Fast and sensitive method for phosphorus determination in dairy products

Anna Gliszczynska-Świgło1 · Iga Rybicka1

Received: 12 December 2020 / Revised: 21 April 2021 / Accepted: 10 May 2021 / Published online: 9 June 2021
© The Author(s) 2021

Abstract
The spectrophotometric molybdenum blue method for phosphorus determination was adapted to a multiwell plate format. The method was sensitive and allowed for the simultaneous determination of phosphorus in many samples. It was cheap and eco-friendly due to application of small volumes of reagents and, therefore, it meets the requirements for “green” or sustainable chemistry. The method’s limit of detection (LOD) is 0.37 μg/mL and its limit of quantification (LOQ) is 1.13 μg/mL. Its linearity is up to 30 μg of phosphorus/mL. The method was applied for the determination of phosphorus in 65 dairy products (yogurts, yogurt drinks, buttermilks, kefirs and homogenized cheeses) of strawberry, peach, forest fruits, vanilla and other flavours. The phosphorus content was 143–226 mg/100 g in flavoured yogurts, 78–204 mg/100 g in yogurt drinks, 89–218 mg/100 g in kefirs, around 195 mg/100 g in buttermilks, and 165–277 mg/100 g in homogenized cheeses. The presented method can be used in the routine quantitative analysis of the total phosphorus content in dairy products.

Keywords Phosphorus · Mineral · Molybdenum blue · UV–Vis · Sustainable chemistry · Dairy

1 Introduction
Phosphorus (P) is present in various biological, agricultural, environmental, pharmaceutical, and food samples (Vallapragada et al. 2011; Adesanwo et al. 2013). P is an essential nutrient naturally found in most of plant and animal foods. It is primarily responsible for the formation of bones and teeth (Mancini et al. 2018). The Nutrient Reference Value (NRV) for P is 700 mg/day for adults (European Parliament and Council 2011) but most of the population studies estimate its intake to be 1300–1800 mg/day (Calvo and Uribarri 2013; Mancini et al. 2018). It is not as high as the tolerable upper limit (4000 mg/day) (Institute of Medicine, Food and Nutrition Board 1997) but some authors claim that its content in food products is underestimated and therefore the intake level is also underestimated (Calvo et al. 2013; Borgi 2019). This is especially relevant because P is also added to food products in a form of different food additives, which have a higher bioavailability than P from natural sources (Fukagawa et al. 2011). The excess of P intake may significantly disrupt the hormonal regulation of P contributing to disordered mineral metabolism, vascular calcification, bone loss and impaired kidney function as shown for healthy young adults (Calvo and Uribarri 2013). Other studies also associated a high intake of P with the increased risk of type 2 diabetes or effects on bone traits (Mancini et al. 2018). Therefore, the level of this element should be monitored in food.

Minerals are mostly determined using atomic spectroscopy, but because of the high LOD for P [e.g. 75 mg/L for atomic absorption spectroscopy in a flame (F-AAS)] (Perkin-Elmer 2011) another method must be applied for its determination. Several analytical methods have been proposed for the determination of P in food samples, including UV–Vis spectrophotometric methods (Jastrzębska 2009; Kurzawa et al. 2009), chromatography (Ruiz-Calero and Galceran 2005; Sekiguchi et al. 2000), electrophoresis (Dušek et al. 2003), X-ray fluorescence (Jastrzębska et al. 2003), electrothermal AAS (ETAAS) (Coskun and Akman 2005), inductively coupled plasma with optical emission spectrometry (ICP-OES) (Hwang et al. 2015), ICP with mass spectrometry (ICP-MS) (Cozzolino et al. 2008), and P-NMR (Cade-Menun 2005). The development of an analytical method usually results in obtaining a more sensitive, faster, simpler, cheaper, more informative or more environmentally
sustainable method (Bergquist and Turner 2018). Methods that use less toxic or smaller amounts of reagents or solvents additionally meet the requirements of so-called “green” or sustainable chemistry (Koel and Kaljurand 2006). Thus, the aim of this study was to adapt the molybdenum blue method to a 48-microwell plate format. This method is based on the formation of molybdophosphoric acid from orthophosphate and molybdate under acidic conditions followed by reduction to molybdenum blue (Murphy and Riley 1962). The intensity of blue colour is proportional to the amount of P in solution. In the study, we used a novel approach based on the simultaneous determination of P in many samples that can be used in a precise and accurate routine analysis of the mineral in dairy products. The proposed method was applied to various dairy samples to provide up-to-date data on P content in food products.

2 Materials and methods

2.1 Materials

Randomly selected flavoured dairy products of different brands and producers including yogurts (n = 21), yogurt drinks (n = 11), kefirs (n = 10), buttermilks (n = 5) and homogenized cheeses (n = 18) were purchased in local shops in Poland in 2018 (Table S1; Supplementary Material). ERM-BD150 Skimmed Milk Powder (European Commission’s Joint Research Centre, Geel, Belgium) was selected as certified reference material (CRM) to verify the accuracy of the analytical procedure.

2.2 Samples preparation

Heterogeneous products were homogenized using Polytron PT 1300 D (Kinematica AG, Luzern, Switzerland) at 13,500 g and mineralized using microwave digestion as described by Lesniewicz et al. (2010). The mineralization was conducted in a microwave oven (CEM Corporation Mars 6, Matthews, USA). After cooling, samples were filled to 50 mL with demineralized water (Hydrolab, Wiślina, Poland). The mineralization of each sample was conducted in triplicates. The blank sample was prepared under the same conditions.

2.3 Certified reference material’s preparation

The determination of P in CRM was performed for dried material. For this purpose, the CRM was dried in the oven at 102 °C ± 0.5 °C until constant mass. Subsequently, the CRM was mineralized as described in the previous section.

2.4 Reagent preparation

The solution of 5% ammonium molybdate (molybdate-sulfuric [VI] acid) was prepared by mixing 5 g of anhydrous ammonium molybdate dissolved in 60 mL of demineralized water with a solution of 15 mL of concentrated sulfuric acid in 40 mL of demineralized water. The reagent was stored in an amber bottle at 4 °C. Its stability was confirmed for four weeks.

The solution of 0.5% hydroquinone (benzene-1,4-diol) was prepared by dissolving 0.5 g hydroquinone (benzene-1,4-diol) in 100 mL of demineralized water with 10 μL of concentrated sulphuric acid. The reagent was stored in an amber bottle at 4 °C. Its stability was confirmed for four weeks.

The solution of 20% sodium sulphite (Na2SO3) was prepared freshly (daily). P for AAS (1000 mg/L of water; TraceCERT, Supelco, Sigma-Aldrich, Saint Louis, MO, USA) was used as a standard.

2.5 Spectrophotometric determination of phosphorus

A 48-microwell plate (flat bottom) (Nunclon Delta Surface, Thermo Fisher Scientific, Roskilde, Demark) was used to determine the content of total P in mineralized samples. Each well contained 0.08 mL of a sample and 0.08 mL of water (or 0.16 mL of water as a blank), 0.08 mL of 5% molybdate-sulfuric (VI) acid solution, 0.08 mL of 0.5% hydroquinone (benzene-1,4-diol), and 0.08 mL of 20% sodium sulphite. The plate was left for 30 min in the dark and the absorbance was measured at 823 nm using BioTek PowerWave XS2 microplate spectrophotometer (Biokom, Warsaw, Poland). Three determinations were performed for each digest of the sample.

Quantification of P was performed using an external standard method. The six-point calibration curves were prepared with standard solution (0–30 μg of P/mL of sample). The LOD and LOQ were calculated from the standard deviations of the blank samples (n = 20) and the slopes of the calibration curves (n = 6).

2.6 Statistical analysis

The data in Fig. 1 and Table S1 (Supplementary Material) were presented as mean ± SD for each sample. Statistical analyses were carried out using Statistica 13.3 (2017) (StatSoft, Inc., Tulsa, OK, USA). All data were submitted to one-way analysis of variance (ANOVA). The significance of differences between mean values was determined by the least significant differences test (LSD) or Tukey test with
unequal sample size (differences between categories and flavours) at α = 0.05.

3 Results and discussion

3.1 Method validation and cost

Analytical characteristics of spectrophotometric molybdenum blue method adapted to a 48-microwell plate included: linearity, precision, accuracy and sensitivity expressed as LOD and LOQ.

Precision of the method was evaluated as the relative standard deviation (RSD) of the intra-day (repeatability, n = 5) and inter-day (reproducibility in three days, n = 15) determinations of P standard solution. The repeatability and reproducibility was not higher than 3.2% and 4.0%, respectively, indicating satisfactory precision.

Quantification of P was performed using an external standard method. The calibration curve for P (y = ax + b) was as follows: a = 0.0326 ± 0.0005 and b = 0.0476 ± 0.0004 (n = 6) with a very good linearity (average r = 0.999; RSD = 0.03%) to at least 30 μg/mL (final concentration in the well, absorbance not higher than 1.1). The LOD and the LOQ for P determination were 0.37 μg/mL and 1.13 μg/mL (9.3 mg/100 g and 27.9 mg/100 g of product), respectively.

The accuracy of the method was confirmed with CRM. The declared content of total P in CRM was 11.0 ± 0.6 g in kg of dried powder, whereas the determined value was 10.9 ± 0.3 g/kg.

The method allowed for a precise analysis of a large number of samples at the same run with relatively low cost. The cost of P determination was calculated for 480 samples taking into account the cost of reagents and plate, and the fact that the same plate can be used at least ten times. The estimated cost of analysis was about € 28/$ 30 (€ 2.8/$ 3.0 per plate) (November, 20, 2020, Poland).

3.2 Total phosphorus content in dairy products

The content of P in different food products can be found in the literature, e.g. in the US or Polish databases.
The content of P in yogurts ranged from 143 mg (blueberry) to 225 mg in 100 g (strawberry and peach). The literature data mostly relate to plain yogurts with the content of P from 88 mg/100 g (Fuente et al. 2003) to 150–170 mg/100 g (Güler 2007; Zamberlin et al. 2012). The content of P in flavoured yogurts found in databases is higher, around 300 mg/100 g, but it is a calculated, not analytically determined, value (USDA 2019). The relatively large discrepancies observed for analytical data and databases confirm the need of a reliable and fast method for P determination underlined by other authors (Calvo et al. 2013; Borgi 2019).

In the group of yogurt drinks, the lowest content of P < 85 mg/100 g, was found in strawberry yogurts and the highest, 180–200 mg/100 g, in strawberry and peach products. From analysed kefirs, the best source of P, delivering 210–220 mg/100 g, were three kefirs: peach, forest fruits and vanilla. Five out of ten kefirs contained only 90–95 mg of P in 100 g (different flavours). The content of P in buttermilks (all of strawberry flavour) was much higher than in yogurt drinks or kefirs. Buttermilks contained about 195 mg/100 g. The literature data for dairy products to drink, especially of different flavours, are very limited. Kunachowicz and co-authors (2015) estimated the content of P in kefirs to be 74 mg/100 g while in buttermilks to be 80 mg/100 g. Lodi and co-authors (2010) who described “fermented milk beverages” showed an even lower content of P (25–50 mg/100 g). In USDA (2019), only several dairy products to drink described in relation to P content were found. The content of P for plain and strawberry kefirs was 96 and 105 mg/100 g, respectively (analytical determinations) and for plain buttermilk was 85 mg/100 g (nutrition survey).

An interesting category in our study were flavoured homogenized cheeses. They are very popular in Poland but less popular in Europe or worldwide. They are made from pasteurized and acidified cow milk, unflavoured or flavoured (preferably), and mostly consumed as a dessert. The content of P in most of the analysed homogenized cheeses ranged from 190–240 mg/100 g. Only two products contained significantly lower amounts of P: 165 mg/100 g (vanilla-strawberry) and 168 mg/100 g (strawberry-peach) which was comparable to the data published by other Polish authors (138–175 mg/100 g) (Kunachowicz et al. 2015). Especially rich in P were four products (strawberry, raspberry and two vanilla) delivering 250–280 mg in 100 g.

It is commonly known that dairy products are a good source of P. In the American diet, 20–30% of P intake is provided by dairy products (Calvo and Uribarri 2013). The products analysed in our study should be regarded as a good source of this mineral. Their portion (200 g) delivered from 22 to 79% of NRV for P. Most of them could have nutrition claims related to P. In the European Union these claims can be added to solid products with the minimal content of 105 mg/100 g (claim “source of”) or 210 mg/100 g (claim “high in”) and to beverages with the minimal content of 52.5 mg (“source of”) or 105 mg in 100 mL (“high in”) (Food Safety Authority of Ireland 2019). On the other hand, the guidance for dietary supplements with P indicate that products containing ≥ 250 mg of P per portion should carry the statement “This amount of phosphorus may cause mild stomach upsets in sensitive individuals” (EFSA 2009). As the P deficiency is occasional in the developed populations, the nutrition claims are rarely presented on labels of food products.

4 Conclusions

In this paper, the spectrophotometric molybdenum blue method was adapted to simultaneous P analysis in many samples using a microplate reader. The method is sensitive, precise, accurate and can be implemented in the routine quantitative analysis of P content in dairy products. The method was applied for 65 dairy products from different categories and flavours. The content of P in all analysed yogurts, yogurt drinks, kefirs, buttermilks and homogenized cheeses ranged from 78–277 mg/100 g. In general, the lowest content of P was found in yogurt drinks and kefirs and the highest in buttermilks and homogenized cheeses. The
presented method that allows a fast and reliable analysis of P, and the results for dozens of dairy products are beneficial from both an analytical and nutritional point of view.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00003-021-01329-x.

**Funding** The research did not receive any funds.

**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

**References**

Adesanwo OO, Ige DV, Thibault L, Flaten D, Akinremi W (2013) Comparison of colorimetric and ICP methods of phosphorus determination in soil extracts. Commun Soil Sci Plant Anal 44:3061–3075

Bergquist J, Turner C (2018) Analytical chemistry for a sustainable society – trends and implications. Anal Bioanal Chem 410:3235–3237

Borgi L (2019) Inclusion of phosphorus in the nutrition facts label. Clin J Am Soc Nephrol 6(2):239–240

Güler Z (2007) Levels of 24 minerals in local goat milk, its strained yoghurt and salted yoghurt (tuzlu yogurt). Small Rumin Res 71:130–137

Hwang J, Jang HW, Namgung B, Oh M, Kim S, Seo D, Kim SN, Choi Y, Jiang NSMO (2015) Determination of phosphorus in foods by inductively coupled plasma optical emission spectrometry (ICP-OES). Food Eng Prog 19:161–166

Institute of Medicine, Food and Nutrition Board (1997) Dietary reference intakes for calcium, phosphorus, magnesium, vitamin D, and fluoride. National Academies Press, Washington

Jastrzębska A (2009) Modifications of spectrophotometric methods for total phosphorus determination in meat samples. Chem Pap 63:47–54

Jastrzębska A, Brudka B, Szmyńska T, Szłyk E (2003) Determination of phosphorus in food samples by X-ray fluorescence spectrometry and standard spectrophotometric method. Food Chem 83:463–467

Koel M, Kaljurand M (2006) Application of the principles of green chemistry in analytical chemistry. Pure Appl Chem 78:1993–2002

Kunachowicz H, Nadolina I, Przygoda B, Iwanow K (2005) Tabele składu i wartości odżywczej żywności (in Polish), Food composition tables, PZWL, Warsaw, Poland

Kurzawa M, Jastrzębska A, Szłyk E (2009) Application of fluorimetric methods for selected additives determination in food products. Czech J Food Sci 27:S337–S341

Lesniewicz A, Wroź A, Wojcik A, Zyrnicki W (2010) Mineral and nutritional analysis of Polish infant formulas. J Food Compos Anal 23:424–431

Lodi CS, Sassaki KT, Fraiz FC, Delbem ACB, Martinhon CCR (2010) Evaluation of some properties of fermented milk beverages that affect the demineralization of dental enamel. Braz Oral Res 24(1):95–101

Mancini FR, Affret A, Dow C, Balkau B, Clavel-Chapelon F, Bonnet F, Boutron-Ruault MC, Fagerazzi G (2018) High dietary phosphorus intake is associated with an increased risk of type 2 diabetes in the large prospective E3N cohort study. Clin Nutr 37:1625–1630

Murphy J, Riley JR (1962) A modified single solution method for the determination of phosphate in natural water. Anal Chim Acta 27:31–36

Perkin-Elmer (2011) Atomic spectroscopy. A guide to selecting the appropriate technique and system. https://www.perkinelmer.com/Content/relatedmaterials/brochures/bro_worldleaderaicpmscpms.pdf. Accessed 14 Nov 2020

Ruiz-Calero V, Galceran MT (2005) Ion chromatographic separations of phosphorus species: a review. Talanta 66:376–410
Sekiguchi Y, Matsunaga A, Yamamoto A, Inoue YJ (2000) Analysis of condensed phosphates in food products by ion chromatography with an on-line hydroxide eluent generator. J Chromatogr A 881:639–644

USDA U.S. Department of Agriculture, Agricultural Research Service (2019) FoodData Central, https://fdc.nal.usda.gov/. Accessed 14 Nov 2020

Vallapragada VV, Inti G, Ramulu JS (2011) A validated inductively coupled plasma-optical emission spectrometry (ICP-OES) method to estimate free calcium and phosphorus in in vitro phosphate binding study of eliphos tablets. Am J Anal Chem 2:718–725

Zamberlin Š, Antunac N, Havranek J, Samaržija D (2012) Mineral elements in milk and dairy products. Mljekarstvo 62:111–125

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.