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Quantification of retinyl palmitate, thiamine, niacin, pyridoxine, folic acid, cyanocobalamin, zinc, and iron by chromatographic methods in fortified kernels and fortified rice

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ABSTRACT

This study presents the optimization and validation of methods for the analysis of retinol, thiamine, niacin, pyridoxine, folic acid, cyanocobalamin, zinc, and iron in fortified kernels (coated and extruded) and in fortified rice. The analyses were performed by HPLC-UV/FLD/MS and ICP-OES. The optimized methods showed good resolution of the analyte peaks, excellent recovery (87–108%), reproducibility with relative standard deviation (SD) of analyte content between 1.8 and 11% and high correlation coefficient of the calibration curves ($R^2 > 0.997$). Limit of detection was from 2.8 E-4 mg/kg for pyridoxine to 1.26 mg/kg for zinc and limit of quantification was from 9.2 E-4 mg/kg for pyridoxine to 4.21 mg/kg for zinc. Thereby the optimized methods demonstrated reliability and sensitivity in the detection and quantification of these micronutrients and that they are suitable for routine analysis of fortified kernels (coated and extruded) and fortified rice.

KEYWORDS

vitamins, minerals, rice, HPLC, ICP-OES

INTRODUCTION

Fortification of staple food, such as rice, can be an excellent tool to improve the nutrition and health especially in low- and middle-income countries, where micronutrient deficiency burden is an extensive problem [1]. Rice is the dominant staple food crop for around 3 billion people worldwide, providing up to 70% of their daily energy intake [1]. Given its level of consumption, large-scale rice fortification is a viable and cost-effective opportunity to deliver micronutrients through daily diets to a very large number of people. World Food Program (WFP) strongly recommends fortification of rice with iron and conditional recommends the fortification of rice with vitamin A and folic acid, as part of a healthy diet [2]. Different methods to fortify rice with vitamins and minerals have been developed and used in the past decade, of which three are mostly used: 1) warm/hot extrusion, 2) cold extrusion and 3) coating [3]. These three methods result in kernels with a high concentration of vitamins and minerals, the so-called fortified kernels. Fortified kernels are then mixed with unfortified head rice, typically in 1:50–1:200 ratios, to produce the final fortified rice that is ready for consumption [1]. Fortified kernels can be made from different technologies, this study includes kernels made by extrusion or coating. To produce fortified kernels using extrusion, dough made of non-fortified rice flour, premix of vitamins and minerals and water is used. The

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dough is processed through an extruder and shaped into a grain-like product that resembles rice kernels. Coated fortified rice kernels are produced by applying a layer of rinse resistant coating containing premix of vitamins and minerals on non-fortified rice [3].

To be able to use rice in a fortification strategy, it is essential that reliable standard methods for the analysis of these micronutrients in this fairly new matrix are available. However, the determination of the vitamins is complicated due to chemical instability, very diverse chemical structure and properties and the complexity of the matrix [4]. It is essential to use methods with high accuracy and sensitivity in order to obtain reliable results.

All the methods used in this study build on reference methods for analyzing vitamins and minerals in food. Although fortified rice and fortified kernels have, to our knowledge, not been validated on the chosen methods previously, fortified rice is a complex matrix to analyze due to the naturally high content of starch in rice and they can be protected by coating. Both will challenge the extraction of the vitamins and minerals from the premix kernels. The validation results are used to judge the suitability of the methods for the matrices, reliability as well as consistency pertaining to analytical results.

The present study aimed to optimize and validate the analysis of retinyl palmitate, thiamine, niacin, pyridoxine, folic acid, cyanocobalamin, iron and zinc by different analytical methods in fortified rice and fortified kernels (extruded and coated). The selected vitamins and minerals are the constituents of the premix used to produce the fortified kernels.

EXPERIMENTAL

Raw material

The fortified kernels were kindly donated by two different commercial suppliers, and were one extruded and one coated type.

The non-fortified rice used in this study is white rice, *Oryza sativa*, i.e. long thin milled rice, which was obtained from the local market under the name jasmine rice (Vejen, Denmark).

Mixing and sampling

The two different fortified kernels (extruded and coated) were blended with non-fortified rice resulting in two different fortified rice. To make fortified rice, blending ratio range 1:100 was used. All analyses were carried out on a minimum of 200 g portions of fortified rice (i.e., 2 g of fortified kernels mixed with 198 g of non-fortified rice). Premix kernels and non-fortified rice were mixed for 5 min using a TURBULA® Powder Blender, in a glass container (Glen Mills, Inc, Clifton, NJ, USA), in order to guarantee homogenous mixing of samples. Samples were grinded on Retsch ultra centrifugal mill ZM200, with fixed titanium ring

sieves sized 0.25 mm. Before sample extraction, the grinded samples were protected against oxygen by the addition of gaseous nitrogen. Milling, grinding and analysis of vitamins were performed in an UV protected laboratory. All analyses were performed in ISO17025 accredited laboratories.

Chemical reagents

All-trans-retinol palmitate (USP), thiamine hydrochloride ($\geq 99\%$), nicotinic acid ($\geq 99\%$), nicotinamide ($\geq 99\%$), pyridoxine hydrochloride ($\geq 99\%$), folic acid ($\geq 97\%$, water $\leq 9\%$), and cyanocobalamin ($\geq 98\%$) were purchased from Sigma Aldrich/Merck (Darmstadt, Germany), folic acid- $[^{13}\text{C}_5]$ ($\geq 95\%$, isotope incorporation $\geq 99\%$) was purchased from IsoScience (Ambler, PA, USA). All other chemicals were purchased from the certified suppliers and were of analytical reagent grade. Water used for all analyses was MilliQ grade.

All the enzymes used during the validation, retinyl palmitate assay: papain from carica papaya (≥ 3 U/mg) and taka diastase (1.5 U/mg), pyridoxine assay: phosphatase (≥ 0.4 U/mg), folic acid assay: α -amylase from porcine pancreas (≥ 10 U/mg), papain from carica papaya (≥ 3 U/mg), cyanocobalamin assay: α -amylase from aspergillus oryzae (~ 1.5 U/mg), were purchased from Sigma Aldrich/Merck (Darmstadt, Germany).

Extraction of retinyl palmitate. Finely ground samples of fortified rice and fortified kernels, 2.5 g and 0.5 g respectively, were weighed into a 50 mL graduated beaker. Thereafter, enzymatically treated with 5 mL enzyme solution (0.1% hydroquinone and 4% sodium acetate mixed in water with pH adjusted to 5.0, and 2% papain and 2% taka diastase added) for 30 min at 37 °C while shaking. Samples were then cooled to room temperature. Thereafter, 20 mL acidified methanol (2% glacial acetic acid) and 10.00 mL isooctane were added and mixed. Sample extracts were centrifuged for 10 min at 3,500 rpm. An aliquot of the supernatant was filtered through a 0.45 μm syringe filter to a HPLC vial. The fortified kernel extracts were diluted 1:2 with isooctane before filtration.

Extraction of thiamin. Finely ground samples of fortified rice and fortified kernels, 5.0 g and 0.5 g respectively, were weighed into a 100 mL graduated beaker. 25 mL of 0.1 M HCl was then added and the beaker was capped. Samples were mixed and autoclaved for 30 min at 121 °C water bath. Thereafter cooled to room temperature and the sample was made to 50 mL by adding 0.1 M HCl. The fortified kernel extracts were diluted 1:10 with 0.1 M HCl. Sample extract was filtered through 0.45 μm PVDF syringe to a HPLC vial.

Extraction of nicotinic acid and nicotinamide. Finely ground samples of fortified rice and fortified kernels, 3.0 g and 0.5 g respectively, were weighed into a 100 mL graduated tube. Thereafter, 50 mL of 0.1 M HCl was added and the samples were autoclaved for 1 h at 100 °C. Extracts were then cooled to room temperature and 2.5 mL of 2.5 M

sodium acetate solution was added, followed by pH adjustment to 4.5 ± 0.1 with 2.5 M sodium acetate solution. Extracts were diluted to 100 mL with water and thereafter filtered through $0.45 \mu\text{m}$ PVDF syringe to a HPLC vial.

Extraction of pyridoxine. Finely ground samples of fortified rice and fortified kernels, 5.0 g and 0.5 g respectively, were weighed into a 50 mL graduated beaker. 25 mL of 0.05 M sodium acetate solution and 2.5 mL of 1.0 M glyoxylic acid monohydrate were then added and pH was adjusted to 4.5 ± 0.1 with 2.5 M sodium acetate and 400 μL ferrous sulfate heptahydrate solution (0.0132 M in 0.05 M sodium acetate) and 1.0 mL acid phosphatase solution (2% in water) was added followed by incubation for 16–20 h at 37 °C, while shaking. Sample extracts were cooled to room temperature, and diluted to 50 mL with water. The fortified kernel extracts were further diluted 1:50 with water. 5.00 mL sample extract was transferred into 50 mL graduated beaker, 3.0 mL NABH_4 solution (0.15 M in 0.2 M NaOH) was then added and mixed, thereafter, 500 μL glacial acetic acid was added and the extracts were filtered through a folded filter paper into a HPLC vial.

Extraction of folic acid. Finely ground samples of fortified rice and fortified kernels, 1.0 g and 0.5 g respectively, were weighed into a 100 mL beaker. Samples were reconstituted with 30 mL water for 15 min at 40 °C water bath. 20 mL α -amylase solution (0.4% in water) was then added and the samples were digested at 40 °C for 15 min, while shaking. Thereafter, the samples were diluted with 40 mL extraction buffer (1.42% Na_2HPO_4 , 2% ascorbic acid, 0.1% dithiothreitol (DTT), pH 6) and incubated for 30 min at 90 °C water bath while shaking. Samples were then cooled to approximately 30–35 °C, and 2 mL protease solution (0.47% in water) was added and digested for 16–20 h at 60 °C while mixing. Extracts were then cooled to room temperature, diluted to 100 mL with water and filtered through a folded filter paper. The fortified kernel extracts were diluted 1:20 with water. 9.00 mL filtrate was transferred into a 10 mL beaker, and 1.00 mL of 100 $\mu\text{g/g}$ $^{13}\text{C}_5$ -folic acid was added and mixed. The filtrate was purified by solute SAX columns (Biotage, Wales, UK). Columns were activated by 4 mL acetonitrile and equilibrated with 10 mL extraction buffer. Thereafter, 3.00 mL sample extract was loaded, cartridge was washed with 6 mL extraction buffer and eluted with 4 mL eluting solution (60:30:10, acetonitrile:extraction buffer:glacial acetic acid). Eluate was evaporated at 50 °C under nitrogen and reconstituted in 1.50 mL reconstitution solution (1% ascorbic acid, 0.5% DTT in water) and filtered through $0.45 \mu\text{m}$ PVDF syringe filter into a HPLC vial.

Extraction of cyanocobalamin. Finely ground samples of fortified rice and fortified kernels, 3.0 g and 0.5 g respectively, were weighed into a 100 mL beaker. 0.05 g α -amylase and 75–80 mL water at 40 ± 5 °C were mixed on shaking table for 30 min and added to the samples. Thereafter, 1 mL of 1% sodium cyanide solution was added

and the samples were incubated at 37 °C for 30 min, and 10 mL of 1.7 M sodium acetate was added and the samples were autoclaved at 100 °C for 30 min. Samples were then cooled to room temperature and filtered. 9 mL sample extract is purified on EASI-extract vitamin B12 immunoaffinity columns (r-biopharm AG, Germany). Cartridge was washed with 10 mL water and the B12 thereafter eluted by 3.5 mL of methanol. Purified sample extracts were concentrated 10 times by evaporating and reconstitution in a solution of 90:10 mobile phase A:mobile phase B. Extraction and immunoaffinity cleanup were following the conditions of Kircher et al [6].

Extraction of iron and zinc. 0.4 g of homogenized sample was weighed into a digestion vessel and 3 mL of conc. nitric acid, 0.5 mL of conc. hydrochloric acid were added to the sample. Digestion vessels were closed according to the manufactures' instructions. Samples were digested with an increase in temperature to 200 °C during 10 min, hold at 200 °C for 15 min, and then cooled down according to the manufactures' instructions. Thereafter, the clear digest was transferred into a 50 mL volumetric tube and filled with 50 mL of MQ water.

Preparation of standards and intermediate standards

Calibration standards retinyl palmitate. Standard solution of retinyl palmitate was prepared by dissolving 35 mg of analytical standard in isooctane in a 25 mL volumetric flask. In order to test linearity of the method, six calibration standards with concentrations ranging from 0.2–17 $\mu\text{g/mL}$ in isooctane were prepared.

Calibration standards thiamine. Standard solution of thiamine hydrochloride was prepared by dissolving 25 mg of analytical standard in 0.1 M HCl in a 250 mL volumetric flask. Six calibration standards with concentrations ranging from 0.016 to 1.6 $\mu\text{g/mL}$ in 0.1 M HCl were prepared.

Calibration standards nicotinic acid and nicotinamide. Standard solutions of nicotinic acid and nicotinamide were prepared by dissolving 50 mg of each analytical standard in water in two separate 50 mL volumetric flasks. Six calibration standards with concentrations ranging from 0.05 to 5 $\mu\text{g/mL}$ for both nicotinic acid and nicotinamide in water were prepared.

Calibration standards pyridoxine. Standard solution of pyridoxine was prepared by dissolving 150 mg of analytical standard in water in a 300 mL volumetric flask. Six calibration standards with concentrations ranging from 0.03 to 3.2 $\mu\text{g/mL}$ in 0.1 M HCl were prepared.

Intermediate standards folic acid. Standard solution of folic acid was prepared by dissolving 10 mg of analytical standard into a solution of sodium hydroxide 0.1 M 5% (v/v)-ethanol 20% (v/v) in a 100 mL volumetric flask. Intermediate standards of folic acid with concentrations 5 and 0.075 $\mu\text{g/mL}$

were prepared in aqueous solution of sodium hydroxide ascorbic acid 1% (w/v), DTT 0.5% (w/v).

Internal standard of $^{13}\text{C}_5$ -folic acid. Standard solution of $^{13}\text{C}_5$ -folic acid was prepared by dissolving 1 mg of $^{13}\text{C}_5$ -folic acid in aqueous solution of sodium hydroxide ascorbic acid 1% (w/v), DTT 0.5% (w/v) in a 10 mL volumetric flask. Intermediate internal standard solution was prepared with a concentration of 0.25 $\mu\text{g/mL}$ in the same solvent.

Calibration standards folic acid. Six calibration standards with concentrations ranging from 1.5 to 400 ng/mL for folic acid in an aqueous solution of sodium hydroxide ascorbic acid 1% (w/v), DTT 0.5% (w/v) were prepared. Final concentration of internal standard was 50 ng/mL for all calibration standards.

Calibration standards cyanocobalamin. Standard solution of cyanocobalamin was prepared by dissolving 20 mg of analytical standard in water in a 100 mL volumetric flask. Six calibration standards with concentrations ranging from 5 to 4,160 $\mu\text{g/mL}$ in 90:10 mobile phase A:mobile phase B were prepared.

Calibration standards iron and zinc. Multielement stock solution containing 90 mg/L of iron and 30 mg/L of zinc was used to prepare calibration standards with concentrations ranging from 0.1875 to 3 mg/L for zinc and from 0.5625 to 9 mg/L for iron in a solution of 0.1% HCl and 0.6% nitric acid in water.

Chromatographic conditions

HPLC-UV analysis of retinyl palmitate. A Thermo Scientific Ultimate 3000 with UV detector (Thermo Scientific, Massachusetts, USA) equipped with a sphereclone NH_2 80 Å 5 μm , 4.6 $\times$ 150 mm column was used for quantitative analysis. 25 μL of the sample extract was injected. The oven temperature was set at 40 °C and the flow rate was 1.5 mL/min. Start conditions of 95% mobile phase A (*n*-hexane):5% mobile phase B (75:25:0.3 *n*-hexane:methyl-*t*-butyl ether:methanol) were maintained for 3 min followed directly by linear gradient in 9 min and then to 95% mobile phase B; isocratic condition of 95% mobile phase B was maintained for 2 min. This was followed by 95% mobile phase A for 1 min, and hold isocratically for 5 min. Detection was carried out by UV detection at 325 nm. Conditions as described by McMahon et al [5] (Fig. 1).

HPLC-FLD analysis of thiamine. A Thermo Scientific Ultimate 3000 with FLD detector (Thermo Scientific, Massachusetts, USA) equipped with a Phenomenex Kinetex C18 2.6 μm , 4.6 $\times$ 100 mm column was used for quantitative analyses. 5 μL of the sample extract was injected. The oven temperature was set at 50 °C and the flow rate was 0.8 mL/min. Start conditions were 80% mobile phase A (aqueous phosphate buffer 1.10 g Sodium-1-heptasulfonate and 1.36 g KH_2PO_4 , 1,000 mL

water, pH 3.0 $\pm$ 0.1) and 20% mobile phase B (methanol) followed directly by linear gradient to 47% B in 9 min and then to 98% mobile phase B for 0.1 min; isocratic conditions of 98% solvent B were maintained for 1.9 min. This was followed by 20% mobile phase B for 0.1 min. Thereafter, isocratic conditions of 30% mobile phase B were maintained for 3.9 min. Detection was carried out by fluorescence detection (excitation 368 nm, emission 440 nm) after postcolumn reaction where thiamine was oxidized to thiochrome by 1% potassium hexacyanoferrate in 5% w/v NaOH solution. The flow post-column reagent was 0.3 mL/min, with 25 μL post column liner T mixer (Fig. 1).

HPLC-FLD analysis of nicotinic acid and nicotinamide. A Thermo Scientific Ultimate 3000 with UV detector (Thermo Scientific, Massachusetts, USA) equipped with a Thermo Hypersil Gold aQ C18, 3 μm , 4.6 $\times$ 250 mm column was used for the quantitative analysis. 25 μL of the sample extract was injected. The oven temperature was set at 30 °C and the flow rate was 1.2 mL/min. Isocratic condition was 100% mobile phase A (9.54 g potassium dihydrogenphosphate, 7.6 mL hydrogen peroxide and 1.0 mL 0.005 M aqueous copper sulfate, total volume 1,000 mL). The mobile phase was fused with nitrogen during the chromatographic run to prevent bubble formation, and the run time was maintained at 25 min. Detection was carried out with FLD detection, excitation at 322 nm and emission at 380 nm, after postcolumn derivatization with hydrogen peroxide, catalyzed by Cu (II) ions in a knitted open tubular polytetrafluoroethylene reactor coil (5 m, 0.5 mm, 1.6 mm) wound around a UV black-light-blue lamp (604 $\times$ 28 mm) under UV 365 nm (Fig. 1).

HPLC-FLD analysis of pyridoxine. A Thermo Scientific Ultimate 3000 with FLD detector (Thermo Scientific, Massachusetts, USA) equipped with a Phenomenex Kinetex XB-C18 100 Å, 2.6 μm , 4.6 $\times$ 150 mm column was used for quantitative analysis. 15 μL of sample extract was injected. The oven temperature was set at 25 °C and the flow rate was 1.0 mL/min. Isocratic condition was 100% mobile phase A (mobile phase A consisted of buffer solution added 6.5% acetonitrile). Buffer solution consisted of 11.0 g potassium dihydrogen phosphate, 0.53 g octane sulfonic acid sodium salt, 300 μL trimethylamine and 1.30 mL 85% ortho phosphoric acid diluted to 900 mL with water. pH was adjusted to 2.75 $\pm$ 0.05 with a 85% ortho phosphoric acid and 5 M KOH, thereafter diluted to 1,000 mL with water. Chromatographic run time was maintained at 17 min. Detection was performed by fluorescence detection with excitation at 290 nm and emission at 395 nm (Fig. 1).

LC-MS/MS analysis of folic acid. A Thermo Scientific Ultimate 3000 (Thermo Scientific, Massachusetts, USA) equipped with a Waters UPLC HSS T3, 1.8 μm , 2.1 $\times$ 150 mm column was used for quantitative analysis. 5 μL of reconstituted extract was injected. The oven temperature

was set at 25 °C and the flow rate was 0.25 mL/min. Isocratic conditions of 100% mobile phase A (0.5% formic acid in water) were maintained for 0.38 min, followed by 10% mobile phase B (acetonitrile) for 0.08 min. Isocratic conditions of 10% solvent B were maintained for 1.07 min followed by linear gradient to 25% mobile phase B for 2.67 min. Thereafter, followed directly to 99% mobile phase B in 0.08 min and maintained for 1.45 min, and returned to the start conditions in 0.07 min, maintained for 5.7 min. Detection was carried out by mass spectrometry performed on an 4500 Triple Quad (AB Sciex, MA, USA) in positive ESI mode. ESI capillary voltage (IS) was set at 4500 V. Source Temperature (TEM) was set at 400 °C and Collision Gas (CAD) was set to 9 psi. The Curtain gas (CUR) was set to 30 psi, Ion Source Gas 1 (GS1) to 50 psi and Ion Source Gas 2 (GS2) to 60 psi. Entrance Potential (EP) was fixed at 10 V. Declustering Potential (DP), Collision Energy (CE) and Collision Cell Exit Potential (CXP) were compound dependent and described in the Multi Reaction Monitor (MRM) table (Table 1, Fig. 1).

HPLC-UV analysis of cyanocobalamin. A Thermo Scientific Ultimate 3000 with UV detector (Thermo Scientific, Massachusetts, USA) equipped with a Phenomenex, Gemini NX C18 3 µm, 3.00 × 150 mm column was used for quantitative analysis. 100 µL of the reconstituted extract was injected. The oven temperature was set at 25 °C and the flow rate was 0.25 mL/min. Start conditions of 90% mobile phase A (0.025% TFA in water) 10% mobile phase B (0.025% TFA in acetonitrile) were maintained for 0.5 min followed directly by linear gradient in 3.5 min to 25% mobile phase B. Thereafter, linear gradient in 1 min to 90% mobile phase B; isocratic condition of 90% mobile phase B was held for min. This was followed by 90% mobile phase A for 2 min, and maintained isocratically for 5 min. The detection was carried out by UV detection at 361 nm. The chromatographic conditions and detection were following the Kircher et al [6].

ICP-OES analysis of iron and zinc. An Agilent 5100 SVDV (Santa Clara, CA) was used for the quantitative analysis. Fe was quantified at the spectral line 238.204 nm, with a secondary line at 259.94 nm. Zn was quantified at the spectral line 206.200 nm, with a secondary line at 213.857 nm. Fitted background correction was used for both the elements. Analysis was performed with the

Fig. 1. Representative chromatograms for retinyl palmitate (A), thiamine (B), nicotinic acid and nicotinamide (C), pyridoxine (D), folic acid (E) and cyanocobalamin (F) in fortified rice extracts

following specifications: forward power: 1200 W; nebulizer: concentric glass and sea spray; argon flow: 0.65 L/min; replicate time: 5 s; number of replicates: 3; viewing mode: axial.

RESULTS AND DISCUSSION

The methods used for the determination of retinyl palmitate and vitamin B12 were following the AOAC official methods [5, 6], only for the vitamin A, an additional enzymatic treatment with amylase was added to break down the starch of the rice. The methods were originally developed for the determination of vitamin A and B12 in milk and soy based products [5, 6]. The principle of the method for determination of retinyl palmitate is enzymatic digestion with amylase and papain, thereafter retinyl palmitate was extracted with isooctane. The sample was analyzed by a normal phase HPLC. Retinyl palmitate was detected with UV at 325 nm and quantified by external calibration curves. For vitamin B12, the samples were first digested with amylase, thereafter natural forms of vitamin B12 were converted to cyanocobalamin by sodium cyanide. Samples were incubated for 30 min at 37 °C. Cyanocobalamin was then extracted with a sodium acetate buffer at 100 °C, pH was adjusted and the extracts were concentrated on immunoaffinity columns. The samples were analyzed on reverse phase HPLC and detected by UV at 361 nm and cyanocobalamin was quantified against external calibration curve. The methods used for the determination of thiamine, niacin and pyridoxine were based on EN reference methods [7–11]. The methods were originally developed for the determination of vitamin B1, B3, and B6 in foodstuff. The analytical procedures have been modified to optimize the performance of the methods for the matrices fortified rice and premix

Table 1. Multi Reaction Monitor table

ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell time (ms)	DP (V)	CE (V)	CXP (V)
FA ^a	442.1	295.0	50.0	58.0	22.0	14.0
FA ^b	442.1	176.1	50.0	58.0	52.0	7.0
ISTD FA ^a	447.1	295.0	50.0	31.0	20.0	17.0
ISTD FA ^b	447.1	176.0	50.0	31.0	60.0	13.0

^aMarks the quantifier ions.

^bMarks the qualifier ions.

kernels. Premix kernels were not covered by the original scope of the methods and therefore it was necessary to verify that the methods are suitable for the determination of vitamin B1, B3, and B6. In the EN reference method [7], an additional enzymatic treatment was performed to convert phosphorylated forms of thiamine to free thiamine. This treatment is not necessary in this study as the thiamine of interest was added to the matrix as thiamine HCl and not bound within the matrix. The method used for the determination of folic acid was based on an AOAC official method [12]. The method was developed for the determination of folic acid in milk matrices. It was observed that the official method did not give reliable results for fortified rice and fortified kernels and changes were necessary to obtain precise and accurate results. The quantification of folic acid was challenged by the high starch content and the coating of the micronutrients. To avoid gel formation, due to the starch content, high amount of α -amylase was added to digest the starch resulting in higher results for the fortified rice. The conditions for the protease were optimized to break down the coating applied to the premix kernels giving better precision of the method. The methods used for determination of iron and zinc were based on the European Standard method EN 13805:2014 for digestion of samples, and ICP-OES for quantification of iron and zinc in the digest. To improve the recovery of iron, a small amount of hydrochloric acid was added in addition to the nitric acid used for digestion. This addition was not a modification of EN13805 as the use of hydrochloric acid was mentioned as an option in the standard.

In this study, the fortified kernels are mixed with raw rice in the ratio 1:100. The estimated and found content of the fortified kernels and fortified rice are shown in Table 2. All the results are within the expected range, except the content of folic acid in the coated kernels and the fortified rice made by coated kernels. These matrices were also analyzed at an external laboratory, using a different method. These results confirmed the found content of folic acid in the coated kernels. Therefore, it is plausible that the content determined in the present study is close to the true content of folic acid in the coated kernels. Non-fortified rice contains a natural content of iron, zinc, and water-soluble vitamins

including thiamine (B1), riboflavin (B2), pyridoxine (B6), niacin (B3) and cyanocobalamin (B12) and low content of the fat-soluble vitamin retinol (A) [13]. The natural content of vitamins is low compared to the fortification level and therefore only small variations in the 1:100 ratios between fortified kernels and fortified rice are observed, for minerals higher natural content is found in rice, this explains the larger variations in the 1:100 ratios between fortified kernels and fortified rice.

Method validation

For all parameters, specificity, linearity, precision, LOD and LOQ and recovery were conducted during the validations following NMKL procedure 4, 2009 [14]. All samples and standards are analyzed under the same chromatographic/spectroscopic conditions. Specificity of the methods were evaluated from chromatograms obtained in the analysis of standards, blanks and samples. There were no interfering peaks with the same retention time as the analytes. Moreover the peak purity data was inspected for methods analyzed with UV and for the ion ratio between folic acid and internal standard. For zinc and iron, no interfering peaks or shoulders were seen at the chosen wavelengths. This proves that the methods are specific for the analytes.

Determination of linearity range of the compounds was performed in duplicate of at least 6 different concentrations of standard solution of each analyte. Either the peak area or the area ratio for folic acid was used for linear regression analysis. The coefficient of determination (R^2) and the residuals obtained were used to assess the linearity of each analyte. All methods were linear according to the results obtained by mean of the calibration curves in the chosen concentration range. The results obtained in all the assay analyses presented a linear correlation between the injected concentration and its peak area with correlation coefficients (R^2) greater than 0.997 and significance (F) lesser than 0.005. The concentration range in which the linearity has been evaluated is shown Table 3. LOD and LOQ were determined by 10 individual prepared samples of either raw rice or rice spiked with low content of the analyte (retinyl

Table 2. Estimated and found content (mean value, $n = 20$) of micronutrients in fortified kernels and fortified rice

Analyte/ matrice	Estimated content		Content found in this study			
	Fortified kernels (mg/kg)	Fortified rice (mg/kg)	Extruded kernels (mg/kg)	Coated kernels (mg/kg)	Rice extruded kernels (mg/kg)	Rice coated kernels (mg/kg)
Vitamin A	195–312	1.95–3.12	196	173	2.11	1.71
Vitamin B1	650–975	6.50–9.75	636	700	7.54	7.93
Vitamin B3	9,100–10,920	91–109	9,980	9,920	100	103
Vitamin B6	780–1,170	7.80–11.70	767	772	8.70	8.55
Folic acid	169–253	1.69–2.53	192	122	1.84	1.05
Vitamin B12	1.30–1.95	0.013–0.020	1.41	2.75	0.018	0.026
Iron	4,000–4,800	40–48	3,779	4,591	39.88	49.11
Zinc	6,000–7,200	60–72	5,009	5,948	64.75	74.75

Table 3. Results obtained in the evaluation of the linearity, LOD and LOQ tests.

Analyte	Linearity range ($\mu\text{g/mL}$)	R^2	Regression equation	LOD (mg/kg)	LOQ (mg/kg)
Retinyl palmitate	0.11–26.5	0.9999	$Y = 1.513x - 0.007$	0.01	0.02
Thiamine	0.0151–1.5132	0.9998	$Y = 3.0E05x - 417$	0.04	0.15
Nicotinic acid	0.010–7.240	0.9973	$Y = 1.1E06x + 5,714$	0.49	1.63
Nicotinamide	0.098–6.884	0.9970	$Y = 2.4E06x + 21,860$	0.05	0.15
Pyridoxine	0.3–3.3	0.9999	$Y = 1.9E05x + 152$	$2.8E-4$	$9.2E-4$
Folic acid	0.0016–0.651	0.9996	$Y = 1.4E - 02x + 0.014$	0.004	0.012
Cyanocobalamin	0.005–4	0.9999	$Y = 6.50x + 0.0662$	0.01	0.02
Iron	10–1.125	0.9980	$Y = 3.6E04x - 516.1$	0.94	3.14
Zinc	5–1.000	0.9979	$Y = 2.9E04x + 1,557$	1.26	4.21

palmitate). The LOD were determined as 3 times the standard deviation (SD) and LOQ as 10 times the SD. Table 3 summarizes the obtained LOD and LOQ for all analytes.

The repeatability refers to the degree of agreement of the results when all conditions are maintained constant, with same analyte, reagents, equipment, instrument and day [15]. All data sets are evaluated by one way analysis of variance (ANOVA). From this analysis, S_R and S_r were calculated, by the means of the results of between-day variance and within-day variance. The repeatability is expressed as the SD (S_r) of four determinations for each matrix. The samples are analyzed as individual preparations but determinations are conducted simultaneously on same analytical instrument, and these conditions are assumed as individual determinations. For the vitamins, the SD of the repeatability is determined on five different days as a part of the determination of the within-laboratory variability (intermediate precision) in a total of 20 determinations for each matrix. For iron and zinc, the replicates were distributed as eight replicates on two different days, in total 16 replicates. Two different analytes have performed the analysis, with different reagents, standards, columns and with different equipment, where possible. The SD of the intermediate precision (S_R) shows the inter-day performance of the methods. Results presented in Table 4

show that the relative SD of the repeatability (S_r) and SD of the intermediate precision (S_R) for all analytes on all four matrices are within acceptable range. The precision on the repeatability as a function of the concentration of each analyte is for all parameters within the expected range according to AOAC guideline [16].

Recovery tests were performed for all analytes, by the addition of standard solution to fortified kernels and fortified rice in two different levels. Analyses were performed in triplicate. The percentage recovery was calculated from the concentration of unspiked sample, added concentration of analyte and final observed concentration of the analyte in the sample. Table 5 shows the mean recovery ($n = 3$) at two different spiking levels equivalent to approximately +50 and +100% of the natural content of the matrix. All recoveries are within the range of 87.2–108.6% that is satisfying the spiking results. Although it can be observed that all the results in the recovery experiment for folic acid are below 100%, these results could indicate that the method has a negative bias for folic acid and this negative bias needs to be regarded when evaluating the uncertainty of the method. For iron and zinc, the accuracy of the methods was further tested by analysis of certified reference material for food element analysis, brown rice flour NMIJ CRM7532-A, the reference material had a mean recovery of 101% ($n = 16$) for iron and 97% ($n = 16$) for zinc.

Table 4. Relative standard deviation of the repeatability (S_r) and intermediate precision (S_R) for the four matrices. Fortified rice made with extruded kernels, fortified rice made with coated kernels, extruded kernels and coated kernels

	Fortified rice extruded kernels		Fortified rice coated kernels		Extruded kernels		Coated kernels	
	S_r (%)	S_R (%)	S_r (%)	S_R (%)	S_r (%)	S_R (%)	S_r (%)	S_R (%)
Retinyl palmitate	2.6	6.3	3.3	5.8	3.1	7.4	1.7	7.8
Thiamine	3.0	3.9	4.2	4.2	0.9	1.8	1.4	1.9
Niacin	3.8	5.1	3.7	4.3	2.8	6.4	3.0	4.4
Pyridoxine	3.0	4.6	2.6	3.7	2.0	2.8	2.0	3.2
Folic acid	3.3	5.0	5.6	7.2	3.8	4.9	3.0	3.5
Cyanocobalamin	5.9	5.5	10.1	11.4	1.1	4.0	5.4	5.5
Iron	2.7	4.1	5.2	5.3	3.9	4.8	1.3	5.4
Zinc	3.1	4.0	5.0	5.2	3.6	6.1	3.0	3.2

Table 5. Recovery of spiking experiment. Spiking is performed at two different levels for each analyte, each spiking experiment is determined in triplicate and the means are shown in this table

Spiking level/analyte	Fortified rice extruded kernels		Fortified rice coated kernels		Extruded kernels		Coated kernels	
	1	2	1	2	1	2	1	2
Retinyl palmitate	102.4%	103.0%	102.0%	104.9%	102.5%	99.1%	99.7%	101.7%
Thiamine	102.8%	100.8%	100.1%	100.3%	97.3%	99.2%	99.9%	99.7%
Niacin	95.1%	97.6%	100.3%	106.7%	101.1%	103.0%	99.3%	102.0%
Pyridoxine	102.4%	103.1%	100.4%	102.4%	103.1%	101.1%	99.4%	100.9%
Folic acid	98.1%	92.8%	93.7%	87.2%	96.0%	87.9%	94.0%	89.1%
Cyanocobalamin	99.4%	96.3%	100.9%	97.7%	98.6%	99.7%	108.6%	101.1%
Iron	97.3%	95.3%	103.8%	103.8%	100.5%	97.2%	100.9%	96.7%
Zinc	97.8%	97.9%	101.2%	96.0%	104.0%	99.4%	101.3%	96.8%

It has not, to our knowledge not before, been verified that the methods used are suitable for the analysis of retinyl palmitate, thiamine, nicotinamide, pyridoxine, folic acid, and cyanocobalamin in fortified rice and fortified kernels extruded and coated.

CONCLUSION

In the current study, it has been shown that the methods validated are suitable for the analysis of retinyl palmitate, thiamine, niacin, pyridoxine, folic acid, cyanocobalamin, iron, and zinc in fortified kernels and fortified rice. The methods are specific for the analytes, and the measuring areas cover the concentration range of the validated matrices. All the methods showed good reproducibility with intermediate precision at maximum 11% and the validated methods showed low bias with excellent recovery (87–108%).

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