Determination of Thiamin in Cooked Sausages

Felicidad Valls,† Martín A. Checa,† Miguel A. Fernández-Muiño,‡ and M. Teresa Sancho*,‡

Campofrío Alimentación, S.A. Fundación Sonsoles Ballvé, s/n, 09007 Burgos (Castilla y León), Spain, and Departamento de Biotecnología y Ciencia de los Alimentos, Area de Nutrición y Bromatología, Universidad de Burgos, Plaza de Misael Bañuelos García s/n, 09001 Burgos (Castilla y León), Spain

A reliable and rapid high-performance liquid chromatography (HPLC) method has been set up for the determination of total thiamin in difficult sample matrices such as cooked sausages. Different hydrolysis conditions and enzymes were tested to release the vitamin from its phosphate ester. The best data in the enzymatic digestion were obtained by incubating the samples with 6% clara-diastase at 50 °C for 3 h. After oxidation of thiamin to thiochrome, the sample extracts were purified by using a C_{18} Sep-Pak cartridge. Final determination was performed by reversed-phase HPLC with fluorescence detector (excitation 360 nm, emission 430 nm), on a low-cost 25 cm × 4 mm i.d. Spherisorb C_8 cartridge using a mixture of 5 mM phosphate buffer pH 7.0 and acetonitrile (70:30, v/v) as mobile phase. Precision of the method was 1.5% (within a day) and 5.2% (between days). The detection limit was 0.015 mg/100 g. All the recoveries from the different cooked sausages were better than 90% of thiamin hydrochloride added to samples of meats. In the samples analyzed, the mean value for thiamin was between 0.039 and 0.508 mg/100 g fresh weight.

Keywords: *Thiamin; cooked sausages; HPLC*

INTRODUCTION

Thiamin is a water-soluble vitamin consisting of a pyrimidine ring and a thiazole moiety linked by a methylene bridge (Merck Index, 1989). This vitamin is involved in the energy metabolism of proteins, lipids, and carbohydrates. Meat and meat products, especially pork, are good thiamin sources (Basu and Dickerson, 1996).

One of the difficulties in thiamin analysis is the variability in food sample matrices' composition. For this reason, a general method for vitamin analysis is difficult. Thiamin commonly exists in most animal products as its coenzyme, thiamin pyrophosphate. This vitamin is also linked to proteins. Acid and/or enzyme solutions are needed to release thiamin to be measured. Different acid conditions and enzymes have been proposed and several methods for the determination of thiamin in foods have been published (Ang and Moseley, 1980; Ryan and Ingle, 1980; Bognar, 1981; Fellman et al., 1982; Hilker and Clifford, 1982; Ayi et al., 1985; Bettendorff et al., 1986; Bötticher and Bötticher, 1986; Defibaugh, 1987; Finglas and Faulks, 1987; AOAC, 1990; Bailey and Finglas, 1990; Vidal-Valverde and Reche, 1990; Bauer et al., 1991; Sander et al., 1991; Bognar, 1992; Laschi-Loquerie et al., 1992; Ollilainen et al., 1993; Hägg, 1994). Microbiological and fluorometric assays have been substituted by high-performance liquid chromatography (HPLC) with C_{18} and C_8 bonded stationary phases; different mobile phases have been used, including organic solvents (Bognar, 1981; Bailey and Finglas, 1990; Bauer et al., 1991; Laschi-Loquerie et al., 1992), ion-pair (Ayi et al., 1985; Vidal-Valverde and Reche, 1990; Bognar, 1992), and organic–aqueous buffer mixtures (Bettendorff et al., 1986; Bötticher and Bötticher, 1986; Sander et al., 1991; Ollilainen et al., 1993). Various researchers (Hilker and Clifford, 1982; Ayi et al., 1985; Vidal-Valverde and Reche, 1990) directly determined the vitamin as thiamin by UV spectrophotometry, but the low vitamin content and the high quantities of interfering substances make the direct chromatographic determination of the sample extracts difficult. This problem is solved by conversion of thiamin to fluorescent thiochrome, making it possible to measure thiochrome by using a fluorescence detector. The use of fluorescence reduces the range of interfering factors, increasing reproducibility and sensitivity (Fellman et al., 1982; Finglas and Faulks, 1987).

Because of the increase in human's consumption of cooked sausages, the purpose of this study was, at first, to determine the thiamin content of these foods to evaluate the possible interest in human nutrition due to this vitamin. However, previously published analytical methods for thiamin determination gave poor results for when applied to cooked sausages, so we tried to optimize the experimental conditions which would result in a proper, accurate, sensitive, and low-cost analytical method in these matrices.

EXPERIMENTAL PROCEDURES

Apparatus and Liquid Chromatograph Conditions. A model HP1090 high-performance liquid chromatograph (Hewlett-Packard) equipped with an HP1046 fluorometric detector (Hewlett-Packard) was used. The chromatographic column was a 25 cm × 4 mm i.d. stainless steel cartridge (Teknokroma) packed with Spherisorb C_8 5 μm. A 10-μL volume of eluate was chromatographed, using a mixture of 70% phosphate buffer (5mM, pH 7.0) and 30% acetonitrile as mobile phase, isocratically pumped at a flow rate of 0.650 mL/min. The oven temperature was 35 °C. The determination of thiamin as thiochrome was measured at an excitation wavelength of 360 nm and an emission wavelength of 430 nm.

* Author to whom correspondence should be sent (telephone, 34-47-25-8813, fax, 34-47-25-8831, e-mail, mtsancho@ubu.es).
† S.A. Fundación Sonsoles Ballvé.
‡ Universidad de Burgos.

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Reagents. Thiamin hydrochloride was obtained from Merck (Germany). Papain was obtained from Sigma Chemical (St. Louis, MO), pepsin was obtained from Merck (Germany), and clara-diastase (α-amylase, cellulase, invertase, peptidase, phosphatase and sulfatase) was obtained from Fluka (Switzerland). Acetonitrile and methanol were HPLC grade, and all other reagents were analytical grade.

Samples. Six commercially purchased cooked sausages were analyzed: "lunch", "chopped pork", "chopped beef", "chopped turkey", "vitamined chopped", and "sicilian mortadella". All of them are composed of meats, fat, water, sugars, salt, different spices, and some additives such as preservatives. They mainly differ in meat composition (more or less quantities of pork, beef, and turkey meats, depending on the meat product) and grinding degree.

Sample Preparation. Thiamin was extracted by the Hägg (1994) method modified for our purpose. Ten grams of finely ground samples was weighed in duplicate into a 250-mL Erlenmeyer flasks. Sixty milliliters of 0.1 N HCl was added to the homogenized sample, and the mixtures were then stirred. The contents were autoclaved at 120 °C for 20 min (Sander et al., 1991). After the contents were cooled to room temperature, the pH was adjusted to 4.0−4.5 with 2.5 M sodium acetate. Five milliliters of freshly prepared aqueous enzyme solution (6% (w/v) clara-diastase) was added, and the samples were incubated at 50 °C for 3 h. To precipitate the proteins, 2 mL of 50% (w/v) trichloroacetic acid was added and the samples were heated on a steam bath at 90 °C for 15 min. After the flasks were cooled to room temperature, the samples were brought to 100 mL with distilled water and then filtered through Albet No. 1305 filter paper. The filtrates were then refrigerated until used the following day.

Thiamin Oxidation and Purification. Thiamin was oxidized to its fluorescent derivative, thiochrome, by adding 5 mL of 1% potassium ferricyanide in 15% aqueous NaOH (AOAC, 1990) to 10 mL of the filtered extract. The solution was shaken and left to stand for exactly 1 min, and later, 1 mL of concentrated H_3PO_4 was added to minimize formation of thiamin disulfide (Ryan and Ingle, 1980). The oxidized extract was passed through an activated (with 2 mL of methanol and 2 mL of water) C_{18} Sep-Pak cartridge (Waters Associates). Interfering substances were removed with 3 mL of 5% methanol/95% 5 mM phosphate buffer at pH 7.0. Thiamin was eluted with 3 mL of methanol and was diluted to 5 mL with methanol, and filtered through a Millipore filter (0.45 μm) into amber vials for HPLC analysis.

Procedure with Standard Solutions. A stock solution of 1000 μg/mL thiamin hydrochloride in 0.1 N HCl was prepared and stored in darkness in a refrigerator. Working standard solutions (5 and 25 μg/mL) were prepared daily. Aliquots of these solutions were treated as samples. The resulting peak areas were plotted against concentration (from 0.5 to 7.5 μg) for the calibration curve. The vitamin content of the sample extracts was obtained by interpolation on the standard curve.

RESULTS AND DISCUSSION

In view of the problems observed by researcher about the different efficacies of the enzymes for hydrolysis (Defibaugh, 1987; Hägg, 1994), three enzymes (papain, pepsin, and clara-diastase) have been tested in the present study; three different concentrations (3, 6, and 9%) and three incubation times (2, 3, and 4 h at 50 °C) have been also studied. The choice of enzymes, the amounts used, and the incubation times were based on the literature. The results showed significant differences depending on the enzymes used. The best data in the enzymatic digestion were obtained by incubating the samples with 6% clara-diastase at 50 °C for 3 h. This enzyme was also the most effective in the assay made by Hägg (1994). Table 1 shows the effect of different enzyme preparations on the determination of thiamin in three representative cooked sausages (according to their composition). Papain gave a result that was 34.7% and pepsin 47.6% of that obtained with clara-diastase.

Table 1. Effect of Different Enzyme Preparations on Thiamin Determination[a]

	sample[c] (mg/100 g)		
enzyme[b]	lunch	chopped beef	chopped turkey
papain	0.177 ± 0.018	0.054 ± 0.004	0.014 ± 0.002
pepsin	0.231 ± 0.021	0.075 ± 0.005	0.020 ± 0.003
clara-diastase	0.499 ± 0.030	0.157 ± 0.007	0.041 ± 0.004
no enzyme	0.204 ± 0.026	0.066 ± 0.004	0.017 ± 0.002

[a] Thiamin was determined on a fresh weight basis. [b] Enzyme treatment (6%, 50 °C, 3 h). [c] Data are expressed as the mean ± S. D., $n = 6$.

Another important thiamin analytical problem is the separation of this vitamin from interferences in difficult sample matrices such as cooked sausages in a reasonably short time. As the purification step of the official methods (AOAC, 1990) gave poor results, different purification and concentration conditions have been tested on the basis of the solid-phase chromatography with a C_{18} Sep-Pak cartridge. Good purification was obtained by removing interfering substances with 3 mL of 5% methanol/95% 5 mM phosphate buffer at pH 7.0 and eluting thiamin with 3 mL of methanol. Purification and concentration with the solid-phase extraction technique before the chromatographic separation was essential to improve the detection sensitivity.

Reversed-phase HPLC techniques offer good results and advantages for analyzing water-soluble vitamins such as thiamin. In this work, a Spherisorb C_8 cartridge stationary phase has been employed. This column has the advantages of lower cost and versatility, but, as far as we know, it has not been used by other authors for thiamin determination. Important variations of thiamin retention times on the column were observed, depending on organic concentration as well as mobile phase pH. Mixtures of methanol/water (70:30, v/v) and acetonitrile/water (60:40, v/v) were used as mobile phases in isocratic elution to optimize the separation conditions. Acetonitrile was chosen as the organic solvent because of a reduction in retention time. The amount of acetonitrile in the eluent was also tested to regulate the elution of the vitamin. The influence of pH on the chromatographic separation was studied (pH 2.7, 5.2, and 7.0), and the observation of the results showed that the intensity of thiochrome fluorescence depended on pH and reached a higher level at pH 7.0. A mixture of acetonitrile and phosphate buffer pH 7.0 in proportion of 30:70, v/v, was used as the optimum mobile phase for achieving an adequate separation of thiochrome. Optimum fluorescence wavelengths were determined by scanning excitation and emission wavelengths of the thiochrome in the mobile phase. Figure 1 shows two chromatograms of thiamin as thiochrome in the standard (A) and in a lunch sample (B). The thiochrome peak is well-resolved in only 5 min without any interference problem and returned to baseline before the next analysis.

The standard curve was prepared between days (for 12 times over 3 months) to verify the applicability of this method to the quantitation of thiamin. It was linear from 0.5 to 7.5 μg, which is adequate for the concentration range in the products analyzed. Linear-regression coefficients were around 0.9996. Results showed that it is important to generate a new standard curve with

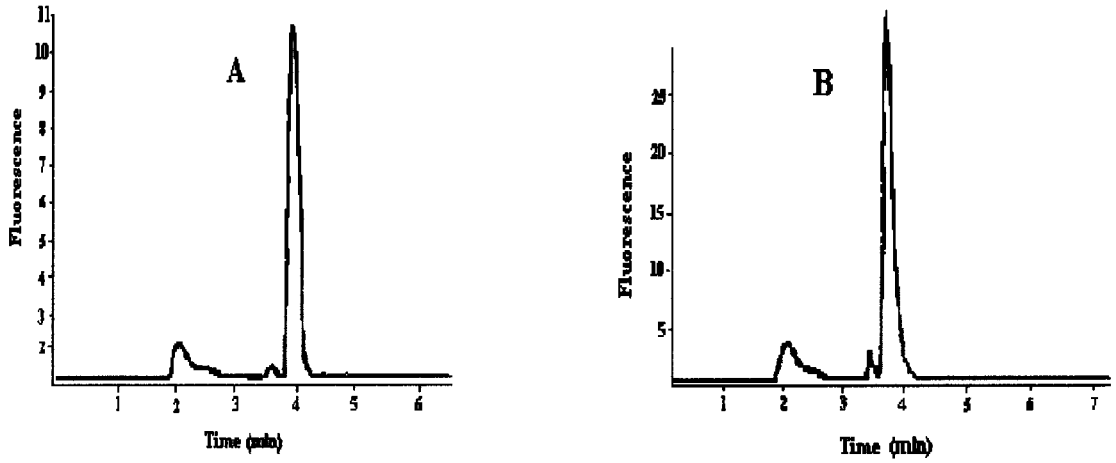

Figure 1. (A) Chromatogram of thiamin hydrochloride standard (thiochrome) (2 μg; 3.929 min) and (B) chromatogram of thiamin determination as thiochrome of a "lunch" sample (0.503 mg/100 g; 3.771 min). See text for chromatographic conditions.

Table 2. Calibration Data for Thiamin[a]

retention time (min)	amount (μg)	area
3.929	0.50	32.90
	1.00	68.11
	2.00	139.83
	2.50	187.87
	5.00	359.46
	7.50	562.05

[a] $r^2 = 0.9996$; linear regression $= -4.654 + 74.746$ (amount).

every batch when samples are analyzed because the slope of the curve changes accordingly. Table 2 shows the calibration data for thiamin. The detection limit of thiamin as thiochrome was estimated, giving a result of 0.015 (mg/100 g) using signal-to-noise ratio of 3. It was low enough to allow the measure of natural contents in the samples studied.

The precision expressed by coefficient of variation was tested. The variation within a day was 1.5% when the thiamin content was determined in six aliquots of the same sample studied in parallel. The variation between days (10 samples analyzed in duplicate over 3 months) was 5.2% on the average.

The recovery of this method was tested by adding different amounts of thiamin to two representative samples before extraction (Table 3), and the percent recovery ranged from 91.0% to 93.7%.

In the present study the thiamin content was determined according to the HPLC method above developed in six commercially cooked sausages named: "lunch", "chopped pork", "chopped beef", "chopped turkey", "vitamined chopped", and "Sicilian mortadella" collected weekly from a food factory during a 4-month period. Table 4 summarizes the thiamin content in the food samples studied. Each value represents an average of 20 samples analyzed in duplicate. Thiamin content in the samples was constant during the study period; however, the values differed widely among different meat products. Thiamin content varies depending on the composition of the samples assayed. It has been observed that meat products with higher proportions of pork (lunch and vitamined chopped) have higher levels of thiamin. In contrast, chopped turkey is a poor source of this vitamin.

Researching the best extraction thiamin method in cooked sausages is interesting, especially when there are procedures for thiamin analysis in different foods with very different analytical results, which have not been thoroughly studied, compared and discussed; so there is not a good reference method for thiamin analysis in these matrices.

Furthermore, it is very important to achieve good thiamin purification, since a lot of interferences have been found in the matrices studied. The interferences cannot be removed by applying the official methods, so if a proper, realiable, and good analytical result is desirable, the application of those previous methods for these foods is obviously unsuitable.

Finally, researching several stationary phases, we have observed that the inexpensive Spherisorb C_8 cartridge (which had not been before used for thiamin analysis), is good for determining the vitamine (and we think that looking for a low cost is also important in analytical procedures).

Table 3. Recoveries of Thiamin Added to Meat Samples by High-Performance Liquid Chromatographic Method

	before addition (mg/100 g)	amount added (mg/100 g)	amount found (mg/100 g)	recovery[a] (%)
lunch	0.448	0.250	0.688	93.2
	0.445	0.250	0.685	92.0
	0.457	0.250	0.691	94.4
	0.463	0.500	0.924	93.8
	0.455	0.500	0.929	94.8
	0.461	0.500	0.926	94.2
mean ± SD	0.455 ± 0.007			93.7 ± 1.0
chopped turkey	0.041	0.025	0.063	88.0
	0.038	0.025	0.064	92.0
	0.043	0.025	0.064	92.0
	0.040	0.050	0.087	92.0
	0.045	0.050	0.087	92.0
	0.039	0.050	0.086	90.0
mean ± SD	0.041 ± 0.003			91.0 ± 1.7

[a] Percent recovery = (amount found − mean value)/amount added × 100.

Table 4. Thiamin Contents in Cooked Sausages

meat samples[a]	N[b]	water content (% ± SD)	thiamin content (mg/100 g FW[c] ± SD)
lunch	20	64.75 ± 0.63	0.508 ± 0.032
chopped pork	20	70.70 ± 0.53	0.172 ± 0.010
chopped beef	20	70.51 ± 0.57	0.153 ± 0.008
chopped turkey	20	71.81 ± 0.45	0.039 ± 0.004
vitamined chopped	20	70.60 ± 0.56	0.410 ± 0.033
Sicilian mortadella	20	59.53 ± 0.72	0.141 ± 0.006

[a] All samples were analyzed in duplicate. [b] The number of different samples. [c] The fresh weight.

The rest of the manuscript describes several improvements of previous HPLC−fluorescence methodology for thiamin determination, but these improvements are not the main focus of the paper.

In conclusion, the proposed HPLC method allows the rapid, efficient and interference-free separation of total thiamin in difficult food matrices, as cooked sausages. The precision, accuracy, and sensitivity of this method were extremely satisfactory.

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