Detection of B₆ Vitamers in Grain Products: Experimental and Computational Studies

Anna Lebiedzińska¹ · Marcin L. Marszał² · Małgorzata Grembecka¹ · Jakub Czaja¹ · Piotr Szefer¹ · Jadwiga Kuta³ · Brady D. Garabato³ · Paweł M. Kozłowski^{1,3}

Received: 19 May 2017 / Accepted: 20 September 2017 / Published online: 1 October 2017
© The Author(s) 2017. This article is an open access publication

Abstract A reversed phase high-performance liquid chromatography method (HPLC) with coulometric electrochemical detection has been applied and validated for the simultaneous analysis of pyridoxamine (PM), pyridoxal (PL), and pyridoxine (PN) in cereal products. Isocratic separation was achieved using a C₁₈ column with a mobile phase consisting of methanol-phosphate buffer (10:90) and 0.018 M trimethylamine adjusted to pH 3.55 with 85% orthophosphoric acid. The limits of detection for PM, PL, and PN were 0.28, 0.36, and 0.43 ng mL⁻¹, respectively, with vitamins recoveries ranging from 90.4 to 98.1%. The applied method for the analysis of B₆ vitamins naturally present in grain products, offers a simple and fast sample preparation without derivatization. To understand vitamer separation further, methods of computational chemistry were employed. Specifically, density functional theory (DFT) was used to determine electrostatic potentials, as well as the charges of each vitamer. A number of correlations were established between these properties and elution order.

Keywords Cereal products · Pyridoxal · Pyridoxine · Pyridoxamine · HPLC · Coulometric detection · Quantum-chemical calculations · Density functional theory

Introduction

The B₆ vitamers (Fig. 1) are a group of water-soluble forms of vitamin B₆, which are chemically quite distinct compounds. Vitamin B₆ represents a group of highly bioavailable 3-hydroxy-2-methylpyridine derivatives that exist in food as phosphorylated and non-phosphorylated forms (Ball 2006; Eitenmiller et al. 2008). Vitamin B₆ are also enzymatic co-factors required for over 140 biochemical reactions involved in amino acid biosynthesis and degradation, as well as sugar, and fatty acid metabolism (Ball 2006). Vitamin B₆ is necessary in the synthesis of both serotonin and GABA neurotransmitters that control depression, pain perception, and anxiety. Most vitamin B₆-dependent enzymes catalyze reactions including transamination, decarboxylation, dehydration, desulfhydration, racemization, cleavage, and synthesis, among other processes including the activation of glycine during the initial stages of heme production (Bui and Small 2012; Denslow et al. 2005; Sorolla et al., 2010). Interestingly, vitamin B₆ has recently been found to be a potent antioxidant, with the particular ability to quench reactive oxygen species such as superoxide, and singlet oxygen (Tambasco-Studart et al. 2005). Recent studies have shown that DNA damage resulting from deficiencies of micronutrients such as vitamins B₆, and B₁₂ are likely to be major contributing factors to cancer, as vitamin B₆ plays a crucial role in 1-carbon metabolism, which involve DNA synthesis, and methylation (Larsson et al. 2005; Liu et al. 2008).

Although all organisms are dependent on vitamin B₆, only microorganisms and plants are able to synthesize it *de novo*.

Paweł M. Kozłowski is a visiting professor at the Department of Food Sciences, Medical University of Gdańsk.

✉ Anna Lebiedzińska
aleb@gumed.edu.pl

✉ Paweł M. Kozłowski
pawel@louisville.edu

¹ Department of Food Sciences, Medical University of Gdańsk, Al. Gen. J. Hallera 107, 80-416 Gdańsk, Poland

² Department of Toxicology, Medical University of Gdańsk, Al. Gen. J. Hallera 107, 80-416 Gdańsk, Poland

³ Department of Chemistry, University of Louisville, 2330 South Brook Street, Louisville, KY 40292, USA

Fig. 1 Molecular structure of B₆ vitamers: **a** pyridoxamine (PM), **b** pyridoxal (PL), and **c** pyridoxine (PN) (C—gray, O—red, N—blue, H—white)

Mammals, including humans have a *salvage* pathway, lack the enzymes for *the novo* synthesis of B₆ and rely on uptake of the vitamin from food, with plants as the most important source for human nutrition (Hellmann and Mooney 2010; di Salvo et al. 2012).

The B₆ vitamers exist in various forms of pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM) (Fig. 1), which during metabolic conversion becomes phosphorylated at the 5-hydroxymethyl position by a kinase required as a co-factor in enzymatic reactions. In plants, PN can exist in quite significant amounts as a β -glucoside. Pyridoxine is the major

vitamer in cereal products where it may also exist as the glucosylated adduct, 5'-O-(β -D glucopyranosyl) pyridoxine, which is of limited bioavailability (Gregory and Kirk 1981). Animal products contain more bioavailable forms of vitamin B₆ compared to plant food sources, which comprise approximately 50% of human bioavailability (Gregory 2012). The bioavailability of PN in plant-based foods varies considerably, ranging from 0 to 80% (Kabir et al. 1983).

Pyridoxal-5'-phosphate (PLP), on the other hand, has been reported to be the most biologically active form of vitamin B₆ and functions as a co-factor crucial for numerous enzymatic reactions (di Salvo et al. 2012; Eitenmiller et al., 2008; van der Ham et al. 2012).

The high variability of B₆ vitamins and their low concentrations in food products cause difficulties in analysis. Moreover, grains and grain products are food matrixes that show special analytical difficulties, as they are starchy products that contain several interfering compounds (Lebiedzińska et al. 2008). These reasons are why the majority of methods target determination of total vitamin B₆ content in grain product analysis.

Traditionally, the Association of Official Analytical Chemists (AOAC 2003) recommends microbiological assays of B₆, which are perceived as time-consuming. Moreover, chemical and microbial methods previously used failed to accurately measure the nutritionally active vitamin forms, mainly due to varying extraction efficiency and stability of the vitamers. The advantage of chromatographic methods is thus the possibility to characterize vitamer forms individually in foods (Eitenmiller et al. 2008; Gregory 2012). Therefore, new methods should be developed that allow this instead of the approach including conversion of vitamers to a single analyte forms.

A variety of LC methods are reported for the determination of vitamin B₆ in pharmaceuticals (Jin et al. 2012) and in food products (Ciulu et al. 2011; Gliszczynska-Świgło and Rybicka 2015; Langer and Lodge 2014; Santos et al. 2012). Most separation and detection procedures are reversed-phase high-performance liquid chromatography (HPLC) techniques (Eitenmiller et al. 2008) coupled with fluorescent detections (Bui and Small 2012; Gatti and Gioia 2005; Gliszczynska-Świgło and Rybicka 2015; Langer and Lodge 2014), UV (Ciulu et al. 2011; Jin et al. 2012; Langer and Lodge 2014), and MS (Hälvin et al. 2013; van der Ham et al. 2012). Methods using electrochemical detection (ED) have also been proposed but applications to samples other than pharmaceuticals supplements and animal foods are few (Lebiedzińska et al. 2007; Marszał et al. 2005).

Although several analytical methods have been developed for the determination of individual vitamin B₆ compounds, only few may be applied to the analysis of grain samples. Thus, there is a need for reliable, accurate, and precise analytical methods for vitamins in cereal foods as it is essential for determining the dietary intake of vitamin B₆. The aim of the

present work is thus to determine B₆ vitamers present in cereal products using HPLC coupled with ED detection. To further understand the elution profile and the nature of separation, computational chemistry, specifically density functional theory, was applied to predict the electrostatic properties of each vitamin under consideration and was positively correlated with retention times.

Experimental

Instrumentation

Chromatographic analyses of pyridoxamine (PM), pyridoxal (PL), and pyridoxine (PN) in cereal products were performed on an HPLC system that consisted of a pump P 580 (Dionex, USA) and a sample injector (Rheodyne) with a 20- μ L loop, coupled with Coulochem II electrochemical detector (ESA, USA) and diode array UVD 340S (Dionex, USA). Chromatographic separation and quantification were achieved on a Chromeleon Chromatography Management System using Supelco (Germany) C18, 5 μ m (250 mm $\times$ 4.6 mm).

Reagents and Solutions

All reagents were of analytical grade. Pyridoxine hydrochloride, pyridoxamine hydrochloride (purity $\geq$ 99.0%), and pyridoxal hydrochloride (purity $\geq$ 99.5%) were provided by Fluka (Biochemica). Water-soluble papain (from *Carica papaya*) and diastase (from fungi) were obtained from Merck (catalog no. 103604 and 107144, respectively); α -amylase (from *Aspergillus oryzae*) was purchased from Sigma-Aldrich (catalog no. A-6211). Trimethylamine hydrochloride and sodium phosphate dibasic heptahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA) while orthophosphoric acid (85% w/w) was obtained from Riedel-de Haën (Seelze, Germany). Methanol, HPLC grade, was supplied by Baker (HPLC Analyzed). Purified water was obtained from Compact Mili-Q system (Millipore, Milford, MA, USA). The phosphate buffer (pH 3.55) was prepared using 0.05 mol L⁻¹ sodium phosphate dibasic heptahydrate and 85% orthophosphoric acid. Enzymes were dissolved in distilled water to a concentration of 100 mg mL⁻¹. Vitamin standards were prepared by dissolving 0.010 g of each vitamin in 100 mL of ultrapure water to 0.100 mg mL⁻¹. Solutions were stored separately in the dark at 4 °C. Standards were prepared daily by appropriate dilution of individual stocks from the mobile phase.

Samples and Reference Materials

The products analyzed in this study including wheat bread and wholemeal flour were randomly purchased from the local

market in Gdansk (Poland). Firstly, they were homogenized and subsampled (50–100 g) and then stored at – 18 °C until analysis. The certified reference material wholemeal flour (CRM 121), which was obtained from the Community Bureau of Reference, BCR (Belgium), was also subsampled (5 g) and stored frozen.

Sample Preparation

Samples were first hydrolyzed with mineral acids and then enzymatic treatments were applied to effectively liberate the B₆ vitamers analyzed from the sample matrix and to correctly determine their concentration. Phosphorylated vitamin esters were converted to free vitamin forms using the enzyme mixtures. The extraction procedure applied in this study was based on a combination of acid digestion and enzymatic hydrolysis, according to the study of Esteve et al. (1998) and recommended by the AOAC (2003).

Extraction Procedure

Samples of cereal products, i.e., bread, flour, and wholemeal flour (CRM 121) were weighed accurately (2.0 ± 0.0001 g) and left for 30 min in an autoclave at 121 °C with 50 mL of 0.1 M HCl previously added. Then, the sample was cooled and pH adjusted to 4.5 with 2.5 M sodium acetate buffer and mixture of papain (40 mg) and diastase (100 mg) per 1 g of sample was added. An enzyme blank was always used to correct for the addition of vitamins from the enzyme suspension. After enzyme incubation at 37 °C for 18 h, samples were heated to 100 °C for 5 min to inactivate the enzyme and diluted to 100 mL with distilled water. Prior to HPLC analysis, samples were filtered through a Whatman 1 Chr filter paper. All preparations were conducted under dim light, and samples were kept in the dark in an amber flask at + 4 °C (24 h), then at 18 °C until analysis.

Chromatographic Conditions

Isocratic separation of B₆ vitamers was performed on an LC 18 column of 5 μ m (25 cm $\times$ 4.6 mm) which was thermostated at 25 °C. The mobile phase was filtered through a 0.22- μ m membrane filter and vacuum degassed prior to use, consisting of methanol-phosphate buffer (10:90), and 0.018 M trimethylamine reagent adjusted to pH 3.55 with 85% orthophosphoric acid. The volume of injection was 20 μ L.

Pyridoxamine, pyridoxal, and pyridoxine were detected using a Coulochem II (model 5020A, ESA, USA) electrochemical detector equipped with a dual analytical cell (model 5010), with guard cell (model 5020). The guard cell was connected in-line before the injection port and was used to eliminate the interference with baseline stability. The detector response was set to give a full-scale detection for a 1- and 50- μ A

current output received from the analytical cell. The HPLC was operated in a constant flow mode, and the flow rate was kept at 1 mL min⁻¹. The peak area of the electrochemical signal at the porous graphite electrode was used for quantitative analysis.

Statistics and Visualization

Statistical analyses such as linear regression and tests for intercept, slope, and intra- and inter-day were conducted with the use of Statistica for Windows (version 10, Statsoft, 1984–2011, Cracow, Poland) software-package.

Computational Methodology

All quantum chemical calculations reported in this study were carried out in the gas phase using the B3LYP hybrid functional and the 6-311g(d) basis set as implemented in Gaussian 09 suite of programs for electronic structure calculations (Gaussian 09 n.d). The primary aim of these calculations was to determine the electrostatic potentials of PM, PL, and PN vitamers. Geometries of each vitamer were taken from the PubChem Compound Database, (CIDs: 1052, 1050, and 1054) and used for single-point energy calculations. In addition to electrostatic potentials for each vitamer, Mulliken and natural charges were also computed.

Results

Validation

In order to guarantee accurate, precise, and linear results, the B₆ vitamer concentration ranges were adjusted to their levels in food. All calculations were based on the calibration graphs constructed as a function of vitamer standard concentrations (ng mL⁻¹) vs. peak area. The calibration graphs were characterized with linearity and a high correlation coefficient ($r \geq 0.998$). All parameters obtained for regression equations are given in Table 1. The CRM material (CRM 121) linearity was established within the concentration ranges of 1–50, 5.0–350, and 1.5–200 ng mL⁻¹ for PM, PL, and PN, respectively.

The limits of detection (LOD) for pyridoxamine, pyridoxal, and pyridoxine were 0.28, 0.36, and 0.43 ng mL⁻¹, respectively, with the detector sensitivity set at 1 μ A. LODs were calculated within three standard deviations of background noise, with the standard finally diluted in the same buffer as the food sample, with a limit of quantification (LOQ) of $10 \times \text{SD}$. The LOQ values obtained for pyridoxamine, pyridoxal, and pyridoxine were 1.00, 1.20, and 1.50 ng mL⁻¹, respectively (detector sensitivity 1 μ A).

The 1-day repeatability was evaluated by 10-fold measuring the same wholemeal flour sample (CRM121), where

intermediate precision was determined by analyzing the same sample during five consequent days. All validation results are reported in Table 1.

The accuracy of the method was checked using the certified material CRM 121 wholemeal flour, which was used to verify the accuracy of results and monitor method performance. The analytical results obtained for CRM 121 wholemeal flour were highly satisfactory, and the recovery (as a measure of accuracy) for the B₆ vitamin amounted to 99% and RSD (as a measure of precision) equaled 1.88%. The exemplary chromatogram obtained for wholemeal flour is shown in Fig. 2a.

Quantitative Analysis of the B₆ Vitamins in the Commercial Plant Food Products

The proposed analytical approach was verified by analysis of commercially available plant food products (Fig. 2b). Vitamin B₆ concentration estimate was performed based on calibration curves obtained as a function of concentration (ng mL⁻¹) vs. peak area for pyridoxamine, pyridoxal, and pyridoxine, Table 2.

Computational Modeling Using Density Functional Theory

To provide a better understanding of separation order based on chromatographic data (Fig. 2a, b), molecular properties were determined from single-point energy calculations. Figure 3 provides a graphical summary of electrostatic potential surfaces for PM, PL, and PN. Electrostatic potentials provide a simple, conceptual means to understand surface interactions with separation medium. Mulliken and natural charges also provide a complementary and quantitative understanding. Because all molecules interact with the same medium, it is expected that elution order should correlate to the electrostatic potential differences between functional groups.

As shown in Table 3, a direct relationship can be seen between the order of elution and the sum of electrostatic potentials, where lower potentials correlate to higher retention times. Mulliken and natural charges are also shown to increase as expected for PM, PL, and PN, respectively. Separation of B₆ vitamer fragments is quite different from that previously reported for vitamin B₁₂ derivatives (cobalamins). In contrast to B₆ vitamers, cobalamins cannot be simply separated (Lebiedzińska et al. 2007). Based on DFT calculations, this may be attributed to the much larger size of B₁₂ compared to B₆, leading to an electrostatic potential distribution that is more disperse, where functional group modifications of the outer coordination sphere have relatively little effect on the electrostatic properties. Based on this analysis, it is expected that all cobalamins should interact in a similar way with the separation surface, while the B₆ vitamers will produce notable electrostatic changes with even a small modification of the structure, due to their smaller size.

Table 1 Validation parameters of the method

Analytical parameter	Pyridoxamine	Pyridoxal	Pyridoxine
Concentration range (ng mL ⁻¹)	1.0–50.0	5.0–350.0	1.5–200.0
Intercept	0.0162	0.142	0.085
Slope $\pm$ SD (μ g mL ⁻¹)	17.7 $\pm$ 0.7	10.6 $\pm$ 0.6	53.4 $\pm$ 0.8
Coefficient of correlation (<i>r</i>)	0.9989	0.9992	0.9991
Limit of detection (ng mL ⁻¹)	0.28	0.36	0.43
Limit of quantification (ng mL ⁻¹)	1.0	1.2	1.5
Repeatability ^a SD (%)	2.4	3.1	1.2
Intermediate precision ^b SD (%)	4.6	5.1	3.5

^a The repeatability data of each vitamin were obtained by analyzing 10-fold the same wholemeal flour samples

^b Represents the intermediate precision that was obtained by analyzing each vitamin in wholemeal flour samples during sequential 5 days

Discussion

Comprehensive vitamin B₆ analysis is required for food analysis, although differentiation between unphosphorylated vitamers and the 5-phosphoric acid esters is usually not. The principle method of extraction is release of B₆ vitamins, their glucosated forms from proteins, and conversion of the

phosphorylated compounds to pyridoxine, pyridoxamine, and pyridoxal (Lebiedzińska et al. 2008). The critical part of the analysis is the enzymatic treatment necessary to liberate protein- and glucosated phosphate-bound vitamins (Lebiedzińska et al. 2008). Recent research (Ball 2006; Gregory 2012) has shown that PN-glycoside has a much lower bioavailability than pyridoxine, and it is therefore reasonable to assay all B₆ vitamers separately.

Sample extractions with mineral acids or enzymatic digestions of food matrices are analytical procedures most frequently used before quantitative measurement of vitamin B₆ vitamers (Eitenmiller et al. 2008). Glucosidase side activity of enzyme preparations such as diastase is high enough to hydrolyze glucosidic linkages in bound vitamin forms. A microbiological procedure, which involves an acid hydrolysis and enzymatic steps during sample extraction, is used in most reports (Sampson et al. 1995; Sampson et al. 1996). However, in this procedure, any PN-glycoside (PNG) in the sample is converted into PN (Gregory 2012). Consequently, much of the literature may report PNG present in plant foods as PN. Plant foods contain substantial fractions of vitamin B₆ as PN-glucoside, and thus, such methods may lead to over estimation of nutritional content since the resulting data does not reflect the B₆ content, or the partial bioavailability of PN-glucoside. Most vitamin B extraction procedures use HCl hydrolysis because vitamins are stable in acidic environments and break protein complexes, effectively liberating vitamins from cellular material in most sample matrices.

However, Ollilainen et al. (2001) has shown that vitamin B₆ compounds may be measured in their intact forms, and the original distribution of vitamers can be preserved during the analytical procedure, which included cold perchloric acid extraction followed by enzymatic digestions before the liquid chromatographic analysis. Moreover, Gatti and Gioia (2005) have developed an HPLC method with fluorescence detection for simultaneous analysis of B₆ vitamers and B₂ in commercial vitaminized milk and human breast milk. Other methods of detection of vitamin B₆ are based on redox

Fig. 2 **a** Chromatogram of vitamin B in reference materials CRM 121 (wholemeal flour) 1—PM (t_R 3.7 min, 0.08 μ g mL⁻¹), 2—PL (t_R 4.7 min, 0.54 μ g mL⁻¹), 3—PN (t_R 5.6 min, 0.12 μ g mL⁻¹), ED sensitivity 50 μ A. **b** Chromatogram of vitamin B in wheat bread sample (A) 2—PL (t_R 4.7 min, 4.4 ng mL⁻¹), 3—PN (t_R 5.6 min, 32.2 ng mL⁻¹)

Table 2 The concentration of pyridoxamine, pyridoxal, and pyridoxine in plant food samples; values represent the mean for six samples and standard deviations (SD)

Food product	Vitamins B6		
	Pyridoxamine HCl (mg 100 g ⁻¹)	Pyridoxal HCl	Pyridoxine HCl
Wheat bread	0.010 ± 0.001	0.015 ± 0.002	0.161 ± 0.009
Wholemeal spelt flour ^a	0.065 ± 0.004	0.063 ± 0.001	0.227 ± 0.008
Wholemeal spelt flour ^b	0.035 ± 0.007	0.136 ± 0.002	0.127 ± 0.006
Spelt flour 630	0.017 ± 0.009	0.058 ± 0.001	0.110 ± 0.012
Flour mix for bread rolls	0.102 ± 0.001	0.059 ± 0.002	0.155 ± 0.005

^{a, b} Different degrees of milling spelt flour

Fig. 3 Electrostatic potential surfaces (negative potential in orange, positive in yellow) of **a** pyridoxamine (PM), **b** pyridoxal (PL), and **c** pyridoxine (PN)

reactions with electrochemical detectors, such as amperometric and coulometric. Early assays followed the oxidation of PN at a carbon paste electrode. Flow injection and sequential injection techniques as well as the availability of modified glassy carbon electrodes have also added speed and improved selectivity to electrochemical assays for vitamin B₆ (Desai et al. 2008; Eitenmiller and Landen 1999; Eitenmiller et al. 2008).

Nishimura et al. (2008) have established a method for the determination of individual vitamin B₆ compounds on the basis that the B₆ vitamers are converted by enzymes into highly fluorescent 4-pyridoxolactone. HCl hydrolysis is also used to convert the phosphoester forms into their corresponding free forms. Seven vitamin B₆ compounds were determined in animal, fish, and vegetable food using the enzymatic-based HPLC method developed by Do et al. (2012).

In our extraction process, the 5'phosphate forms, as well as pyridoxine- β -glucoside were hydrolyzed with HCl and determined as their free forms in a few minutes, using data from computational chemistry analysis. Separation of B₆ vitamers is quite different from previously reported for vitamin B₁₂ derivatives (cobalamins) separations. In contrast to B₆, cobalamins cannot be simply separated (Lebiedzińska et al. 2007). As mentioned, based on DFT calculations, this may be attributed to the much larger size of B₁₂ in comparison to B₆. While the larger coordination sphere of the cobalamins results in an essentially uniform interaction with the separation medium, greater differences of electrostatic potential distribution of the B₆ vitamers result in an elution profile that is well separated, due to their smaller size.

Coulometric detection enabled us determination of B₆ free forms in a few minutes and provided higher sensitivity and selectivity than UV detection. It was due to the lack of interferences with other substances and the ability of ED to resolve peaks according to their voltammetric properties. The UV and ED detectors as well as FL one are rather inexpensive, but ED and FL

Table 3 Electrostatic potential, Mulliken charges, and natural charges on the relevant groups

Molecule	Group	Sum of electrostatic potential on relevant groups
Pyridoxamine	–CH ₂ NH ₂	– 37.4792
Pyridoxal	–CHO	– 38.0729
Pyridoxine	–CH ₂ OH	– 40.2608
Molecule	Group	Sum of Mulliken charges on a group
Pyridoxamine	–CH ₂ NH ₂	– 0.2715
Pyridoxal	–CHO	– 0.0806
Pyridoxine	–CH ₂ OH	– 0.0577
Molecule	Group	Sum of natural charges on a group
Pyridoxamine	–CH ₂ NH ₂	0.0396
Pyridoxal	–CHO	0.0388
Pyridoxine	–CH ₂ OH	0.0347

characterize with clean blank and sample backgrounds and very high sensitivities.

Conclusions

The growing interest in interactions between diet and health requires a more precise analytical methodology for determination of vitamins in food products, which is essential for the calculation of dietary intake of vitamin B₆ from food. Thus, the outlined method of B₆ vitamer analysis is important to consumers as well as the scientific community. The presented method characterized with the satisfying sensitivity and selectivity and low limits of detection.

Finally, a unique aspect of this study is the combined theoretical approach used to determine elution order based on analysis of computationally predicted electrostatic potentials. Density functional theory was implemented to determine the charge characters of each fragment, and it was concluded that due to the relatively small size of B₆, lower electrostatic potentials, and consequently higher atomic charges, correlate to longer retention times. According to our knowledge, this is the first report using computational chemistry combined with HPLC-ED. Our research has confirmed that grain products can constitute an important source of all three vitamers of B₆ vitamin, including pyridoxine hydrochloride, and that vitamer concentrations can be routinely estimated using this method.

Funding Information Visiting professorship of Pawel M. Kozłowski at the Medical University of Gdansk was partially supported by the Ministry of Science and Higher Education of the Republic of Poland, from the quality-promoting subsidy under the Leading National Research Centre (KNOW) program for the years 2012–2017.

Compliance with Ethical Standards This article does not contain any studies with human or animal subjects.

Conflict of Interest Anna Lebedzińska declares that she has no conflict of interest. Marcin L. Marszał declares that he has no conflict of

interest. Małgorzata Grembecka declares that she has no conflict of interest. Jakub Czaja declares that he has no conflict of interest. Piotr Szefer declares that he has no conflict of interest. Jadwiga Kuta declares that she has no conflict of interest. Brady D. Garabato declares that he has no conflict of interest. Pawel M. Kozłowski declares that he has no conflict of interest.

Informed Consent Not applicable.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

- AOAC (2003) Official Methods of Analysis of AOAC (Association of Official Analytical Chemists) International (18th ed.). AOAC International, Arlington, VA, USA
- Ball GMF (2006) Vitamins in foods. Analysis, bioavailability and stability. CRC, Press, Boca Raton
- Bui LTT, Small DM (2012) The stability of pyridoxine hydrochloride used as a fortificant in Asian wheat flour noodles. *Food Chem* 130(4):841–846
- Ciulu M, Solinas S, Floris I, Panzanelli A, Pilo MI, Piu PC, Spano N, Sanna G (2011) RP-HPLC determination of water-soluble vitamins in honey. *Talanta* 83:925–929
- Denslow SA, Walls AA, Daub ME (2005) Regulation of biosynthetic genes and antioxidant properties of vitamin B₆ vitamers during plant defense responses. *Physiol Mol Plant Pathol* 66:244–255
- Desai PB, Kotkar RM, Srivastava AK (2008) Electrochemical behavior of pyridoxine hydrochloride (vitamin B₆) at carbon paste electrode modified with crown ethers. *J Solid State Electrochem* 12(9):1067–1075
- Do HTV, Ide Y, Mugo AN, Yagi T (2012) All-enzymatic HPLC method for determination of individual and total contents of vitamin B₆ in foods. *Food Nutr Res* 56:5409. <https://doi.org/10.3402/fnr.v56i0.5409>
- Eitenmiller RR, Landen WO Jr (1999) Vitamin analysis for the health and food sciences. CRC Press, Boca Raton

- Eitenmiller RR, Ye L, Landen WO Jr (2008) Vitamin analysis for the health and food sciences. CRC Press Taylor and Francis Group, Boca Raton
- Esteve MJ, Farré R, Frígola A, García-Cantabella JM (1998) Determination of vitamin B₆ (pyridoxamine, pyridoxal and pyridoxine) in pork meat and pork meat products by liquid chromatography. *J Chromatogr A* 795:383–387
- Gatti R, Gioia MG (2005) Liquid chromatographic determination with fluorescence detection of B₆ vitamers and riboflavin in milk and pharmaceuticals. *Anal Chim Acta* 538:35–41
- Gaussian 09. M. J. G. Frisch, W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. G. Mennucci, A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. E. M. Hada, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski and D. J. Fox
- Gliszczynska-Świgło A, Rybicka I (2015) Simultaneous determination of caffeine and water-soluble vitamins in energy drinks by HPLC with photodiode array and fluorescence detection. *Food Anal Methods* 8: 139–146
- Gregory JF (2012) Accounting for differences in the bioactivity and bioavailability of vitamers. *Food Nutr Res* 56:5809. <https://doi.org/10.3402/fnr.v56i0.5809>
- Gregory JF, Kirk JR (1981) The bioavailability of vitamin B₆ in foods. *Nutr Rev* 39:1–8
- Hälvén K, Paalme T, Nisamedtinov I (2013) Comparison of different extraction methods for simultaneous determination of B complex vitamins in nutritional yeast using LC/MS-TOF and stable isotope dilution assay. *Anal Bioanal Chem* 405:1213–1222
- van der Ham M, Albersen M, de Koning TJ, Visser G, Middendorp A, Bosma M, Verhoeven-Duif NM, de Sain-van der Velden MGM (2012) Quantification of vitamin B₆ vitamers in human cerebrospinal fluid by ultra performance liquid chromatography-tandem mass spectrometry. *Anal Chim Acta* 712:108–114
- Hellmann H, Mooney S (2010) Vitamin B₆: A Molecule for Human Health. *Molecules* 15:442–459
- Jin P, Xia L, Li Z, Che N, Zou D, Hu X (2012) Rapid determination of thiamine, riboflavin, niacin amide, pantothenic acid, pyridoxine, folic acid and ascorbic acid in vitamins and minerals tablets by high-performance liquid chromatography with diode array detector. *J Pharm Biomed Anal* 70:151–157
- Kabir H, Leklem JE, Miller LT (1983) Relationship of the glycosylated vitamin B₆ content of foods to vitamin B₆ bioavailability in humans. *Nutr Rep Int* 28:709–716
- Langer S, Lodge JK (2014) Determination of selected water-soluble vitamins using hydrophilic chromatography: a comparison of phodiode array, fluorescence and coulometric detection, and validation in a breakfast cereal matrix. *J Chromatogr B* 960:73–81
- Larsson SC, Giovannucci E, Wolk A (2005) Vitamin B₆ intake, alcohol consumption, and colorectal cancer: a longitudinal population-based cohort of women. *Gastroenterology* 128(7):1830–1837
- Lebiedzińska A, Marszał M, Kuta J, Szefer P (2007) Reversed-phase high-performance liquid chromatography method with coulometric electrochemical and ultraviolet detection for the quantification of vitamins B₁ (thiamine), B₆ (pyridoxamine, pyridoxal and pyridoxine) and B₁₂ in animal and plant foods. *J Chromatogr A* 1173:71–80
- Lebiedzińska A, Dąbrowska M, Szefer P, Marszał M (2008) High-performance liquid chromatography method for the determination of folic acid in fortified food products. *Toxicol Mech Methods* 18: 463–467
- Liu S-Q, Cao M-L, Dong S-L (2008) Electrochemical and ultraviolet-visible spectroscopic studies on the interaction of deoxyribonucleic acid with vitamin B₆. *Bioelectrochemistry* 74(1):164–169
- Marszał M, Lebiedzińska A, Czarnowski W, Szefer P (2005) High-performance liquid chromatography method for the simultaneous determination of thiamine hydrochloride, pyridoxine hydrochloride and cyanocobalamin in pharmaceutical formulations using coulometric electrochemical and ultraviolet detection. *J Chromatogr A* 1094:91–98
- Nishimura S, Nagano S, Cral CA, Yokochi N, Yoshikane Y, Ge F, Yagi T (2008) Determination of individual vitamin B₆ compounds based on enzymatic conversion to 4-pyridoxolactone. *J Nutr Sci Vitaminol* 54:18–24
- Ollilainen V, Finglas PM, van den Berg H, de Froidmont-Görtz I (2001) Certification of B-group vitamins (B₁, B₂, B₆ and B₁₂) in four food reference materials. *J Agric Food Chem* 49:315–321
- di Salvo ML, Budisa N, Contestabile R (2012) PLP-dependent enzymes: a powerful tool for metabolic synthesis of non-canonical amino acids <http://www.beilstein-institut.de/Bozen2012/Proceedings/Budisa/Budisa.pdf>
- Sampson DA, Eoff LA, Yan XL, Lorenz K (1995) Analysis of free and glycosylated vitamin B₆ in wheat by high-performance liquid chromatography. *Cereal Chem* 72(2):217–221
- Sampson DA, Wen Q-B, Lorenz K (1996) Vitamin B₆ and pyridoxine glucoside content of wheat and wheat flours. *Cereal Chem* 73(6): 770–774
- Santos J, Mendiola JA, Oliveira MBPP, Ibáñez Herrero M (2012) Sequential determination of fat- and water-soluble vitamins in green leafy vegetables during storage. *J Chromatogr A* 1261:179–188
- Sorolla MA, Rodríguez-Colman MJ, Tamarit J, Ortega Z, Lucas JJ, Ferrer I, Ros J, Cabisco E (2010) Protein oxidation in Huntington disease affects energy production and vitamin B₆ metabolism. *Free Radic Biol Med* 49:612–621
- Tambasco-Studart M, Titiz O, Raschle T, Forster G, Amrhein N, Fitzpatrick TB (2005) Vitamin B₆ biosynthesis in higher plants. *Proc Natl Acad Sci U S A* 102(38):13687–13692