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Methods for Analysis of Thiamin and Folic Acid by HPLC–DAD in Fortified Rice Pure and Mixed to Milled Rice Before and After Different Cooking Techniques

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Summary. This study aimed to optimize and validate methods for the analysis of thiamin and folic acid in fortified rice, pure and mixed to the milled rice (raw and cooked). The analysis was performed by high-performance liquid chromatography coupled to a diode array detector (HPLC–DAD). Different mobile phases were tested. Different ratios of organic modifier, pH ranges, triethylamine concentrations, and flow rates were used. For the validation, tests of recovery, repeatability, linearity, limit of detection (LOD), and limit of quantification (LOQ) were performed. The optimized methods showed good resolution of vitamins' peaks, excellent recovery (82.6 to 104%), repeatability with relative standard deviation of peak areas, and retention times less than 10% and high coefficients of determination (0.9998 for thiamin and 0.9997 for folic acid). The LOD and LOQ were 0.00193 μg and 0.0193 μg for thiamin and 0.000934 μg and 0.00934 μg for folic acid. The optimized methods demonstrated reliability and sensitivity in the detection and quantification of these vitamins in fortified rice, pure and mixed to milled rice (raw and cooked). Furthermore, the methods were performed in isocratic mode, with short run time (<13 min), reflecting positively on the economy of reagents and analysis times.

Key Words: vitamins, food fortification, cooking, analytical method, chromatography

Introduction

Food fortification constitutes a major global strategy for the prevention of micronutrient deficiencies in many populations [1]. According to the Food and Agriculture Organization [2], rice is one of the most commonly produced cereal in the world and is a food commonly consumed by much of

the world's population, especially in developing countries, like Brazil. The worldwide rice production is approximately 606 million tons, to which Brazil contributes with 13,140,900 tons (2.17% of world production) and stands among the top 10 producers [3].

Rice has enormous potential as a vehicle for micronutrient fortification, since rice is a staple food for more than 3 billion people worldwide [4]. Previous attempts involving the fortification of rice flour were unsuccessful, due to the habit of washing and cooking rice with excess water, which results in the leaching of micronutrient used for enrichment. However, a new technology was created (Ultra Rice® — UR®) that overcame this barrier. Broken and cracked grains, which typically comprise 20% to 30% of the production and are generally destined for animal feed, can be transformed into rice flour, combined with a binder and other nutrients, and refurbished by extrusion as reconstituted rice grains with the same size, shape, and texture of conventional rice [5]. Fortified rice can be mixed with conventional rice in proportions ranging from 1:50 to 1:200 in order to obtain rice preparations that convey higher concentrations of these nutrients and contribute to the control of nutritional deficiencies, especially among vulnerable groups [6].

The knowledge of the concentrations of nutrients in foods, including vitamins, is essential for the development of eating plans and studies for the analysis of nutrient intake and nutritional adequacy [7].

Thus, it is essential that reliable methods for the analysis of micronutrients are available, especially for vitamins, in fortified rice, pure and mixed to the milled rice before and after the different cooking techniques that are routinely used.

However, the determination of B-group vitamins in foods is very difficult due to the chemical instability, diverse chemical structures and properties of vitamins, and complexity of food matrices. The choice of the method of analysis depends on the accuracy and sensitivity required. In addition, intrinsic differences between the various types of food matrices and the presence of interfering compounds imply the need for the optimization of specific techniques of extraction and analysis [8, 9].

Analytical methods such as microbiological [10] and fluorimetric [11] were used by the Program for Appropriate Technology in Health (PATH) to analyze, respectively, folic acid and thiamine in fortified rice (unpublished data). However, analytical methods for determination of these vitamins in fortified rice, especially fortified rice cooked, using reliable, rapid, economic, and easy applicability methods such as high-performance liquid

chromatography coupled to diode array detector (HPLC–DAD) were not found.

Thus, the present study aimed to optimize and validate methods for the analysis of thiamin and folic acid by high-performance liquid chromatography coupled to diode array detector in fortified rice, pure and mixed to milled rice before and after different cooking techniques used routinely in Brazil.

Materials and Methods

Raw Material

A kind of rice fortified with iron, zinc, thiamin, and folic acid (Ultra Rice®) was used, which was produced from rice flour by a pasta manufacturer located in Indaiatuba, São Paulo, Brazil, after authorization by the Program for Appropriate Technology in Health (PATH). Long, thin milled rice, which was obtained in the local market (Viçosa, Minas Gerais, Brazil), was also used.

Cooking Methods

Three methods were used on a laboratory scale (stir-frying, boiling, and cooking in a microwave oven), and one method was used in a food service (FS) (boiling).

For cooking methods on a laboratory scale, the prepared amount aimed to feed a family of four people. In the FS, the prevision was to attend approximately 666 people, with a total of 4000 meals served daily at lunchtime.

In the method used on the laboratory scale, the mixture of fortified rice and milled rice at a ratio of 1:100 was performed by weighing 2 g of fortified rice and 198 g of milled rice (not washed) on a semidigital analytical balance. Thereafter, the mixing was carried out manually in a glass container. For the cooking method in the FS, 0.6 kg of fortified rice and 59.4 kg of milled rice (previously washed in tap water) were weighed in a semidigital analytical balance. Mixing of the grains was performed after adding to the cauldron.

Optimization of Methods for Extraction and Analysis of Vitamins

Analyses were performed in five repetitions for extraction and in duplicate for the injection of each extract. During the chemical analysis, samples and extracts were kept protected from sunlight and artificial light using amber glassware, foil and blackout curtains, and under the protection of oxygen, through the use of glassware with lids.

For the analysis of vitamins in raw grains of fortified rice and in those mixed to the raw milled rice, the samples were crushed in an analytic mill, whereas for analysis in fortified rice mixed to milled rice after cooking, the samples were homogenized in a domestic food processor.

Extraction of thiamin

To optimize the method of extraction of thiamin, the methodology described by Anyakora et al. [12] was initially used, with the following modifications: (a) adjusting the pH of extraction solution to a range between 2 and 4 (pH range more stable for thiamin) [13]; (b) centrifugation for solid waste decantation and filtration for cleaning the extract; (c) reduction of weight of the samples for about 1 g of raw fortified rice and 2 g of fortified rice mixed to milled rice before and after cooking; and (d) reducing the volume of extraction solution to approximately 22 mL (for definition of these quantities, a shorter centrifugation and the ability of the solvent to extract the compounds of interest were taken into account).

For the extraction of thiamin, about 1 g of raw fortified rice and 2 g of raw and cooked mixture were weighed in a digital analytical balance. Twenty-two milliliters of extraction solution was added, which was composed of sodium salt of hexane sulfonic acid (5 mM) and glacial acetic acid (1%), with the pH adjusted to 3.5 with KOH (10 M). The sample was homogenized in a microgrinder for 3 min, and centrifuged at 1789 g in a centrifuge for 7 min (raw fortified rice), 12 min (raw mixture), and 15 min (cooked mixture). The supernatant was filtered with the aid of a vacuum pump in a Buchner funnel using filter paper nº JP41 J. The filtrate was transferred to a 25-mL volumetric flask, and the final volume was completed with extraction solution. The extract was stored in a tightly closed amber flask under refrigeration (4 °C ± 1 °C) until analysis, which occurred after 1 h at most.

Extraction of folic acid

To optimize the extraction method for folic acid, the same procedures used for centrifugation and filtration in the extraction of thiamin were also used. The extraction solution used was based on the methodology described by Della Lucia et al. [7], using approximately 1 g of raw fortified rice and 3 g of the raw and cooked mixture.

For the extraction of folic acid, about 1 g of raw fortified rice and 3 g of the raw and cooked mixture were weighed on a digital analytical balance. Twenty-two milliliters of extraction solution was added, which was composed of phosphate buffer (0.1 M), at pH 6.0, adjusted with KOH (10 M) [7]. The sample was homogenized in a microgrinder for about 3 min and centrifuged at 1789 g in a centrifuge for 7 min (raw fortified rice), 15 min (raw mixture), and 18 min (cooked mixture). The supernatant was filtered with aid of a vacuum pump in a Buchner funnel, using a filter paper nº JP41 J. The filtrate was transferred to a 25-mL volumetric flask, and the final volume was completed with extraction solution. The extract was stored in a tightly closed amber flask tightly under refrigeration (4 °C ± 1 °C) until analysis, which occurred after 1 h at most.

Preparation of Standards

Stock solutions of standards of folic acid (pteroyl-L-glutamic acid) and thiamin (thiamin mononitrate) were prepared (100 μg mL^{-1}) in HCl 0.1 M and NaOH 0.1 M, respectively, which were used to prepare solutions with different concentrations of the compounds.

Conditions Tested for Analysis by HPLC–DAD

For the analysis of folic acid and thiamin, a high-performance liquid chromatography (HPLC) system was used (Shimadzu SCL 10AT VP), consisting of high-pressure pump (LC-10AT VP), automatic sampler with loop of 500 μL (SIL-10AF), a diode array detector (SPD-M10A), and an oven (Shimadzu CT0-10AVP). To thiamine and folic acid analysis, it was not necessary to connect the oven. However, the room temperature was controlled using an air conditioner (19 ± 1 °C).

Different mobile phase compositions were tested for the analysis of thiamin *(Table I)* and folic acid *(Table II)*. Furthermore, different flow rates

were tested for the analysis of folic acid. Analyses were performed in raw grains of fortified rice and in the raw and cooked mixture.

Table I. Mobile phases and flow tested for the analysis of thiamin in raw fortified rice (A), fortified rice added to raw milled rice (C), and fortified rice added to milled rice after cooking (B and D)

Condition	Sodium salt of sulfonic hexane acid	Glacial acetic acid	Sodium salt of sulfonic hexane acid + glacial acetic acid–methanol	Triethyl-amine	pH	Flow	Injec-tion volume
Anyakora et al. (2008)	5 mM	0.01%	70:30	–	–	1 mL min^{-1}	–
Dong et al. (1988)	4–7 mM	1%	80–87.5:12.5–20	0.1–0.13%	2.8–3.2	–	–
A	5 mM	0.01%	70:30	0.09%	–	1 mL min^{-1}	30 μL
B	6 mM	0.5%	70:30	0.05%	–	1 mL min^{-1}	200 μL
C	5 mM	1%	85:15	0.1%	2.8	1 mL min^{-1}	200 μL
D	5 mM	1%	75:25	0.1%	3.5	1 mL min^{-1}	200 μL

For analysis, the methodologies described by Anyakora et al. [12] and Dong et al. [16] with adaptations were initially used.

The chromatographic condition described below has been optimized for the analysis of thiamin: mobile phase consisting of a solution of sodium salt of hexane sulfonic acid (5 mM) and glacial acetic acid (1%) in ultrapure water–methanol (75:25), with the addition of triethylamine (0.1%) and pH adjusted to 3.5 with KOH (10 M); column RP18 (Phenomenex Gemini, 250 × 4.6 mm, 5 μm) equipped with a guard column (Phenomenex ODS; 4 mm × 3 mm). The analysis was performed in isocratic mode with a flow rate of 1 mL min^{-1} and run time of 10 min. Chromatograms were obtained at 247 nm.

The chromatographic conditions optimized for folic acid, as shown in *Table II*, were based on the procedure described by Anyakora et al. [12] and Dong et al. [16] with minor modifications as follows: mobile phase consisting of solution of sodium salt of heptane sulfonic acid (5 mM) and glacial acetic acid (1%) in ultrapure water–methanol (80:20), with addition of

triethylamine (0.1%) and pH adjusted to 5.0 with KOH 10 M; column RP18 (Phenomenex Gemini, 250 × 4.6 mm, 5 μm), equipped with a guard column (Phenomenex ODS; 4 mm × 3 mm). The analysis was performed in isocratic mode with a flow rate of 0.7 mL min^{-1} and a run time of 12 min. Chromatograms were obtained at 282 nm.

Table II. Mobile phases and flow tested for analysis of folic acid in raw fortified rice (A and D), fortified rice added to raw milled rice (B), and fortified rice added to milled rice after cooking (C, E, and F)

Condition	Sodium salt of sulfonic heptane acid	Glacial acetic acid	Sodium salt of sulfonic heptane acid + glacial acetic acid–methanol	Sodium salt of sulfonic heptane acid + glacial acetic acid–acetonitrile	Triethyl-amine	pH	Flow	Injection volume
Anyakora et al. (2008)*	–	0.01%	–	–	–	–	1 mL min^{-1}	–
Dong et al. (1988)	5 mM	1%	85:15	93:7	0.1–0.13%	2.8–3.2	–	–
A	5 mM	1%	–	90:10	0.05%	3.5	1.2 mL min^{-1}	30 μL
B	5 mM	1%	–	85:15	0.05%	3.0	0.8 mL min^{-1}	200 μL
C	5 mM	1%	–	80:20	0.05%	3.0	0.5 mL min^{-1}	200 μL
D	5 mM	1%	75:25	–	0.1%	3.5	1 mL min^{-1}	30 μL
E	5 mM	1%	70:30	–	0.1%	5.0	0.6 mL min^{-1}	200 μL
F	5 mM	1%	80:20	–	0.1%	5.0	0.7 mL min^{-1}	200 μL

Identification and Quantification of Folic Acid and Thiamin

Qualitative identification of the compounds was performed comparing the retention times obtained for standards and samples analyzed under the same conditions. Furthermore, folic acid and thiamin were identified by co-chromatography, and the purity of the peaks was confirmed by HPLC analysis, observing the presence of a single peak for each standard.

The concentrations of standards were checked by spectrophotometry and corrected using the following equations:

To thiamin: C (μg mL^{-1}) = ABS × 10^4 / $E^{1\%}_{1\ cm}$, where C = concentration; ABS = maximum absorbance (read at 247 nm); $E^{1\%}_{1\ cm}$ = molar absorptivity coefficient (410, solvent HCl 0.1 N) [14].

To folic acid: $A = E^{1\%}_{1\ cm} \times C \times W$, where A = maximum absorbance (read at 282 nm); $E^{1\%}_{1\ cm}$ = molar absorptivity coefficient (23.8, solvent NaOH 0.1 M) [15]; C = molar concentration; W = width of the quartz (1 cm).

Analytical curves were used to quantify the compounds. Solutions were prepared with increasing concentrations of the standards in the respective extraction solutions for each vitamin solution.

The analytical curves for thiamin and folic acid have been prepared taking into account the concentration of these vitamins in raw fortified rice and in fortified rice mixed to milled rice after cooking. Standard solutions were injected in duplicate. For thiamin, six increasing concentrations were injected (0.014 μg mL^{-1}, 0.149 μg mL^{-1}, 0.375 μg mL^{-1}, 3.732 μg mL^{-1}, 5.597 μg mL^{-1}, and 7.463 μg mL^{-1}). For folic acid, seven increasing concentrations were injected (0.00477 μg mL^{-1}, 0.00954 μg mL^{-1}, 0.0477 μg mL^{-1}, 0.09545 μg mL^{-1}, 0.33407 μg mL^{-1}, 0.71587 μg mL^{-1}, and 0.9545 μg mL^{-1}).

The quantification of thiamin and folic acid in raw fortified rice and in fortified rice mixed to milled rice before and after cooking was made from analytical curves and regression equations (thiamin: $Y = 2{,}381{,}473.1982\,X + 122{,}852.4402$; $R^2 = 0.9998$; folic acid: $Y = 4{,}736{,}910.1846\,X - 8543.3495$; $R^2 = 0.9997$). The calculation of the real concentration was obtained from the dilutions made.

Validation of Analytical Methods

Recovery tests of thiamin and folic acid were conducted by the addition of the standards in raw fortified rice and preparations containing fortified rice added to milled rice after cooking. The percentage of recovery was calculated using the formula: % recovery = (final concentration of vitamin) − (concentration of vitamin added) / (initial concentration of vitamin) × 100. Analyses were performed in triplicate.

The determination of the linearity range of compounds was performed by injection, in duplicate, of six increasing concentrations of standard solutions of each vitamin, using the same chromatographic conditions used for the analysis of the extracts. The data obtained for the peak areas were used for linear regression analysis. The coefficient of determination (R^2) obtained in each case was used to assess linearity [17].

The evaluation of the limit of detection (LOD) was performed by successive dilutions of the standards of folic acid and thiamin, followed by determination of the smallest detectable amount as being three times the amplitude of the baseline noise. The limit of quantification (LOQ) was considered as 10 times the LOD [18].

The repeatability test was carried out by extraction and analysis of thiamin and folic acid, in quintuplicate, of raw fortified rice and fortified rice mixed to milled rice after cooking. The evaluation of repeatability was performed by calculating the relative standard deviation (RSD) of the peak areas and retention times of the analyzed components [15].

Experimental Design and Statistical Analysis

To analyze the content of thiamin and folic acid, a completely randomized design was used with four treatments and five repetitions for the extraction of thiamin and folic acid; the analysis was performed in duplicate (α = 5%). Data were subjected to analysis of variance (ANOVA), and means were compared using the Duncan's test. Statistical analysis was performed using the Statistical Analysis System [19], licensed to the Federal University of Viçosa.

Results and Discussion

Optimized Methods for Analysis of Thiamin and Folic Acid

Thiamin

The typical chromatograms of conditions used for analysis of thiamin are shown in *Fig. 1*. Initially, the condition described by Anyakora et al. [12] for the analysis of B-group vitamins in cereals was tested: the mobile phase was composed of solution of sodium salt of hexane sulfonic acid (5 mM) and glacial acetic acid (0.01%)–methanol (70:30). In this condition, a compound was eluted very close to thiamin, interfering with the quality and resolution of the analysis (condition A = *Fig. 1A*), invalidating its use.

Similar results to that described above were observed in condition B *(Fig. 1B)*, whose mobile phase was composed of a solution of sodium salt of sulfonic hexane acid (6 mM) and glacial acetic acid (0.5%)–methanol (70:30), with the addition of triethylamine (0.05%). In this condition, an interfering compound was also eluted close to thiamin. The increase in the concentra-

tion of the ion-pair reagent and glacial acetic acid did not change the retention time of thiamin in relation to the condition A.

From condition C, the concentration of the ion-pair reagent (5 mM), in glacial acetic acid (1%) and triethylamine (0.1%) was kept constant according to the optimal conditions for the analysis of water soluble vitamins, as described by Dong et al. [16]. The proportion of methanol–sodium salt of sulfonic hexane acid and glacial acetic acid changed, as did the pH of the mobile phase. In condition C *(Fig. 1C)*, a mobile phase composed of a solution of sodium salt of sulfonic hexane acid and glacial acetic acid–methanol (85:15), pH 2.8 was used, resulting in a peak of thiamin with good resolution. However, as the retention time of thiamin was relatively long (over 10 min), we decided to proceed using the optimization condition for D, from which a shorter time was obtained in the chromatographic analysis of this vitamin. This reduction was achieved by increasing the volume of methanol (organic modifier) and varying the pH of the mobile phase.

The optimal condition for analyzing thiamin was the condition D *(Fig. 1D)*, in which the mobile phase was composed of a solution of sodium salt

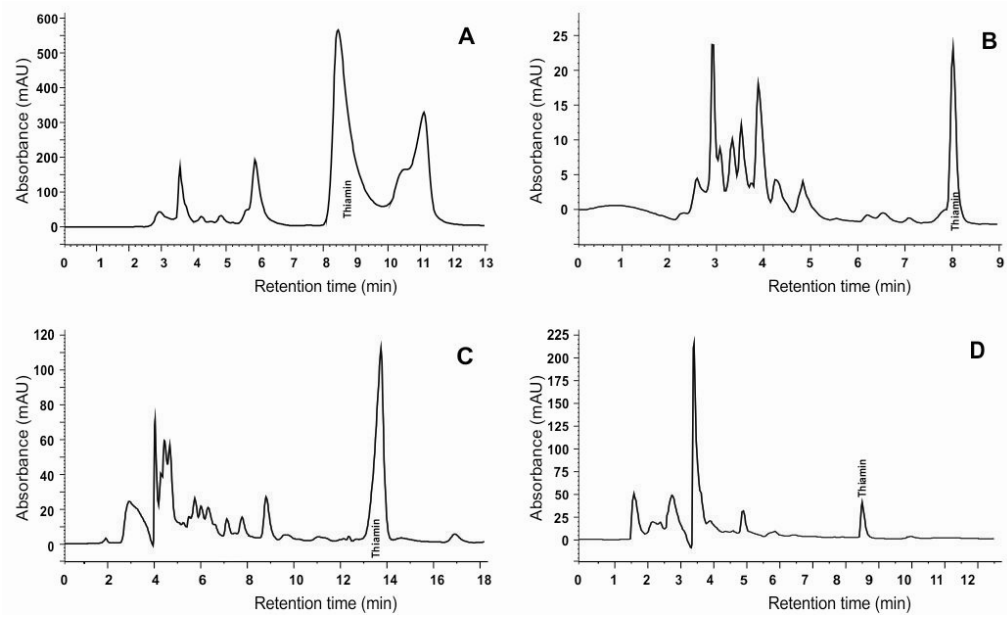

Fig. 1. Typical chromatograms of the conditions tested for analysis of thiamin in raw fortified rice (A), fortified rice mixed to milled rice before cooking (C), and fortified rice mixed to milled rice after cooking (B and D). The chromatographic conditions are given in *Table I* (condition A = *Fig. 2A*; condition B = *Fig. 2B*, condition C = *Fig. 2C*; condition D = *Fig. 2D*)

of sulfonic hexane acid (5 mM) and glacial acetic acid (1%)–methanol (75:25) with the addition of triethylamine (0.1%) and pH adjusted to 3.5 with KOH (10 M). This condition showed good resolution for the thiamin peak and a reduced chromatographic run time, reflecting positively the reliable quantification of the vitamin with a short analysis time. The lowest peak area of thiamin in *Fig. 1D* compared to the others is due to the fact that this condition was tested not only for raw fortified rice, in which we would expect a higher peak area, but also for fortified rice mixed to milled rice after cooking. That is, although the thiamin concentration is lower (further diluted) since fortified rice is added to the milled rice at a ratio of 1:100, this vitamin still suffered the impact of cooking.

Folic acid

The typical chromatograms of conditions used for analysis of folic acid are shown in *Fig. 2*. In the conditions A, B, and C, acetonitrile was used as the organic modifier. In condition A *(Fig. 2A)*, in which the mobile phase was composed of a solution of the sodium salt of heptane sulfonic acid (5 mM) and glacial acetic acid (1%)–acetonitrile (90:10) with the addition of triethylamine (0.05%), pH 3.5 adjusted with KOH (10 M) and a flow rate of 1.2 mL min^{-1}, a good resolution, was obtained for the folic acid peak. However, this condition was not used due to the relatively long chromatographic run (approximately 20 min).

In condition B *(Fig. 2B)*, a mobile phase composed of a solution of sodium salt of heptane sulfonic acid (5 mM) and glacial acetic acid (1%)–acetonitrile (85:15) with the addition of triethylamine (0.05%), pH 3.0 adjusted with KOH (10 M) and a flow rate of 0.8 mL min^{-1} was used, which resulted in a reduction of the retention time of folic acid to about 8 min. However, some interfering compounds co-eluted with this vitamin, leading to the condition not being used. In condition C, the concentration of acetonitrile in the mobile phase was increased from 15% to 20% and the flow rate was reduced from 0.8 to 0.5 mL. As a result, a nearly complete separation of folic acid was observed, but this was not yet deemed satisfactory *(Fig. 2C)*.

In conditions D, E, and F, methanol was used as the organic modifier instead of acetonitrile. In condition D *(Fig. 2D)*, in which the mobile phase was composed of solution of sodium salt of heptane sulfonic acid (5 mM) and glacial acetic acid (1%)–methanol (75:25), with the addition of triethylamine (0.1%), pH 3.5 adjusted with KOH (10 M) and a flow rate of 1 mL min^{-1} was used; although the peak of folic acid showed good resolution, the chromatographic run time was much longer (>24 min).

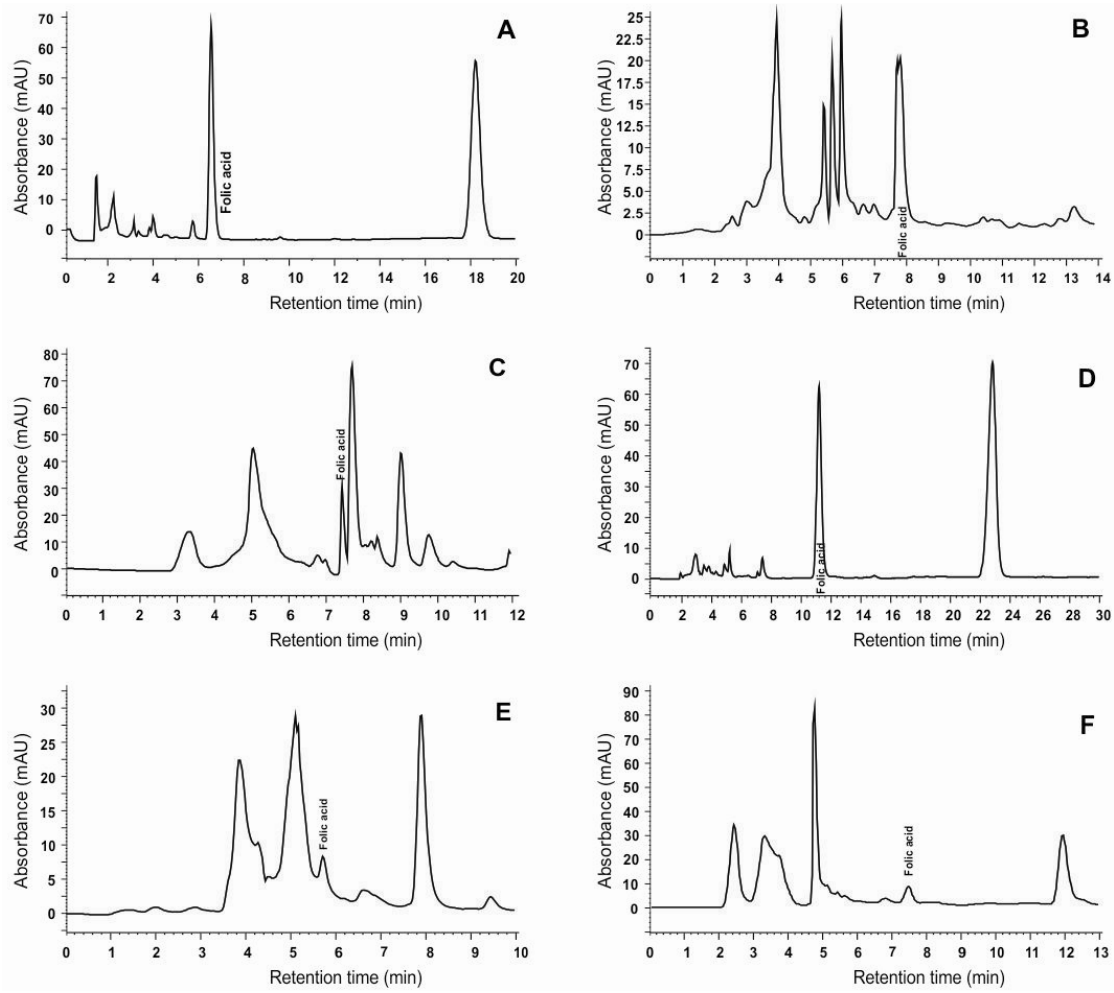

Fig. 2. Typical chromatograms of the conditions tested for analysis of folic acid in raw fortified rice (A and D), fortified rice mixed to milled rice before cooking (B), and fortified rice mixed to milled rice after cooking (C, E, and F). The chromatographic conditions are shown in *Table II* (condition A = *Fig. 3A*, condition B = *Fig. 3B*; condition C = *Fig. 3C*; condition D = *Fig. 3D*; condition E = *Fig. 3E*; condition F = *Fig. 3F*)

Condition E differs from condition D by the use of 30% methanol in the mobile phase, a pH of 5.0, and a flow rate of 0.6 mL min^{-1}, leading to a reduction in the retention time of folic acid by approximately 5.5 min. However, other compounds eluted close to this vitamin, impairing the resolution and quality of analysis *(Fig. 2E)*.

Finally, the best condition (F) for the analysis of folic acid was obtained when the concentration of methanol in the mobile phase decreased to 20% and the flow rate increased to 0.7 mL min^{-1}, verifying good resolution for the peak of interest and a shorter chromatographic run time (13 min) *(Fig. 2F)*. This condition allowed the reliable quantification of folic acid in samples and reduced analysis time. As described for thiamin, the lower peak area of folic acid in relation to others is due to the fact that the sample of fortified rice was mixed with milled rice and further subjected to cooking, causing a reduction in the content of this vitamin.

Content of Thiamin and Folic Acid in Fortified Rice before and after Cooking

The thiamin content (dry basis) was statistically superior in the preparation cooked in a microwave oven (0.97 mg/100 g) compared to other cooked preparations. The content of folic acid (dry basis) did not show a significant difference among preparations cooked in a microwave oven, stir-frying, and boiling (mean of 0.20 mg/100 g) and was statistically superior to the content found in the preparation cooked in an FS (0.17 mg/100 g) *(Table III)*.

Table III. Mean content (mg/100 g) of thiamin and folic acid in grains of raw fortified rice and fortified rice added to milled rice before and after cooking (raw and cooked mixture)

Sample	Fresh basis		Dry basis	
	Thiamin (mean ± SD)	Folic acid (mean ± SD)	Thiamin (mean ± SD)	Folic acid (mean ± SD)
Raw fortified rice	149.30 ± 1.93	19.35 ± 0.10	161.74 ± 2.09	20.97 ± 0.22
Fortified rice added to raw milled rice	1.49 ± 0.38	0.24 ± 0.01	1.89 ± 0.62	0.27 ± 0.009
Fortified rice added to milled rice cooked in a microwave oven	0.36 ± 0.31	0.077 ± 0.004	0.97 ± 0.08^{a}	0.208 ± 0.01^{a}
Fortified rice added to milled rice stir-fried and boiled	0.25 ± 0.007	0.081 ± 0.002	0.71 ± 0.02^{c}	0.227 ± 0.007^{a}
Fortified rice added to milled rice boiled	0.26 ± 0.01	0.070 ± 0.002	0.79 ± 0.05^{b}	0.213 ± 0.007^{a}
Fortified rice added to milled rice cooked in food service	0.31 ± 0.05	0.074 ± 0.003	0.74 ± 0.13^{bc}	0.174 ± 0.008^{b}

Means of cooked preparations (dry basis) followed by the same letter in the column do not differ by Duncan's test (α = 5%); SD = standard deviation.

In the literature, no data is available regarding the content of folic acid and thiamin in this fortified rice, either raw or cooked (using the same cooking methods on a laboratory scale and in a food service). However, according to PATH [20] (unpublished data), the amount of folic acid and thiamin added to raw fortified rice (fresh base) is, on average, 22.2 mg/100 g and 164.6 mg/100 g of fortified rice, respectively. In our study, we found values very similar to those reported previously, with approximately 19.35 mg of folic acid and 149.3 mg of thiamin/100 g of fortified rice (fresh base). These data reflect reliability for the methodologies optimized for the analysis of thiamin and folic acid in fortified rice.

Validation of Analytical Methods for Folic Acid and Thiamin

Linearity

Folic acid and thiamin showed excellent linearity in the concentration ranges used (thiamin: between 0.014 μg mL^{-1} and 7.463 μg mL^{-1}, folic acid: between 0.00477 μg mL^{-1} and 0.95 μg mL^{-1}). The coefficient of determination for thiamin and folic acid was 0.9998 and 0.9997, respectively. Studies analyzing these vitamins found coefficients of determination for thiamin of 0.9957 [21], 0.9967 [22], 0.9998 [23], and 0.9950 [24] and for folic acid of 0.9838 [22], 0.9997 [25], 0.9980 [26], and 0.9999 [27].

Recovery

The two methodologies presented excellent percentages of recovery of the standards added to the samples, ranging from 82 to 104% for thiamin and 87 to 96% for folic acid *(Table IV)*. These values are within the range of 80 to 110% set by the AOAC [28]. Studies were found in the literature with similar percentages for the recovery of thiamin, of between 73 to 106% [21, 23, 24] and 81 to 103% for folic acid [25, 27, 29].

Table IV. Percentage of recovery of standards of thiamin and folic acid added to raw fortified rice and fortified rice added to milled rice before and after cooking (raw and cooked mixture)

Vitamin	Sample	Initial concentration ($\mu g\ g^{-1}$)[a]	Standard added ($\mu g\ g^{-1}$)	Final concentration ($\mu g\ g^{-1}$)	Recovery[b] (%)
Thiamin	Raw fortified rice	487.60	254.80	776.70	104.62
	Raw mixture	3.90	3.51	6.12	82.59
	Fortified rice added to stir-fried and boiled milled rice	0.40	0.70	1.09	99.09
	Fortified rice added to milled rice cooked in a microwave oven	3.69	1.93	5.26	93.59
Folic Acid	Raw fortified rice	197.70	50	228.60	92.28
	Fortified rice added to stir-fried and boiled milled rice	0.78	0.31	1.03	94.49
	Fortified rice added to boiled milled rice	0.76	0.14	0.87	96.66
	Fortified rice added to the milled rice cooked in a micro-wave oven	0.64	0.32	0.84	87.5

[a]Mean of samples in triplicate.

[b]% of recovery = (final concentration of vitamin) − (added amount of vitamin) / (initial concentration of vitamin) × 100.

Repeatability

The results obtained in this study suggest the reliability of the analysis conditions used in this study *(Table V)*, since the relative standard deviations for the peak area and retention times obtained for each sample were below the limit of 10% [18].

Table V. Repeatability of the analytical methods of thiamin and folic acid in raw fortified rice and fortified rice mixed to milled rice before and after cooking (raw and cooked mixture)

Compound	Sample	RSD of the peak area (%)	RSD of the retention time (%)
Thiamin	Raw fortified rice	2.2	0.1
	Fortified rice added to milled rice boiled	3	0.4
	Fortified rice added to milled rice stir-fried and boiled	7.4	1.2
Folic acid	Raw fortified rice	3.3	0.1
	Fortified rice added to milled rice boiled	8.2	0.1
	Fortified rice added to milled rice stir-fried and boiled	3.9	0.3
	Fortified rice added to milled rice cooked in a microwave oven	4.6	0.9

RSD: relative standard deviation (%)

Limits of detection and quantification

The LOD for thiamin and folic acid found in this study was 0.00193 μg and 0.000934 μg, respectively. The value found for thiamin was lower than the limits of detection found by Hucker et al. [24], Presoto and Almeida-Muradian [25], and San José Rodriguez [22], being 0.02 μg, 0.03 μg, and 0.06 μg, respectively. In relation to folic acid, values of 0.003 μg, 0.0013 μg, and 0.06 μg were found by Boen et al. [26], Lebiedziñska et al. [27], and Alaburda et al. [28], respectively.

The LOQ was 0.0193 μg for thiamin and 0.00934 μg for folic acid. These values show that the optimized methods allow the detection of very small concentrations of thiamin and folic acid in raw fortified rice as well in fortified rice added to milled rice.

Conclusion

The optimized methods demonstrated reliability and sensitivity in the detection and quantification of folic acid and thiamin in fortified rice before and after different cooking methods. The optimized methods showed excellent recovery rates (ranging from 82 to 104% for thiamin and 87 to 96% for folic acid), good repeatability (since the relative standard deviations for the peak area and retention times obtained for each sample were below the limit of 10%), and low limits of detection and quantification, showing high sensitivity and excellent linearity (for thiamin: between 0.014 μg mL^{-1} and 7.463 μg mL^{-1}; for folic acid: between 0.00477 μg mL^{-1} and 0.95 μg mL^{-1}). The coefficient of determination for thiamin and folic acid was 0.9998 and 0.9997, respectively. Furthermore, the methods were performed in isocratic mode, with short run time (<13 min), reflecting positively on the economy of reagents and analysis times.

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