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Abstract. The method of analysis was standardized, and the folic acid content was determined in samples of fortified white rice (Z, G and D) and unfortified (E and O). The analysis was performed in triplicate in samples of 2.0000 g, hydrolysis was performed with phosphate buffer pH 8.82 for one hour and then lowered to pH 7.00 for enzymatic treatment with takadiastase at 65ºC. The extracts were filtered by gravity, purified with nylon syringe filters and ion exchange cartridges (solid phase extraction using strong anion exchange cartridges). The concentration in mg folic acid/kg of rice, in each extract was determined by ultrafast liquid chromatography, with average values for Lot1 of the fortified rice samples, of 44.03, 51.95, 38.08, and unfortified samples of 31.51 and 21.94, and with respect to Lot2 of 41.26, 41.27, 34.54 for fortified ones and 24.92 and 20.94 for non-fortified ones, respectively. The concentration of folic acid obtained by filtration through syringe filters was higher than in the extracts purified by solid phase extraction using strong anion exchange cartridges, evidencing loss of the analyte. The recovery of folic acid in the samples doped and purified by syringe filters was around 65.00%, while by solid phase extraction using strong anion exchange cartridges 43.00%. There is evidence of variation in the folic acid content between different batches of rice of the same brand.

1. Introduction

Rice is one of the most consumed foods in Colombia, Ecuador, Peru and Venezuela, making it a potential vehicle for fortification. It is consumed by 73.8% of the Colombian population, in Venezuela, it is the second most consumed food by the population, after the corn flour. In Peru, white rice is the cereal most consumed by women and children and in Ecuador it is the first food on the monthly consumption expenditure list. Currently, Colombia and Venezuela are voluntarily fortifying rice. In Colombia, some companies fortify with vitamin A, folic acid, and riboflavin; while in Venezuela other micronutrients are added such as thiamine, iron and niacin [1].

Some technological processes, such as the refining of flours and cereals in general, cause significant losses of minerals and vitamins with respect to the content of the whole grain. The elimination of the fat of many foods to reduce their caloric value also leads to the loss of fat-soluble vitamins, such as A or D; therefore, there is a need to fortify food, including rice. Thus, through enrichment, the initial levels of nutrients lost during the handling of food are restored or even exceeded [2]. The processes for the fortification of rice are varied, which can be subjected to processes such as hot extrusion, cold extrusion, coating and sprinkling [3]. Rice is one of the foods with greater presence in the diet of Colombians, so it is important that it meets the appropriate nutritional requirements.

Among the water-soluble vitamins is folic acid (FA) (vitamin B9), also known as folate or folacin, has been shown in several investigations on metabolism, which has important absorption characteristics

in the defense of cancer, heart disease, cerebrovascular accidents and congenital defects. The folate in food is unstable, it is lost during cooking and in foods stored at room temperature as in the case of green leafy vegetables they lose up to 70% of their content in three days [4].

For the analysis of FA, spectrophotometric methods are used, however, high resolution liquid chromatography (HPLC) in reverse phase is one of the most used methodologies for the determination of folic acid, being reliable and quick since it allows to analyze several types of folates with high sensitivity and selectivity [5].

Among the works that report the analysis of vitamin B9 in food, through ultrafast liquid chromatography (UFLC) are found, determination of folate in cereal-grain food products by reversed-phase liquid chromatography [6], determination of folic acid in vegetables [7], evaluation of two quantification methods HPLC and UFLC for water-soluble vitamins [8], determination of iron and folic acid in a milk-type yogurt [9] and the determination of folic acid in flours [10].

The objective of this work was the standardization of the method of analysis of folic acid, which was used for the quantification of the vitamin present in samples of white rice, marketed in chain stores, which report in their nutritional values fortification with the vitamin, it was also applied to samples that did not report fortification, in order to determine if these samples contained the vitamin. The statistical treatment of the data obtained in the analysis performed by UFLC with detection by arrangement of diodes was carried out with software R version 3.2.2 [11].

2. Materials and Methods

2.1. Standardization of the method

The analysis of vitamin B9 in white rice was based on the procedure described by [12], in which a modification was made to the procedure carried out by [11]. For the standardization of the method of analysis, a certified folic acid standard (Sigma Aldrich) was used, with which the calibration curve was elaborated with patterns between 1.000 ppm and 4.000 ppm, and to determine quality parameters of the method as limit of detection (LOD) and quantification (LOQ) were prepared concentration patterns from 0.1000 ppm to 1.000 ppm folic acid.

2.2. Selection of samples

The fortified samples (Z, G and D) and the unfortified samples (E and O) of different brands marketed in the metropolitan area of San José de Cúcuta, Norte de Santander, Colombia, were selected based on the nutritional report of each of them and they were acquired at random in chain stores with a presence in the region. The production lots purchased for the analysis had distribution dates greater than one month to guarantee the difference between batches of each brand and thus make a comparison of the vitamin content between brands and between production batches. The samples that report fortification (Z, G and D) are nationally distributed brands, two of them with their main domicile in the metropolitan area of San José de Cúcuta and the non-fortified ones (E and O) correspond to the chain store's own brands that are present in the region and nationally.

2.3. Analysis and quantification of vitamin B9 in white rice

2.3.1. Sample preparation. Each sample of each batch of white rice to be analyzed was crushed by a food processor, pulverized in porcelain mortar, passed through a 20 mesh stainless steel screen and homogenized; 2.0000 g were taken in triplicate from each of the samples and taken to continuous stirring in heating plate at 250 rpm, with 45 mL of K_2HPO_4 (Merck) 0.1000 M solution at pH between 8.00 and 9.00 for one hour. After the stirring time was removed from the plate and the pH was lowered to 7.00 with HCl (Merck) 0.5000 M, at room temperature. It should be mentioned that the samples must be managed under controlled light conditions to avoid loss of the analyte by photoluminant reaction [12].

2.3.2. Enzymatic hydrolysis. The sample at pH 7.00, was added with 1 mL of Takadiastase (Sigma Aldrich) at a concentration of 25 mg/mL per gram of sample, after the addition of the enzyme the sample was heated to 65 °C and continuous agitation at 250 rpm for 1 hour; after this time the enzyme was inactivated increasing the temperature to 90 °C. During the hydrolysis process temperature monitoring was carried out to avoid inhibition in the activity of the enzyme, as well as the loss of the vitamin due to excessive and prolonged heating. Once the enzyme was inactivated, the sample was adjusted to a volume of 50.00 mL with 0.1000 M K_2HPO_4 solution.

2.3.3. Purification of extracts obtained in enzymatic hydrolysis. After the enzymatic hydrolysis, the samples were filtered by gravity with Whatman No. 40 filter paper, then the filtrates were purified in two ways: one by additional filtration with 0.45 µm Nylon syringe filter and another by solid phase extraction (SPE) using strong anion exchange cartridges (SPE-SAX). For the SPE, the cartridges were conditioned with 3.00 mL of hexane (Merck), 3.00 mL of methanol (Merck), and 5.00 mL of K_2HPO_4 (Merck) 0.1000 M solution at pH 7.00, then 4.00 mL of the filtrate of each sample was passed, then the cartridges were washed with 2.00 mL of 0.0200 M phosphate buffer solution and finally the FA of each sample was eluted at a rate of 0.6 mL/min with a solution of CH_3COONa 0.1000M of pH 4.50 containing 5.0% w/v Na_2HPO_4 and ascorbic acid at 0.05% p/v, the extracts resulting from the elution were stored in amber vials.

2.3.4. Chromatographic analysis of the extracts. Extracts containing FA obtained from rice samples were analyzed in a SHIMADZU Prominence series UFLC chromatograph, with SIL-20AHT autosampler, CTO-20A column heater, SPD-M20A diode array detector and CBM-20A control module, provided with the software Labsolutions of Shimadzu. A 0.01%w/v trifluoroacetic acid: acetonitrile (85:15) solution was used as a mobile phase, with flow rate of 1.4 mL/min to isocratic elution in reverse phase, using C18 column of 4.6 mm x 50 mm and precolumn C18 brand Shimadzu. The detection of FA was performed at a wavelength of 280 nm. Each sample was analyzed in triplicate, both the extracts obtained by syringe filters, and by SPE.

2.3.5. Analysis of data. The data obtained both in the standardization of the method, and in the analysis of the samples, were analyzed to determine the precision of the method by determining the standard deviation and the coefficient of variation of the repeated data for each sample. Using the 3.2.2 version of software R, the comparison of the concentration of folic acid present in all the samples was made, and the analyte concentration between batches of the same brand was also compared, in order to observe if it was present or no, significant difference in vitamin content, taking into account that for probability values (p)> 0.05, it is considered that there is no significant difference between the data.

3. Results

3.1. Standardization of the method

Hernandez Hurtado [12], describes in the validation process a linear interval between 0.1000 ppm and 4.000 ppm of folic acid, however, with the calibration curve elaborated in this work, the analyte was not detected in the 0.1000 ppm standard, therefore FA patterns with higher concentrations were analyzed, observing that the linearity of the method was in the concentration range of 0.2500 ppm to 4.000 ppm, with a correlation coefficient of 0.9979 as shown in Figure 1.

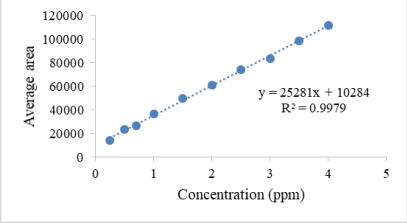

Figure 1. Folic acid calibration curve with concentrations of 0.2500 ppm - 4.000 ppm.

The precision in the measurements of the calibration standards by UFLC, was evidenced with low values of variation coefficients (% CV) (between 0.28 and 3.94%), as shown in Table 1.

Table 1. Data of the calibration curve of FA by UFLC.

Concentration (mg FA/kg of rice)	Average area	Std dev	%CV
0.2500	14,563.67	573.64	3.94
0.5000	23,510.33	851.51	3.62
0.7000	27,008.33	873.11	3.23
1.0000	37,143.00	428.38	1.15
1.5000	50,257.67	807.66	1.61
2.0000	60,919.67	636.58	1.04
2.5000	74,554.33	1,382.55	1.85
3.0000	83,429.00	763.17	0.91
3.5000	98,515.33	812.66	0.82
4.0000	112,000.00	311.88	0.28

3.2. Analysis and quantification of vitamin B9 in white rice

3.2.1. Analysis of data. By means of the equation of the calibration curve of the method (see Figure 1), the concentration of FA in each of the samples, expressed in mg FA/kg of rice, was calculated both for the samples purified by nylon syringe filter and for those obtained by SPE-SAX, the average values, the standard deviation (std dev) and the% CV obtained for each of the brands are shown in Table 2. Each sample of rice from each batch and each extract obtained by the two purification techniques were analyzed in triplicate in the UFLC, obtaining a general matrix of 180 data for all samples analyzed.

As shown in Table 2, for the average values of the FA concentration in the samples purified by syringe filters there are significant variations between each analyzed batch of the same brand, the coefficients of variation range from very low values (1.23% for E1) to values that come out of the ranges acceptable (10.69% for D2 and 10.12% for O1).

For samples purified by SPE-SAX, average values of the concentration are reported with significant variations between each batch analyzed, the variation coefficients mostly exceed the acceptable values, since they exceed 10%, as shown in Table 2. The great dispersion that occurs in the results, could be attributed to the greater manipulation of the extract that must be done with this purification technique.

Table 2. Average values of AF concentration in the extracts obtained by purification by syringe filters and by SPE-SAX.

Rice brand	Lot	Syringe filters			SPE-SAX		
		Concentration (mg FA/kg of rice)	Std dev	%CV	Concentration (mg FA/kg of rice)	Std dev	%CV
Z	Z1	44.4641	3.1224	7.02	24.5275	3.1224	12.73
	Z2	41.6877	3.9829	9.55	21.7511	2.9206	13.59
G	G1	52.3740	3.4119	6.51	32.4374	3.4119	10.51
	G2	41.7060	3.0879	7.40	21.7694	3.0879	14.18
D	D1	38.5162	2.4972	6.48	18.5796	2.4972	13.44
	D2	34.9728	4.0908	10.70	14.1572	1.8706	13.04
E	E1	31.9465	0.3929	1.23	12.0099	0.4928	7.25
	E2	25.3570	1.9956	7.87	5.4204	0.3303	7.39
O	O1	22.3834	2.2674	10.13	2.7524	0.4269	11.75
	O2	21.3834	1.9573	9.15	2.5456	0.3537	12.84

Figure 2 shows the chromatographic profiles of the FA pattern of 4.0000 ppm and those obtained for the extracts of the samples of lot 1 of each of the five brands analyzed. The retention time (tR) of the AF was presented at 0.78 min, a short time that speeds up the analysis times and allows to reduce the volume of solvent used.

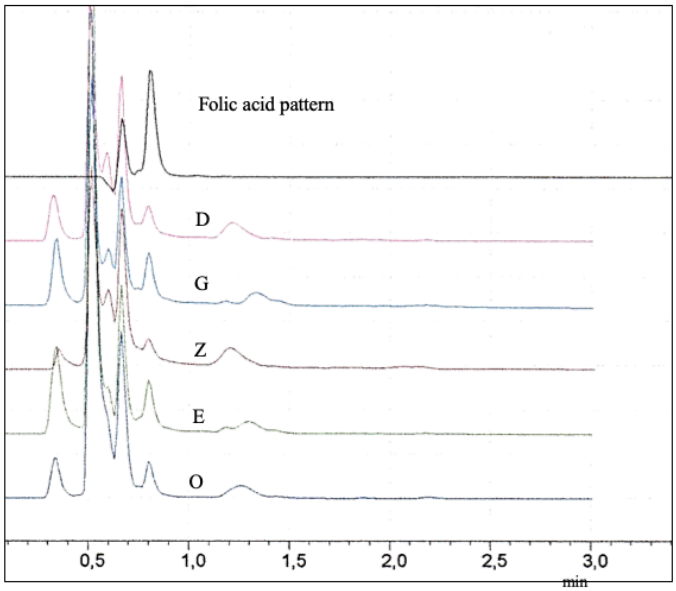

Figure 2. Chromatograms of the folic acid pattern of 4.0000ppm and of the five rice brands analyzed.

The unfortified samples (E1, E2, O1 and O2) were enriched with 2.0000 ppm of FA and processed under the same conditions as the samples without adding and purified by the two methods used. The concentration of AF reported by the purification techniques, as well as the recovery percentage, are shown in Table 3.

Table 3. FA concentration and recovery percentage in extracts purified by syringe filters and SPE-SAX in added samples.

Rice brand	Lot	Concentration (mg FA/kg of rice)		%Recovery	
		Syringe filter	SPE-SAX	Syringe filter	SPE-SAX
E	E1	65.2445	30.6336	66.60	43.73
	E2	56.5904	25.4616	62.47	42.43
O	O1	55.1445	24.3792	65.33	41.50
	O2	53.5180	23.8515	64.27	42.19

3.2.2. Analysis of the data using the R software. The concentration of FA in the samples of all the brands analyzed was compared using software R version 3.2.2., obtaining the box diagram shown in Figure 3.

As shown in Figure 3, the data obtained from the extracts analyzed by filtration by syringe, although they present significant differences, both for the fortified and unfortified samples ($p = 0.015$), these values are not very dispersed. In Table 2, it is observed that the brands that report FA fortification do not show a significant variation in the concentration of the vitamin; however, if there is a slight variation between batches of the same brand. For the unfortified samples a very similar concentration is observed, which corresponds to the natural content of the vitamin in the grain.

In the box diagram of Figure 4, the concentration of FA present between lots 1 of all the brands is compared, as observed there is a marked difference between lots 1 of the unfortified samples (E1 and

O1) with respect to the fortified (G1, D1 and Z1), this is corroborated when examining the value corresponding to the reported concentration for lots 1 shown in Table 2.

Figure 5 shows the box diagram in which the concentration of the FA is compared between the samples of the lots 2 of all the marks, a difference between the lots 2 of the unfortified samples is observed (E1 and O1). Regarding the fortified ones, the G2 and Z2 samples show a similarity of FA, while the D2 shows a difference with respect to the most outstanding, with a lower concentration.

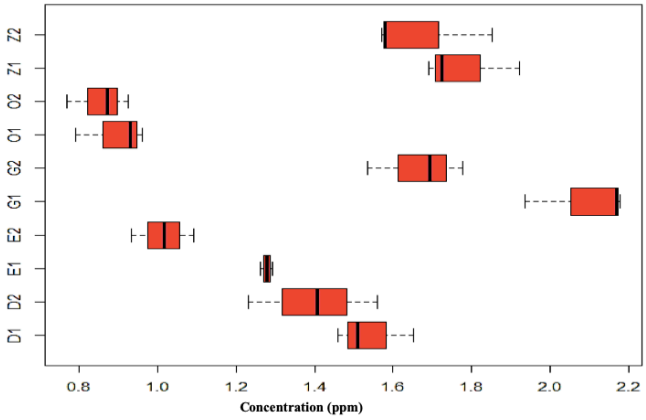

Figure 3. Comparison of folic acid concentration (ppm) between batches of different brands of fortified rice (Z, G and D) and unfortified (E and O).

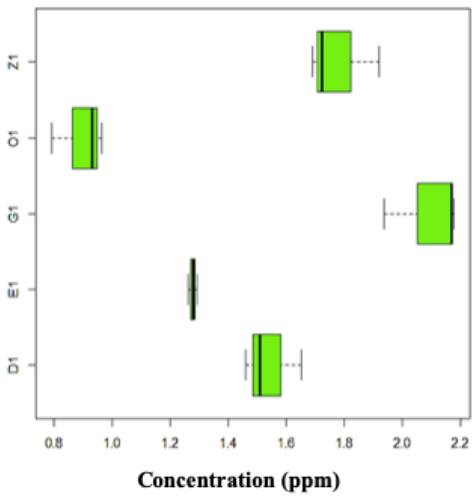

Figure 4. Comparison of folic acid concentration (ppm) between lots 1 of the different brands of rice.

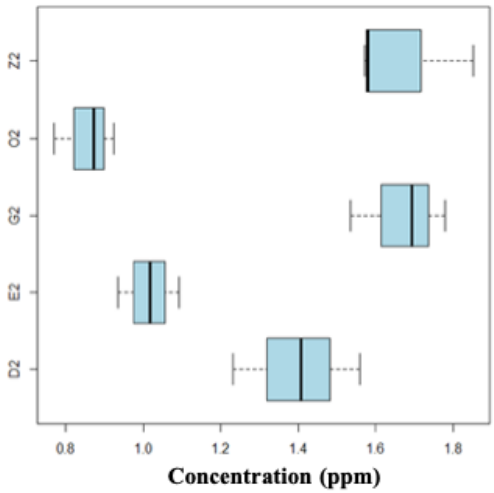

Figure 5. Comparison of folic acid concentration (ppm) between lots 2 of the different brands of rice.

4. Conclusions

For the analysis of folic acid with the standardized method it was established that the linear range is from 2.500 ppm to 4.000 ppm, with a correlation coefficient of 0.9979, establishing the LOD at 2.5000 ppm since at lower concentrations the linearity of the method is lost.

When comparing the content of folic acid between batches of the same brand, in the fortified samples no significant difference is observed, something similar is observed in the non-fortified samples

although the folic acid content of these samples corresponds to the natural content of the vitamin in the grain, however, between fortified and unfortified brands, the difference in content is not so significant, which leads us to think that the amount of folic acid added to these samples is not very high, or in the storage process there are losses of the analyte.

The standardized method sought to improve the cleaning conditions of the sample to avoid interfering substances in the analysis, so two purification techniques were used, filtering by Nylon syringe filters and by SPE-SAX. Between the two techniques, the filtration by syringe presented better results since no loss of analyte was generated by having a percentage of recovery in samples added of 63% while the purification by SPE-SAX, showed a recovery of 43% which indicates loss of the analyte during the process.

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