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Determination of Thiamine in Wheat Flours Using a Validated Isocratic HPLC-Fluorescence Method Previously Optimized by Box–Behnken Design

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Abstract The main purpose of this work was to optimize and validate a new isocratic high-performance liquid chromatography method for thiamine analysis in wheat flours. Thiamine was previously derivatized into thiochrome and determined by fluorescence detection (excitation wavelength, 366 nm; emission wavelength, 435 nm). The mobile phase consisted of buffer phosphate (0.02 M) and organic modifier (methanol 88 %; acetonitrile 12 %). Optimization was done using a Box–Behnken design (three factors at three levels) where organic modifier proportion (A), varying from 20 to 90 %, mobile phase pH (B), varying from 5.8 to 7.8, and flow rate (C), varying from 0.5 to 1 mL min^{-1}, were the studied variables. Peak width was the studied response. Optimal chromatographic conditions that minimized response were A=90 %, B=6.00, and C=1 mL min^{-1}. Using optimal conditions, thiochrome extractions with isobutanol and solid-phase extraction (SPE) employing styrene-divinylbenzene adsorbent cartridges were compared. Method validation, using thiamine standard solutions and both types of thiochrome isolation, included linearity studies, limits of detection and quantification, and calibration and analytical sensitivity quantifications. Standard addition method was employed to quantify thiamine in wheat flours. Good precision results were obtained by both extraction methods. Recoveries ranged from 63 to 65 % for isobutanol and 84 to 101 % for SPE, including sample extraction, thiamine derivatization, thiochrome purification, and chromatographic separation. Taking into account better obtained SPE recoveries, acceptable precision, and isobutanol irritant action on the eyes and skin of operator, SPE using styrene-divinylbenzene adsorbent could be chosen for routine thiamine analysis in wheat flour samples.

Keywords Thiamine · Thiochrome solid-phase extraction · Chromatographic optimization

Introduction

Thiamine also known as vitamin B_1 or aneurin is present not only in almost all cereals, but also in pork and cow meats. It helps human body's cells convert carbohydrates into energy as a coenzyme involved in its cellular metabolism. Thiamine is also essential for functioning of the heart, muscles, and nervous system. It can be found in foods both as free or phosphorylated thiamine (mono-, di-, and triphosphate thiamine). Phosphorylated thiamine is mainly found in meats, whereas free thiamine is almost the only one present in cereals (Ndaw et al. 2000).

In spite of many thiamine analytical methods which have been developed and published, there is still a necessity of a rapid, safe, and reliable determination of vitamin B_1 because of governmental regulations about food nutritional labeling. Vitamin B_1 can be easily determined in pharmaceutical products using high-performance liquid chromatography (HPLC)-UV methods.

In foods, more specific techniques should be applied due to the presence of many interfering compounds, being thiamine derivatization to thiochrome the most common methodology. San José Rodriguez et al. (2012) published an isocratic HPLC-UV method for thiamine analysis, faster than usual, without thiamine derivatization to thiochrome. The mentioned methodology could be used only in fortified cereal foods because of its rather high limit of quantification. So, it would be necessary to develop a reliable and faster methodology to

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analyze lower thiamine concentration such can be found in intermediate extraction rate wheat flours.

Determination of thiamine in different foodstuffs including cereals, flours, and meats has been widely performed by thiochrome determination, as was already mentioned, directly or by HPLC with fluorescence detector, which is highly recommended because of its lower limit of detection (Lynch and Young 2000; Tang et al. 2006; Arella et al. 1996; Moore and Dolan 2003; Poel et al. 2009). In this methodology, thiamine has to be derivatized to thiochrome in order to be detected. Derivatization reaction must be carried out in a highly concentrated alkaline medium, and then, thiochrome is usually extracted by liquid–liquid extraction in isobutanol. This solvent is employed also in spectrofluorometric vitamin B_1 determination in foodstuffs (AOAC 1995). As isobutanol is a moderate skin but severe eye irritant, some authors have extracted and also concentrated thiochrome using solid-phase extraction (SPE) utilizing different cartridges (Ollilainen et al. 1993).

As previously mentioned, different techniques were optimized and published to determine thiamine in cereals and their derivatives. All of them relied on univariate approaches in order to obtain the better resolution of vitamin B_1. If the fact that univariate methods are often time consuming, it would be advisable to use a multivariate chemometric technique to optimize thiamine HPLC determination. Some works have shown a successful HPLC method optimization using response surface methodology (Van de Velde et al. 2012; Mirza and Tan 2001; Ferreira and Bruns 2007).

The aim of the present study was to optimize and validate a thiamine RP-HPLC determination by thiochrome fluorescence detection in wheat flours, using a Box–Behnken experimental design. An additional aim was to study if it was possible to extract thiochrome from its reaction medium by SPE using styrene-divinylbenzene adsorbent cartridges instead of isobutanol utilization.

Materials and Methods

HPLC

HPLC analyses were performed on Konik KNK-500-A Series liquid chromatograph (Konik Instruments, Barcelona, Spain) coupled to a fluorescence detector (Gilson 121, Middleton, WI, USA). Samples were injected into the chromatographic system through a manual injector equipped with a 20-μL loop (Rheodyne, Berkeley, CA, USA).

Reagents and Stock Standard Solution

Standard thiamine hydrochloride was from Sigma-Aldrich Inc. (St. Louis, MO, USA), and its stock solution (1 mg L^{-1}) was prepared in 20 % methanol according to AOAC (1995).

Potassium phosphate dibasic, sodium acetate, sodium sulfite, hydrochloric acid, sodium hydroxide, isobutanol, and potassium ferricyanide (ACS) were acquired from Laboratorios Ciccarelli (Reagents S.A., San Lorenzo, Santa Fe, Argentina). Methanol HPLC grade was from Sintorgan S.A. (Villa Martelli, Buenos Aires, Argentina). α-Amylase enzyme with an activity of 5,000 S.K.B. was from Epecuen S.A. (Valentín Alsina, Buenos Aires, Argentina). Distilled water was purified using an Easy Pure II RF System (Barnstead International, IA, USA), and this purified water was used for preparing all the solutions.

HPLC Conditions

Separations were tried in a reverse phase column Phenomenex Hypersil C18 (100×4.6 mm, 5 μ) attached to a Phenomenex guard column (Phenomenex Inc., CA, USA) at room temperature (25 °C). The mobile phase, under isocratic conditions, consisted of methanol/acetonitrile/buffer phosphate (0.02 M). The mixture methanol/acetonitrile was named "organic modifier" and it was prepared in the proportion of 88:12 (*v*/*v*), as it was suggested by Gauch et al. (1992). The organic modifier proportion in the mobile phase, pH value, and flow rate of mobile phase were varied according to the design optimization conditions, as presented in Table 1. Mobile phase was filtered through a 0.45-μm nylon membrane filter and degassed under vacuum. Fluorescence-selected detector filters were those corresponding to 366 and 435 nm which are the excitation and emission thiochrome wavelengths, respectively.

Thiamine Derivatization Reaction and Isobutanol and SPE Thiochrome Extractions

Thiamine derivatization to thiochrome was done according to Gauch et al. (1992). One milliliter of the solution to be derivatized (thiamine standard or thiamine from extracted flour samples) was added to 1 mL of freshly prepared alkaline potassium ferricyanide solution (1 % (*w*/*v*) in 15 % sodium hydroxide). The mixture was then stirred with a vortex during 8 s and let stand to react for 2 min. After that, 0.1 mL 10 % (*w*/*v*) sodium sulfite was added in order to stop the reaction, and the mixture was subsequently stirred. To extract the obtained thiochrome, liquid–liquid extraction was performed by adding 2 mL isobutanol to the mixture or 1 mL of the mixture was taken for SPE treatment as it is described below.

In SPE thiochrome extraction, a styrene-divinylbenzene adsorbent (Strata™-X) cartridge (6 mL, 200 mg, 800 m g^{-1} (San José Rodríguez et al. 2012), Phenomenex Inc., CA, USA) was conditioned with 4 mL methanol and 4 mL deionized water. The cartridges were attached to a suitable connector, and 1 mL of solution containing thiochrome derivates was added each time. After washings with buffer phosphate pH 8

Table 1 Experimental conditions and response values obtained for each of the 15 test points of the Box–Behnken design

Test	Box–Behnken			Factors and levels			Response
	A	*B*	*C*	*A* = % organic phase	*B* = pH	*C* = flow rate (mL min^{-1})	Peak width (s)
1	−1	−1	0	20	5.8	0.75	22.5
2	1	1	0	90	7.5	0.75	18.0
3	0	−1	−1	55	5.8	0.5	21.5
4	0	0	0	55	6.6	0.75	22.0
5	0	0	0	55	6.6	0.75	24.5
6	1	0	1	90	6.6	1	15.5
7	0	0	0	55	6.6	0.75	24.0
8	0	1	1	55	7.5	1	19.0
9	−1	0	−1	20	6.6	0.5	23.5
10	1	−1	0	90	5.8	0.75	20.0
11	−1	0	1	20	6.6	1	17.5
12	0	1	−1	55	7.5	0.5	32.0
13	1	0	−1	90	6.6	0.5	22.5
14	0	−1	1	55	5.8	1	14.0
15	−1	1	0	20	7.5	0.75	18.00

Experimental runs were performed in random order

and water, the elution was carried out with 2 mL SPE elution solution consisting of 90:10 (*v*/*v*) methanol/buffer phosphate pH 8 into a 25-mL Erlenmeyer attached to cartridges.

Wheat Flour Sample Preparation

Two wheat flour samples were analyzed. Sample 1 consisted of a wheat flour of an intermediate extraction rate provided by a regional milling company, and sample 2 consisted of enriched wheat flour acquired in a local market.

Samples were homogenized and a 4–6-g was weighted in a 250-mL Erlenmeyer flask. Hydrochloric acid was added (50 mL, 0.1 N), and the mixture was digested at 90–95 °C for 50 min. The pH of the cooled mixture was adjusted to 4.5 with sodium acetate 3 mol L^{-1}. Then, the mixture was diluted to 70 mL with deionized water, and 500 mg α-amylase was added and samples were incubated at 37 °C for 18 h. Enzymatic hydrolysis was carried out only with α-amylase, knowing that thiamine is present in its free form in wheat (Ndaw et al. 2000). This enzyme helps to destroy the starch gel formed and so to release the vitamin B_1.

After that, hydrolyzed mixture was centrifuged at 5,000×*g* during 15 min. One milliliter of the obtained clarified sample extract was derivatized at conditions mentioned above, and the obtained thiochrome was extracted with isobutanol or SPE as it was described in "Thiamine Derivatization Reaction and Isobutanol and SPE Thiochrome Extractions" section. Subsequently, thiochrome solutions were diluted with the mobile phase 1:1 (*v*/*v*) and filtered through a 0.45-μm Millipore membrane and were ready to be injected into the HPLC system.

Optimization Procedure

A Box–Behnken design (BBD) using three factors at three levels (coded levels −1, 0, and 1) was used for optimization of thiamine determination by HPLC. BBD consisted of 15 experiments (chromatographic runs) under different conditions (Table 1). Three replicates in the central point were included in order to estimate the experimental error. The involved factors were pH of the mobile phase (uncoded levels 5.8, 6.6, and 7.5), flow rate (uncoded levels 0.5, 0.75, and 1.00 mL min^{-1}), and organic modifier proportion in the mobile phase (uncoded levels 20, 55, and 90 %). Factors and its levels were selected taking into account previous literature on this topic where a wide range of chromatographic conditions were utilized to determine thiamine by HPLC (Ollilainen et al. 1993; Gauch et al. 1992; Fernando and Murphy 1990).

Using the presented experimental design, each experiment was conducted by making duplicate injections of the extracted thiochrome. Response variable was thiamine peak width expressed as seconds.

Thiamine standard used to develop the optimization design was prepared from stock solution (as it was described in "Reagents and Stock Standard Solution" section). The standard was derivatized, extracted with the classical isobutanol liquid–liquid extraction ("Thiamine Derivatization Reaction and Isobutanol and SPE Thiochrome Extractions" section), diluted 1:20 (*v*/*v*) with mobile phase, and finally injected into the HPLC system. The thiamine concentration in each design condition was 0.050 mg L^{-1}.

Validation Procedure

The optimized HPLC method was validated for the determination of thiamine in wheat flours. Linearity, limit of detection and quantification, and calibration and analytical sensitivity were calculated through standard thiamine solutions using isobutanol and SPE thiochrome extractions. Precision and accuracy were assessed using wheat flour samples (prepared as it was described in "Wheat Flour Sample Preparation" section). All experiments were performed in the optimized conditions under isocratic conditions.

Linearity and Other Figures of Merit

Standard thiamine solutions were prepared in triplicates, derivatized, extracted by isobutanol and SPE, and finally diluted with mobile phase to obtain solutions with the following concentrations: 0.01, 0.025, 0.050, 0.075, and 0.100 mg L^{-1}. Integrated peak areas were plotted against

thiamine concentrations, and graphics were used for the linearity test, calibration and sensibility, and detection and quantification limit calculations.

Standard Additions

Thiamine wheat flour quantification was performed through the standard addition method. For each standard addition experience, 0, 10.25, 20.50, and 41.00 μg thiamine hydrochloride standard were added to sample prior to enzymatic acidic hydrolysis and then treated as described in the "Wheat Flour Sample Preparation" section. Final dilution prior to injection in sample 2 with isobutanol thiochrome extraction was 1:3. Final volume after enzymatic acidic hydrolysis in sample 1 with SPE thiochrome extraction was 53.2 mL. With mentioned methodology concentrations of added thiamine at the moment of injection of 0.036, 0.073, and 0.146 mg L^{-1} for sample 1 and 0.018, 0.037, and 0.073 mg L^{-1} for sample 2 with isobutanol thiochrome extraction were achieved. For SPE thiochrome extraction, concentrations of added thiamine at the moment of injection were 0.016, 0.032 and 0.059 mg L^{-1} for sample 1 and 0.013, 0.026 and 0.052 mg L^{-1} for sample 2.

Precision

Four independent analyses of thiamine in wheat flour samples, prepared as described in sample preparation ("Wheat Flour Sample Preparation" section), were analyzed through the full analytical method in order to know the repeatability or intra-day variation method. Results were expressed as relative standard deviation (RSD in percent) (Eq. 1).

$$RSD\ (\%) = \frac{\overline{x}}{SD} \times 100 \quad (1)$$

where $\overline{x}$ and SD are the mean and the standard deviation of the thiamine in wheat flour samples.

Accuracy

Accuracy was evaluated by calculating recoveries from standard addition experiences. Each time, recovery (R) was calculated considering slopes (b) obtained for thiamine calibration curves (b_{SC}) and thiamine standard addition samples (b_{SAM}) (Maroto et al. 2001), as it is expressed in Eq. 2.

$$R(\%) = \frac{b_{SAM}}{b_{SC}} \times 100 \quad (2)$$

Statistical Analysis

Statgraphics Centurion XV 15.2.06 (StatPoint Technologies, Inc., Warrenton, VA, USA) was used to perform ANOVA analysis, to fit the second-order polynomial equation to the experimental data and to obtain the coefficients of the equation. The significance of each term of the model was evaluated referred to the pure error. Previously, tests to verify that the residuals satisfied the assumptions of normality, independence, and randomness were also done. For verification of the model adequacy, the lack of fit and the coefficient of determination (R^2) were calculated.

Results and Discussion

HPLC Condition Optimization

In recent years, chemometric tools have been frequently applied to the optimization of different analytical methods considering their advantages such a reduction in the number of experiments resulting in lower reagent consumption and considerably less laboratory work (Ollilainen et al. 1993; Van de Velde et al. 2012; Mirza and Tan 2001). A three-level Box–Behnken factorial design was used for the optimization of thiamine chromatographic conditions, using isobutanol thiochrome extraction. This kind of response surface methodology (RSM) uses second-order polynomial models to describe responses in the experimental design. The experiments data are shown in Table 1. From ANOVA results (Table 2), flow rate through lineal term and organic modifier proportion through quadratic term were factors that showed significant effects ($p \leq 0.05$). Experimental data for peak width response

Table 2 Analyses of variance of peak width in HPLC-fluorescence thiamine determination

Sum of squares		
Source	*df*	Peak width (s)
A	1	2.0
B	1	13.8
C	1	140.3*
A^2	1	34.6*
AB	1	0.3
AC	1	0.3
B^2	1	5.2
BC	1	7.6
C^2	1	1.7
Residual	5	–
Lack of fit	3	20.0
Pure error	2	1.8
R^2	–	0.76

A organic modifier proportion, *B* mobile phase pH, *C* flow rate
*$p \leq 0.05$

fitted a second-order polynomial equation (Eq. 3). Coefficient of determination was equal to 0.76, indicating that 76 % of variability in the response could be explained by the developed RSM model which did not show lack of fit ($p \leq 0.05$).

$$y = -90.2 + 0.2A + 27.8B + 44.4C + 0.008AB - 0.03AC - 6.5BC - 0.003A^2 - 1.6B^2 - 11.0C^2 \quad (3)$$

where A = organic modifier proportion, B = mobile phase pH, and C = flow rate.

Peak width was narrower (better resolution) at higher flow rate as it could be expected (Fig. 1). Similarly, peak width was narrower at low and high levels of organic modifier proportions (Fig. 1). The last mentioned feature is according to previous literature. Fernando and Murphy (1990) employed a mobile phase with 13 % organic modifier, whereas Tang et al. (2006) and Gauch et al. (1992) utilized 80 and 90 % organic modifier, respectively, to determine thiamine as thiochrome by HPLC in different foods. The mobile phase pH has not shown a significant effect in the peak width. In that regard, different mobile phase pH conditions have been utilized in bibliography from pH 5.5 to 7.5 (AOAC 1995; Gauch et al. 1992).

Optimum chromatographic conditions corresponding to minimum peak width were determined from experimental results and were organic modifier proportion = 90.0, pH = 6.0, and flow rate=1 mL min^{-1}. The predicted peak width calculated by Eq. 3 was approximately 14 s.

A verification test was performed using a derivatized thiamine standard solution with a final concentration of 0.050 mg L^{-1}. Mentioned solution was injected in triplicate and analyzed in optimum chromatographic conditions and showed a peak width of 15±1 s. Therefore, there were no differences ($p \leq 0.05$) between the predicted and experimental peak width results.

Validation Procedure

The method validation was carried out in optimum chromatographic conditions. Linearity was evaluated for thiamine determination using working standard solutions at concentrations mentioned in "Linearity and Other Figures of Merit," being derivatized and then thiochrome was extracted using isobutanol and SPE as mentioned in "Thiamine Derivatization Reaction and Isobutanol and SPE Thiochrome Extractions." Typical chromatograms corresponding to isobutanol and SPE thiochrome extractions with retention times of approximately 1.2 min are shown in Fig. 2. The data homoscedasticity and linearity were analyzed through an F test according to Danzer and Currie (1998). Calibration curve obtained by external thiamine standard with SPE was linear until 0.075 mg L^{-1}. Peak areas (y) were plotted against thiamine concentration at injection (x) and a least square analysis was performed. The coefficients of the linear model are presented in Table 3. Linearity models for isobutanol and SPE thiochrome extraction have not exceeded the tabulated values; therefore, the linearity relationship between peak areas (y, dependent variable) and thiamine concentration (x, independent variable) can be assumed, within the evaluated concentration range.

Detection (LOD) and Quantification Limits (LOQ)

The limits of detection (LOD) and quantification (LOQ) were calculated by the equations:

$$LOD = 3.3S_0 \quad (4)$$

$$LOQ = 10S_0 \quad (5)$$

S_0 is the standard deviation of the predicted analyte concentration in a blank sample which depends on the noise level in the response data, slope of the calibration curve, and analyte concentrations in the calibration experiments (Olivieri and Goicochea 2007; Muñoz de la Peña et al. 2003).

LOD were 0.00375 and 0.00397 mg L^{-1} for isobutanol and SPE thiochrome extraction, respectively. The values of LOQ for isobutanol and SPE extraction were 0.011 and 0.012 mg L^{-1}, respectively.

LOD expressed as milligram of thiamine per kilogram wheat flour were 0.21 and 0.75 for isobutanol and SPE extraction, respectively. Similarly, LOQ expressed as milligram

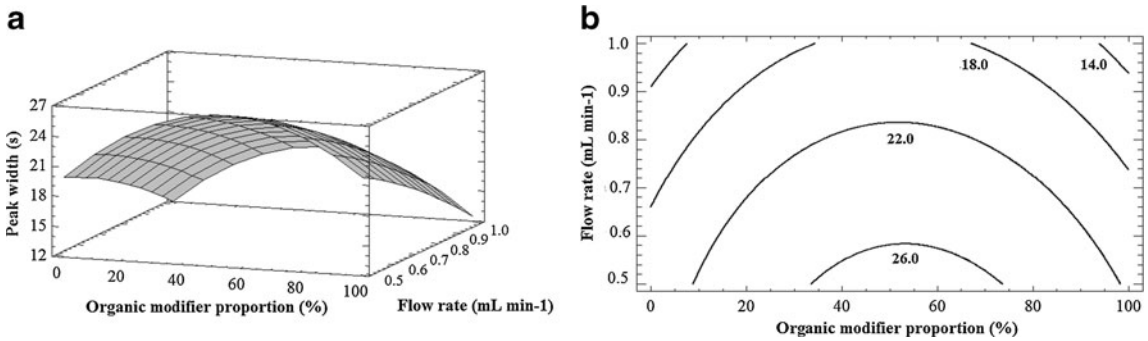

Fig. 1 Effect of organic modifier proportion and flow rate on thiamine peak width at a mobile phase pH = 6.6. Response surface (**a**) and contour plot (**b**)

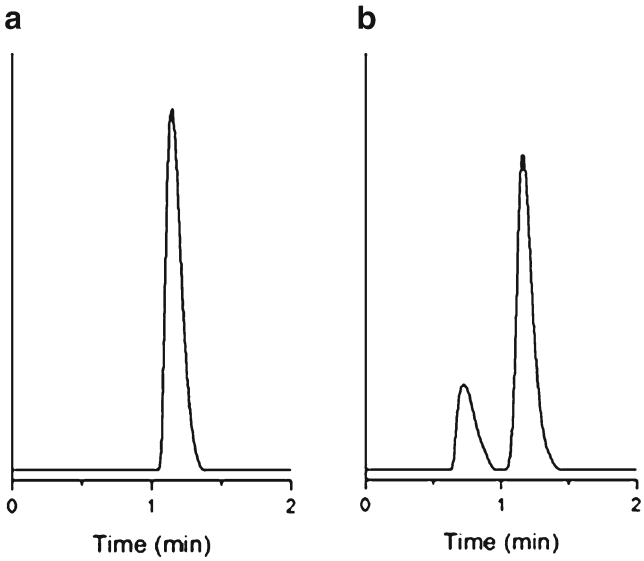

Fig. 2 Typical chromatograms obtained for thiochrome after liquid–liquid extraction in isobutanol (**a**) and solid-phase extraction using styrene-divinylbenzene adsorbent cartridges (**b**)

of thiamine per kilogram wheat flour were 0.64 for isobutanol and 2.3 for SPE. The fact that LOQ values determined in the present experiment were lower than all values of thiamine analyzed in wheat flour samples is a clear evidence of the validity of the method. Recently, an HPLC-UV determination of thiamine in complex cereal foods with an LOD of 6.3 mg thiamine kg^{-1} wheat flour was published. Eventually, it could be used only for the analysis of enriched cereal products (San José Rodríguez et al. 2012).

Calibration and Analytical Sensibility

The calibration sensibility (SEN) is equal to the slope of the calibration line and indicates the variation of response produced by a unit change in analyte concentration. Slopes for isobutanol and SPE thiochrome extraction were equal to 10.48 and 11.37 mg L^{-1}, respectively.

The analytical sensitivity (γ) (Eq. 6) is defined as the relation between the SEN and the instrumental noise (S_y); the last one can be estimated for the standard deviation of residues of the linear regression (Muñoz de la Peña et al. 2003).

$$\gamma = \frac{SEN}{S_{y/x}} \tag{6}$$

The γ parameter is best interpreted in terms of its inverse (γ^{-1}). The γ^{-1} values of thiamine were 1.61 and 1.73 mg L^{-1} for isobutanol and SPE thiochrome extraction, respectively. As the inverse of the analytical sensitive indicates the lowest concentration difference that is noticeable throughout implementation of the method, these results indicate practically similar sensibility between both thiochrome extractions.

Table 3 Statistical analysis of the calibration curves for isobutanol and SPE thiochrome extractions

Item	Results	
	Isobutanol	SPE
F test for homoscedasticity	16.73 (19.00)[a]	9.26 (19.00)[a]
F test for linearity	1.35 (2.89)[b]	0.97 (2.89)[b]
Calibration sensibility	10.48	11.37
Calibration line	$y=10.48x-12.40$	$y=11.37x-8.30$
R^2 value	0.9984	0.9966

The values between parenthesis correspond to critical values at $\alpha=0.05$ and the degrees of freedom

[a] ($\nu_1=2$, $\nu_2=2$)

[b] ($\nu_1=13$, $\nu_2=10$)

Determination of Thiamine in Wheat Flours

Thiamine concentration in wheat flour samples was calculated by standard addition method (SAM) carrying out the thiochrome extractions by isobutanol or SPE. The standard addition method consists in comparing the curve of the reference analyte (reference curve) with the curve of the same analyte spiked into the samples where it must be quantified (in matrix curve). This allows both the absolute quantification of the analyte from the intercept of the in-matrix curve with the x-axis and an estimation of the matrix effect, by comparing the slopes of the reference and in-matrix curves. In the presence of a negligible matrix effect, both curves are parallel, whereas the slopes are divergent when the matrix impairs the analyte detection (Resta et al. 2012). In Figs. 3 and 4, reference and in-matrix curves are shown for sample 1 and sample 2 when thiochrome was extracted by SPE and/or isobutanol.

When derivatized thiochrome from both samples was extracted with isobutanol, they presented matrix effect as could be observed in Fig. 3. When SPE extraction was employed, sample 1 presented matrix effect but sample 2 did not. The last mentioned feature can be seen in Fig. 4, where thiamine standard and sample 2 SAM curves are almost parallel, and their slopes were 11.37 and 11.48, respectively.

Final thiamine wheat flour samples concentrations were calculated taking into account standard addition experiences (Table 4). When SPE extraction was employed, sample 1 presented a thiamine concentration of 2.9±0.1 mg kg^{-1} of wheat flour which corresponds to intermediate extraction rate of wheat flour as the manufacturer had claimed (Ziegler and Greer 1978). Meanwhile, thiamine concentration found in sample 2 by SPE extraction was 6.4±1 mg kg^{-1} of wheat flour, meeting requirements of Argentine legislation for an

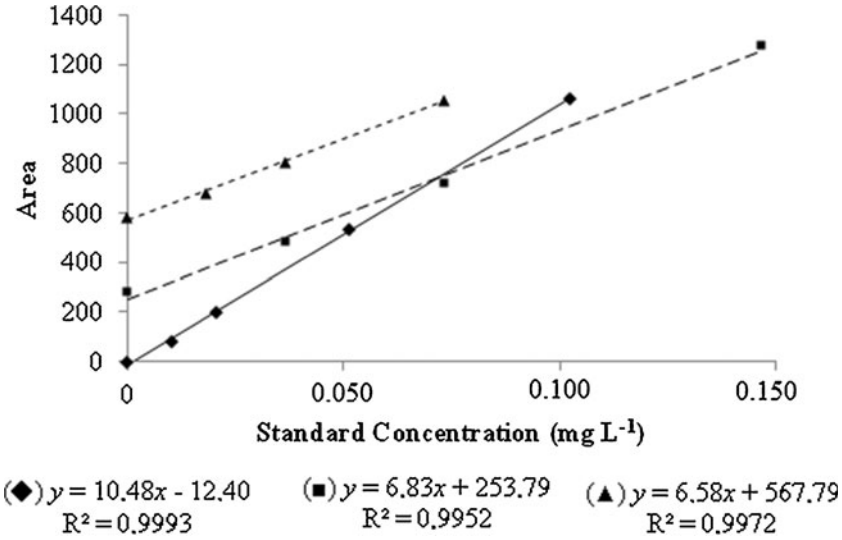

Fig. 3 Calibration curves obtained by external thiamine standard (*black diamond*) and standard addition method (SAM) for sample 1 (*black square*) and sample 2 (*black triangle*) with isobutanol extraction method

enriched wheat flour (Argentine Public Health Ministry 2013).

Precision

For the whole procedure including thiamine extraction, derivatization, thiochrome isolation, and HPLC analysis, two different wheat flour samples and thiamine standards have been analyzed in the same day. For SPE, sample 1 showed an RSD of 3.4 % which is below to the limit of 11 % considered as maximum for analytes around 1 mg kg^{-1} sample by AOAC (1993). Sample 2 showed a 14 % RSD which is above-mentioned limit. It can be taking into account that sample 2 is an enriched sample and that added thiamine could not be homogeneously distributed. In spite of the analysis which has been done four times in the present study, it could be advisable to carry out more repetitions when analyzing enriched samples. Isobutanol thiochrome extraction presented an overall 2 and 15 % RSD for samples 1 and 2, respectively, so both results were below mentioned AOAC limits.

Accuracy

In present conditions, mean recovery percents for isobutanol extraction ranged from 65 % for sample 1 to 63 % for sample 2. In the case of SPE, mean recoveries were 84 % for sample 1 and 101 % for sample 2. SPE recovery values are in the accepted interval by AOAC (1993) for analyte determination ranging from 1 to 10 mg kg^{-1} analyte concentration (80–100 %). The mentioned results showed that styrene-divinylbenzene adsorbent (Strata™-X) cartridge provides very good thiochrome recoveries. Ollilainen et al. (1993) have used C18 cartridges for thiochrome extraction and found that the mean total recovery of thiamine analyzing some food samples and standards was approximately 60 %, including sample extraction, thiochrome derivatization, solid-phase purification, and chromatographic separation. Poel et al. (2009),

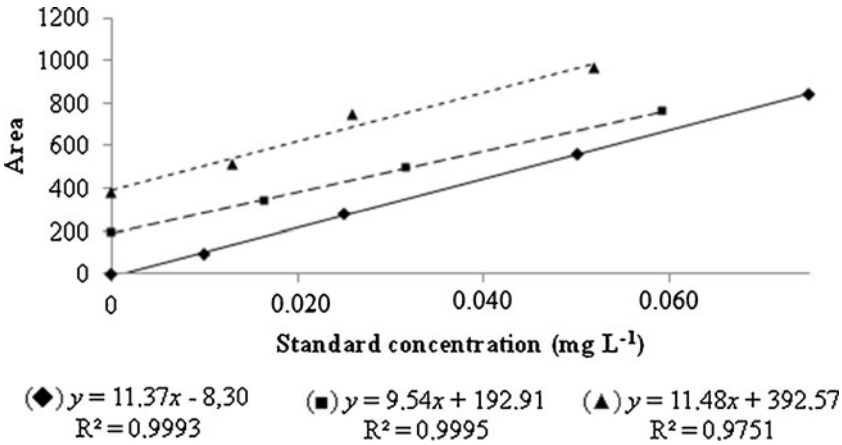

Fig. 4 Calibration curves obtained by external thiamine standard (*black diamond*) and standard addition method (SAM) for sample 1 (*black square*) and sample 2 (*black triangle*) with the solid phase method

Table 4 Thiamine content found in analyzed wheat flours when thiochrome was extracted by isobutanol and by solid-phase extraction (SPE) before HPLC determination with standard addition method (SAM)

	Thiamine ± SD (mg kg^{-1})[a]	
	Isobutanol extraction	SPE
Sample 1	2.5±0.1	2.9±0.1
Sample 2	9.6±1.4	6.4±0.9

SD standard deviation

[a] Average value, $n=2$

applying a gradient elution HPLC method, utilized a strong ion exchange coupled to C-18 end-capped cartridges to isolate thiamine and thiamine phosphate esters as its thiochrome derivates and found 68 to 97.4 % recoveries depending on concentrations and type of thiamine phosphate esters.

Conclusions

A new thiochrome HPLC isocratic method with fluorescence detection for thiamine determination on wheat flour samples was successfully optimized and validated. Derivatized thiamine thiochrome was isolated using liquid–liquid extraction in isobutanol and SPE through styrene-divinylbenzene adsorbent cartridges before HPLC injection. Thiamine concentration in wheat flour samples was calculated by standard addition method, and good precision results were obtained by both thiochrome extractions. Thiamine recoveries ranged from 63 to 65 % for isobutanol and 84 to101% for SPE, including sample extraction, thiamine derivatization, thiochrome purification, and chromatographic separation. Taking into account better obtained SPE recoveries, acceptable precision, and isobutanol irritant action on the eyes and skin of operator, SPE using styrene-divinylbenzene adsorbent could be chosen for routine thiamine analysis in wheat flour samples.

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Conflict of Interest Nicolas Michlig declares that he has no conflict of interest. Franco Van de Velde declares that he has no conflict of interest. Cecilia M. del H. Bernardi declares that she has no conflict of interest. Marcelino R. Freyre died during manuscript preparation. This article does not contain any studies with human or animal subjects.

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