29 eq. to phosphoric acids H⁺) to samples 1–5 and 3 M NaOH (>14 eq. to phosphoric acids H⁺) to samples 6–10. The pH of the sample was checked with a universal indicator and found to be >12. Three parallel samples were prepared from the same source.

The 3¹P-NMR measurements were performed on a Bruker Avance III 400 Mhz NMR spectrometer having a 5 mm BBO-probe, and the samples were changed automatically with a B-ACS-60 sample changer. A signal was recorded at 161.95 MHz. Peaks were referenced to 85% H₃PO₄. An inverse gated decoupling was used; for samples 1–5 in Table 3, pulse angles of 90° and 30° were used, for samples 6–10 in Table 4 and linearity control measurements in the soft drink matrix (Table 2), a pulse angle of 90° and for all other measurements a pulse angle of 30° were used. The sample temperature was 296 K; generally 128 scans, and in cases of standard solution measurements 256 scans, were recorded. Before data collection two dummy scans were taken. 32 K data points were used, and the spectral width was 99,8090 ppm. The receiver gain was set at 2050, the acquisition time at 1 s. and the recycle delay at 20 s. The sample measurement took about 45 minutes for 128 scans.

Control of linearity

The correlation of standard concentrations in an artificial mixture of phosphate and MPA with a 3¹P-NMR signal was determined in D₂O. Four NMR samples with standard concentrations of 2.4 mM, 3.4 mM, 4.5 mM and 6.2 mM for phosphate and 2.5 mM, 3.5 mM, 4.6 mM and 8.5 mM for MPA were prepared as follows: 35 µL, 50 µL, 65 µL and 90 µL of 51.3 mM Na₂HPO₄ stock solution and 35 µL, 50 µL, 65 µL and 120 µL of 53.2 mM MPA stock solution, respectively, were added to 50 µL 3 M NaOH and the volume in each NMR tube was adjusted with D₂O to 750 µL.

To determine the correlation of the MPA concentration with a 3¹P-NMR signal response in the soft drink (Coca-Cola®, 0.1 mM, 0.5 mM, 1.1 mM, 2.1 mM, 4.2 mM, 6.4 mM and 8.5 mM MPA solutions were prepared in the following way: 30 µL, 150 µL of 20-fold dilution of 52.8 mM MPA stock solution (for 0.1 and 0.5 mM), 60 µL, 120 µL of 4-fold dilution of 52.8 mM MPA stock solution (for 1.1 and 2.1 mM) and 60 µL, 90 µL, 120 µL of 52.8 mM MPA stock solution (for 4.2 mM, 6.4 mM and 8.5 mM) were added to 500 µL of degassed Coca-Cola®, and 50 µL of 3 M NaOH and the volume in each NMR tube was adjusted with D₂O to 750 µL.

Results and discussion

Evaluation of an internal standard

There is a mixture of compounds in soft drinks, and the only source of phosphorus is phosphoric acid or its salts. As a triprotic acid, phosphate can exist in several acid-base forms. Therefore, the samples under study were made basic by the addition of NaOH to ensure that in all of the samples phosphorus would be in the form of a phosphate. Methylphosphonic acid (MPA) was chosen as an NMR standard, because of its functionality as a phosphorus acid. One might expect that MPA would behave similarly to the analyte in a soft drink matrix, while having a different chemical shift, which would ensure correct measurement of the standard and analyte signals. Fig. 1 shows the 3¹P-NMR measurements of soft drink with the MPA standard (spectrum A) and an artificial mixture of MPA and phosphate (spectrum B).

In a basified solution of the presented soft drink (Fig. 1A), the phosphate peak appears at 5.06 ppm and the peak of the MPA standard at 21.05 ppm. In an artificial mixture of standard solutions, the MPA and phosphate peaks appear in the same region. A small difference in the chemical shift values in the artificial mixture and in the soft drink matrix was caused by slight differences in the pH of the samples. In our study, these small differences were ignored, as they did not give rise to any difficulties in peak assignments. In spectrum B, one can see a significant difference in the peak heights of the MPA and phosphate; however, their areas are in good agreement with their predetermined concentrations. The phosphate peak is wider, due to the dynamic equilibrium between its acid-base forms. The matrices of soft drinks are different, therefore for some samples 14 equivalents of NaOH compared to phosphorus acid acidic protons is not sufficient for full deprotonation. Increasing the NaOH amount in the sample induced narrowing of the phosphate signal.

Next, the correlation of the concentration of phosphorus compounds under study with their 3¹P-NMR signals was determined; for that, four artificial mixtures of MPA and phosphate were measured (Fig. 2). The concentration range for analyte (phosphate) of 2.4 to 6.2 mM was chosen to cover the actual amount of phosphoric acid in the soft drink samples, taking into account the dilution in the NMR tube. The concentration range for the NMR standard MPA was chosen to
be a little bit wider, from 2.5 to 8.5 mM, allowing flexibility in the MPA standard solution preparation. As the NMR signal is proportional to the number of chemically equivalent nuclei, the conclusions on the signal change due to the concentration change are made via absolute areas of the NMR peak. At the same time care was taken to derive comparable data under the same instrumental conditions.

The determination of the $^{31}$P-NMR signal absolute areas for standard phosphate concentrations showed that the phosphate signal was linear in the concentration range of 2.4 to 6.2 mM (Table 1, Na$_2$HPO$_4$ line, $R^2 = 0.9993$). (Experimental and calculation details are given in the ESI.†) From that, we can conclude that the concentration of analyte and its signal values are in good correlation within the necessary measuring range for soft drinks.

The concentrations of the MPA standard solutions also correlated very well with the $^{31}$P-NMR signal absolute areas, giving a linear response in the concentration range of 2.5 to 8.5 mM (Table 1, MPA line, $R^2 = 0.995$). The measured values of the phosphoric acid standard solutions lie within the confidence limits ($f = 10$, $p = 0.05$) of MPA (Fig. 2), so it can be concluded that these compounds give similar responses in the NMR analysis. With an $F$-test,$^{29}$ it can also be shown that the dispersions of the $^{31}$P-NMR signal absolute areas of MPA and standard phosphate, $F_{emp} = 1.12$, are indistinguishable $F_{crit}(11, 0.05) = 2.82$.

Therefore, the $^{31}$P-NMR signal of MPA is a suitable standard to draw conclusions about the phosphate concentrations in D$_2$O.

As previously noted, the pH of the sample affected the chemical shifts of the phosphorus compounds, so it was necessary to evaluate the linear response range of the $^{31}$P-NMR signal of the MPA in a soft drink matrix. To do that, one cola sample was measured in the presence of different concentrations of MPA, in a range of 0.1 to 8.5 mM (Table 2).

The response to the concentration change of MPA in the soft drink matrix was linear (Table 2, $R^2 = 0.9995$). Therefore, one can conclude that MPA is a good standard to use in cola for the determination of the phosphate concentration.

To check the generality of adequate MPA response in different cola matrices, the absolute areas of the MPA $^{31}$P-NMR signals were measured in different beverages, while having the same MPA concentration (7.0 mM) and the same NMR

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Concentration, mM</th>
<th>Signal</th>
<th>$(a \pm S_a) \times 10^4$</th>
<th>$(b \pm S_b) \times 10^4$</th>
<th>$R^2$ ($n = 12'$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>2.4 to 6.2</td>
<td>Absolute area</td>
<td>23 ± 1</td>
<td>14 ± 5</td>
<td>0.9993</td>
</tr>
<tr>
<td>MPA</td>
<td>2.5 to 8.5</td>
<td>Absolute area</td>
<td>23.9 ± 0.7</td>
<td>9 ± 4</td>
<td>0.995</td>
</tr>
</tbody>
</table>

$^a$ Slope ± standard error for slope. $^b$ Intercept ± standard error for intercept. $^c$ Three parallels were separately prepared for every concentration.
Determination of phosphate concentration

NMR parameters (shimming, temperature, pulse angle, delay time, the number of scans, etc.) affect the absolute value of the signal. Therefore the use of the relative area of analyte to internal standard in quantitative analysis of samples, that may contain unknown additives, is the most practical approach. At the same time the spin relaxation of analyte and internal standard has to be similar. In general a 90° pulse angle gives the highest signal response and is therefore preferable. But in the case of simultaneous analysis of nuclei, that have different relaxation times 30° pulse is more reliable, due to the smaller additive error in the repetitive scans. In different matrices spin relaxation times could be different; therefore no optimization of relaxation delay time was done in the current work. The applicability of MPA as an internal standard was additionally confirmed by comparing phosphate concentrations in five cola-type beverages obtained by the 31P-NMR method applying 90° and 30° pulse angles and the spectrometric method (Table 3).

The concentrations of phosphate determined by the 31P-NMR method were retrieved directly from each sample, and standard MPA peaks, respectively, and $C_{M}$(MPA) is the standard MPA concentration in the NMR tube.

$$C_M(PO_4^{3-}) = \frac{V_{tube}}{V_{soft\;drink}} \times \frac{S(PO_4^{3-})}{S(MPA)}$$ (1)

where $V_{tube}$ is the total volume of solutions in the NMR tube, $V_{soft\;drink}$ is the volume of degassed soft drink that was added to the NMR tube, $S(PO_4^{3-})$ and $S(MPA)$ are the areas of phosphate and standard MPA peaks, respectively, and $C_{M}$(MPA) is the standard MPA concentration in the NMR tube.

The obtained results of the 31P-NMR method, applying 90° and 30° pulse angles, were separately compared with the results of the classical spectrometric method, using Fisher ($F$) and Student ($t$)-tests. In order to confirm the hypothesis, that variances of the two method measurements are indistinguishable, the $F$-test has been applied first. The absolute $F$ values for 31P-NMR methods, applying 90° and 30° pulse angles, were 1.3 and 1.5, respectively. Empirical $F$-values were certainly not higher than the $F_{crit} = 6.4$ critical value for $p = 0.05$, which indicates that the standard deviations are not significantly different. Paired $t$-test showed, that $t$-values for the 31P-NMR method, applying 90° and 30° pulse angles, were 1.4 and 0.4, respectively, and the critical value $t_{crit}(4, 0.05) = 2.8$ confirmed that phosphate concentrations obtained by 31P-NMR measurements cannot be distinguished from the results obtained by the spectrometric method.

The fact that both applied pulse angles gave trustful results indicates that deprotonated methylphosphonic acid and phosphoric acid respond similarly under NMR measurement conditions.

Table 3 Phosphate concentrations in cola-type drinks determined by 31P-NMR and spectrometric methods

<table>
<thead>
<tr>
<th>No</th>
<th>Beverage</th>
<th>Concentration of phosphate, mM</th>
<th>Determined by the 31P-NMR method</th>
<th>Determined by the spectrometric method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pulse angle 90°</td>
<td>Pulse angle 30°</td>
</tr>
<tr>
<td>1</td>
<td>Cola® 1</td>
<td>5.5 ± 0.1</td>
<td>5.7 ± 0.1</td>
<td>5.56 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>Cola® 2</td>
<td>5.7 ± 0.3</td>
<td>6.1 ± 0.3</td>
<td>5.56 ± 0.05</td>
</tr>
<tr>
<td>3</td>
<td>Coca-Cola Zero®</td>
<td>4.5 ± 0.1</td>
<td>4.7 ± 0.1</td>
<td>4.80 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>Pepsi® 1</td>
<td>5.4 ± 0.5</td>
<td>5.4 ± 0.3</td>
<td>5.54 ± 0.07</td>
</tr>
<tr>
<td>5</td>
<td>Dr Pepper®</td>
<td>3.5 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>3.68 ± 0.06</td>
</tr>
</tbody>
</table>

* From the same canned drink after degassing, three parallel samples were prepared and independently measured; the results were calculated by eqn (1) and given with standard deviation of concentrations of parallel samples. * Determined by ISO 6878 (ref. 3) in the accredited Central Lab of Estonian Environmental Research Centre, measured in mg P L⁻¹ and converted to mM.

Table 4 Phosphate concentration in cola-type drink samples 6–10 determined by the 31P-NMR method

<table>
<thead>
<tr>
<th>No</th>
<th>Beverage</th>
<th>Concentration of phosphate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Coca-Cola®</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>Coca-Cola Light®</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>8</td>
<td>Pepsi® 2</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>9</td>
<td>Angry Birds Tropic cola®</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>10</td>
<td>RC cola®</td>
<td>4.4 ± 0.1</td>
</tr>
</tbody>
</table>

* From the same canned drink after degassing, three parallel samples were prepared and independently measured; the results were calculated by eqn (1) and given with standard deviation of concentrations of parallel samples.
solution is usable over a short period of time. On the other hand, the presented NMR method is limited to cola-type drinks to which inorganic phosphorous is added. In cases of beverages containing organophosphorus compounds a different sample preparation method is necessary to get phosphorus concentration into a detectable range and this was not studied in the current work.

Furthermore another five cola-type beverages were analyzed applying the $^{31}$P-NMR method with a 90° pulse angle, the latter was chosen due to the higher signal to noise ratio, compared to measurement with a 30° pulse angle.

Conclusions

Methylphosphonic acid (MPA) is introduced as an internal standard for the determination of the phosphorus content of carbonated beverages (cola drinks) by quantitative $^{31}$P-NMR analysis. The correlation of the concentration of phosphorus compounds under study with their $^{31}$P-NMR signals was linear in heavy water and in a soft drink matrix. Statistical analysis showed, that an NMR standard (MPA) and the analyte (phosphate) give similar responses in the NMR analysis. Therefore, it can be concluded that the presented $^{31}$P-NMR method using MPA as an internal standard is reliable and simple for the determination of the phosphorus content of soft beverages. No calibration is necessary for determination of phosphate content in soft drinks. The phosphorus in cola beverages was analyzed in ten commercially available drinks. The measured concentrations of phosphorus were in good agreement with the values obtained by the standardized spectrometric method. The current work was focused on $^{31}$P-NMR analysis; however MPA as a quantitative NMR standard can in future be applied in $^1$H-NMR and $^{13}$C-NMR analysis, providing a good base for comprehensive quality assessment of cola-type soft drinks.

Acknowledgements

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References

8 Dionex Corporation, Application Note 169, Sunnyvale, CA, 2005.