Determination of Vitamin B$_6$ in Cooked Sausages

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A reverse-phase high-performance liquid chromatography (HPLC) method has been described for the determination of various active forms of vitamin B$_6$ in meat products. Different extracting agents were tested to solubilize fully the analyte for quantification. The best data were obtained by extracting the samples with 5% (w/v) metaphosphoric acid. Separation by HPLC was performed with fluorescence detection (excitation, 290 nm; emission, 395 nm), on a 10 cm × 0.46 cm i.d. Hypersil BDS C$_{18}$ 5 μm column using a mixture of 50 mM phosphate buffer (pH 3.2) and acetonitrile (99:1, v/v) as mobile phase. Precision of the method was 0.5% (within a day) and 4.3% (between days). The detection limits were 0.020 mg/100 g for pyridoxal and pyridoxamine, 0.017 mg/100 g for pyridoxamine phosphate, 0.500 mg/100 g for pyridoxal phosphate, and 0.033 mg/100 g for pyridoxol, with a signal-to-noise ratio of 3. The recovery ranged from 92.0 to 100.0%.

Keywords: *Vitamin B$_6$; pyridoxine; pyridoxal; pyridoxamine; cooked sausages; HPLC*

INTRODUCTION

Vitamin B$_6$ is the generic descriptor for 3-hydroxy-2-methylpyridine derivatives having the biological activity of pyridoxine. It comprises three chemically, metabolically, and functionally related forms (or vitamers), pyridoxine (pyridoxol, PN), pyridoxal (PL), and pyridoxamine (PM), in which alcohol, aldehyde, and amine groups, respectively, are located at the 4-position of the pyridine ring. All three vitamers are interchangeable and therefore are comparably active. These forms are converted in the liver, erythrocytes, and other tissues to pyridoxal phosphate (PLP) and pyridoxamine phosphate (PMP), which serve primarily as coenzymes in transamination reactions. The vitamin is widely distributed in foods, with no food being a particularly rich source. The richest sources are meats and cereals; fruits are generally poor sources (*1*).

It is agreed that PMP and PLP are the main vitamin B$_6$ vitamers in meat. They are bound to protein in muscle tissue (*2*); therefore, for proper quantification of vitamin B$_6$, it is very important to fully solubilize the analyte in the extraction step from foods. For this purpose, a variety of protein denaturants have been used by researchers. Trichloroacetic acid (*3, 4*), sulfosalicylic acid (*5, 6*), and metaphosphoric acid (*7*) were employed. Bognar (*8, 9*) used H$_2$SO$_4$ in conjunction with autoclaving for the destruction of the phosphorylated forms of the vitamin. The selected extraction procedure must be compatible with the final analytical method to yield extracts free of interfering compounds.

The determination of vitamin B$_6$ in complex matrices is a difficult analytical problem because there are six forms that exhibit vitamin B$_6$ activity. Therefore, it is not surprising that numerous authors in the analysis of various vitamin B$_6$ vitamers have applied different

high-performance liquid chromatography (HPLC) procedures. Nevertheless, even when there are efficient, specific, and sensitive procedures for the determination of the vitamin B$_6$ content in foods and complex materials, some methods have disadvantages, such as the incomplete separation of the several compounds (*3, 4, 9, 10*). Separation of vitamin B$_6$ vitamers is well suited to ion exchange (*5, 11−13*), reverse-phase liquid chromatography (*3, 7−9, 14, 15*), or ion-pair reverse-phase chromatography (*4, 6, 16, 17*) because of their pH-dependent ionic nature.

Considering these alternative methods the purpose of this study was to optimize the extraction and the HPLC method by modification and adaptation of methods previously described to properly separate and determine the total amount of B$_6$ vitamers including their phosphorylated metabolites.

MATERIALS AND METHODS

Apparatus and Liquid Chromatograph Conditions. A model HP1090 high-performance liquid chromatograph (Hewlett-Packard) equipped with a Rheodyne 7010 autosampler , an HP 1046 fluorometric detector (Hewlett-Packard), and an HP 3390A integrator (Hewlett-Packard) was used. The chromatographic column was a 10 cm × 0.46 cm i.d. stainless steel column obtained from Shandon HPLC, Life Sciences, S.A., packed with Hypersil BDS C$_{18}$, 5 μm. A 10 μL volume of eluate was chromatographed, using a mixture of 99% potassium phosphate buffer solution (0.05 M, pH 3.2) and 1% acetonitrile as mobile phase, isocratically pumped at a flow rate of 0.800 mL/min. The oven temperature was 35 °C. The detector excitation and emission wavelengths for meat samples were set at 290 and 395 nm, respectively.

Reagents. Reference standards of pyridoxal hydrochloride (PL), pyridoxamine dihydrochloride (PM), pyridoxol hydrochloride (PN), pyridoxal phosphate (PLP), and pyridoxamine phosphate (PMP) were obtained from Sigma Chemical Co. (St. Louis, MO). Metaphosphoric acid also was from Sigma Chemical Co. Ultrapure HPLC water generated by a Milli-RO4 coupled to a Milli-Q water purification system (Millipore, Bedford, MA) was used. All reagents for chromatographic conditions were of HPLC grade, and all other chemical

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Table 1. Effect of Extracting Agent on Vitamin B$_6$ Determination

	sample[a] (mg/100 g)	
extracting agent	chopped beef	Sicilian mortadella
metaphosphoric acid	0.216 ± 0.008	0.130 ± 0.007
trichloroacetic acid	0.208 ± 0.007	0.136 ± 0.009

[a] Data are expressed as the mean ± SD, $n = 6$ in duplicate.

reagents used were of analytical grade. Mobile phase solution was filtered through a 0.45 μm Millipore membrane filter and degassed prior to use.

Samples. Six types of 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. Vitamin B$_6$ was extracted according to the Ang et al. (7) method modified for our purpose. Ten grams of finely ground samples was weighed in duplicate into a 50 mL beaker. Twenty milliliters of freshly prepared metaphosphoric acid solution [5% (w/v)] was added, and the mixtures were then homogenized by stirring at room temperature for 2 min. Contents were transferred to a 100 mL volumetric flask, washing the beaker twice with water and making up to volume with water. Samples were centrifuged for 5 min at 3000 rpm; the upper solution was filtered through Albet No. 1305 filter paper and refiltered through Millipore filters (0.45 μm) into amber vials for liquid chromatography analysis.

Procedure with Standard Solutions. Stock solutions of 100 μg/mL PL, PLP, PM, and PMP and a stock solution of 500 μg/mL PN in 0.1 N hydrochloric acid solution were prepared and stored in darkness in a refrigerator. Working standard solutions (10 and 50 μg/mL for PL, PM, PMP, and PN) were prepared daily before use by suitable dilutions. For PLP, stock solution was used as working standard solution. Aliquots of these solutions were treated as samples. The resulting peak areas of each vitamin were plotted against concentration (from 10 to 100 μg for PL, PM, and PMP; from 100 to 500 μg for PLP; and from 20 to 100 μg for PN) for the calibration curves. The vitamin contents of the sample extracts were obtained by interpolation on the standard curve.

RESULTS AND DISCUSSION

Due to the multiple protein denaturants used for the extraction of vitamin B$_6$ compounds from foods, we decided to optimize the experimental conditions by assaying with metaphosphoric acid and trichloroacetic acid at 5% (w/v) concentration in two different meat products (Sicilian mortadella and vitamined chopped) and a mixture of standard solutions. The results in Table 1 show both extracting agents permit similar extraction of vitamin B$_6$, but we observed that extraction with trichloroacetic acid resulted in an appreciable dephosphorylation of PMP, so we used metaphosphoric acid as an effective extractant for vitamin B$_6$. Metaphosphoric acid extracts may be more readily compatible with certain HPLC analyses than extracts prepared using other extractants (18).

Because of the ionic nature of several vitamin B$_6$ forms, a reverse-phase C$_{18}$ HPLC analysis has been carried out, which separates the vitamers in half the time required for anion-exchange chromatography.

Proper separation was achieved with a Hypersil BDS C$_{18}$ 5 μm stationary phase column. The optimum mobile phase composition consisted of a 1:99 (v/v) mixture of acetonitrile/50 mM phosphate buffer, adjusted to pH 3.2
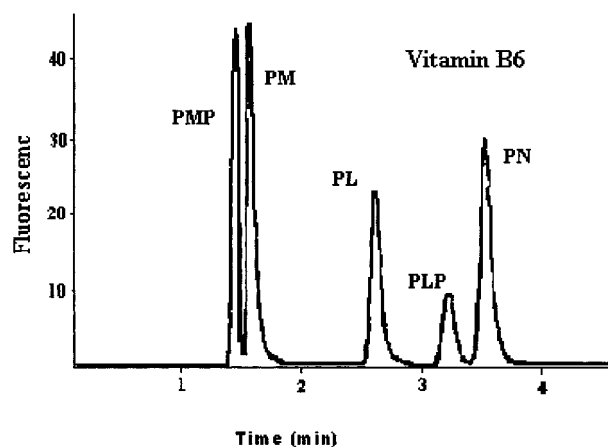

Figure 1. Chromatogram of various forms of vitamin B$_6$ standards. Amounts and retention times of PMP, PM, PL, PLM, and PN were as follows: 100 μg, 1.410 min; 100 μg, 1.527 min; 100 μg, 2.605 min; 500 μg, 3.238 min; and 100 μg, 3.552 min, respectively. Conditions are described in the text.
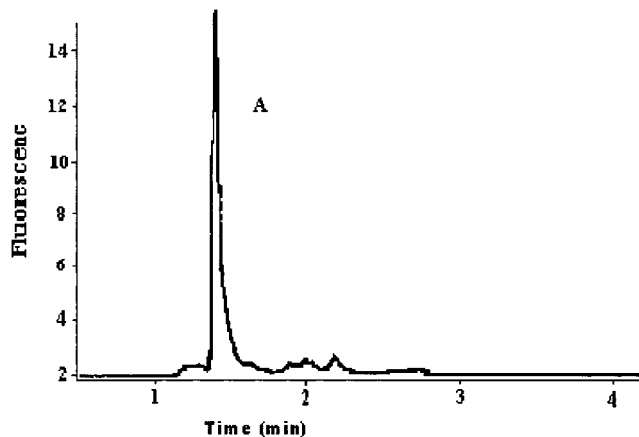

Figure 2. Chromatogram of vitamin B$_6$ (pyridoxamine phosphate) of a chopped pork sample (0.205 mg/100 g; 1.350 min). See text for chromatographic conditions.

with orthophosphoric acid. It was decided to use this value of pH because all vitamers are stable under acidic conditions. The amount of phosphate buffer was regulated to achieve adequate retention time and separation for all compounds without the use of an ion-pair reagent. The vitamers were eluted in ~5.0 min with a flow rate of 0.800 mL/min. Optimum fluorescence wavelengths were determined by scanning excitation and emission wavelengths of the vitamin B$_6$ vitamers in the mobile phase. Figures 1 and 2 show chromatograms of vitamin B$_6$ vitamers in a mixture of standards and in one representative cooked sausages (chopped pork).

The calibration curves were prepared between days (for 12 times over 3 months) for PL, PLP, PM, PMP, and PN to allow the quantification of vitamin B$_6$ components by HPLC. They were linear between 10 and 100 μg for PL, PM, and PMP, between 100 and 500 μg for PLP, and between 20 and 100 μg for PN, which are adequate values for the concentration range in the products studied. Linear regression coefficients were 0.9996 for PL, 0.9967 for PLP, 0.9993 for PM, 0.9997 for PMP, and 0.9998 for PN. Table 2 shows the regression data for vitamin B$_6$ vitamers.

The minimum detectable quantities were 0.020 mg/100 g for PL and PM, 0.017 mg/100 g for PMP, 0.500 mg/100 g for PLP, and 0.033 mg/100 g for PN, with a

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Table 2. Regression Data for Vitamin B$_6$

vitamin B$_6$	retention time (min)	R^2	linear regression
PMP	1.410	0.9997	2.214 + 2.798 (amount)
PM	1.527	0.9993	−0.134 + 2.330 (amount)
PL	2.605	0.9996	−0.587 + 1.397 (amount)
PLM	3.238	0.9967	−1.500 + 0.134 (amount)
PN	3.552	0.9998	−0.211 + 1.802 (amount)

Table 3. Recovery of Vitamin B$_6$ (PMP) Added to Meat Samples by HPLC Method

	before addition (mg/100 g)	amount added (mg/100 g)	amount found (mg/100 g)	recovery[a] (%)
chopped beef	0.212	0.100	0.307	94.0
	0.211	0.100	0.308	95.0
	0.213	0.100	0.305	92.0
mean ± SD				93.7 ± 1.5
	0.213	0.200	0.408	97.5
	0.214	0.200	0.413	100.0
	0.214	0.200	0.410	98.5
mean ± SD	0.213 ± 0.001			98.7 ± 1.3

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

Table 4. Total Vitamin B$_6$ Contents in Cooked Sausages

meat sample[a]	N^b	vitamin B$_6$ content[c] (mg/100 g of FW ± SD)
lunch	20	0.161 ± 0.007
chopped pork	20	0.192 ± 0.009
chopped beef	20	0.211 ± 0.012
chopped turkey	20	0.169 ± 0.005
vitamined chopped	20	0.851 ± 0.030[d]
Sicilian mortadella	20	0.129 ± 0.003

[a] All samples were analyzed in duplicate. [b] N is the number of different samples. [c] PMP values. FW, fresh weight. [d] PMP and PN values.

signal-to-noise ratio of 3. Sensitivity is satisfactory to measure the natural contents in the meat samples studied.

The precision was tested by determining vitamin B$_6$ vitamer contents in six aliquots of the same sample studied in parallel giving a coefficient of variation within a day of 0.5%, and the variation between days (10 samples analyzed in duplicate over 3 months) was 4.3% on average.

The effectiveness of extraction and analysis was evaluated by the recovery of two standard concentrations of PMP (Table 3) added on a representative sample prior to extraction, and the mean percent recovery ranged from 92.0 to 100.0%.

Six commercially cooked sausages (lunch, chopped pork, chopped beef, chopped turkey, vitamined chopped, and Sicilian mortadella) collected weekly from a food factory during a 4 month period were chosen for analysis of vitamin B$_6$ vitamers according to the HPLC method above-described. Table 4 gives results for the vitamin B$_6$ vitamer contents of the meat samples assayed. Each value represents an average of 20 samples analyzed in duplicate. Vitamin B$_6$ vitamer content in each product of the samples tested was constant during the period studied. In all samples except for vitamined chopped, PMP is the vitamer detected and values ranged from 0.129 to 0.211 mg/100 g (in the different studied

sausages). Vitamined chopped contained PMP and PN, with values for PN of 0.651 mg/100 g.

In conclusion, the preparation of a satisfactory method for the extraction and determination of the vitamer compounds individually, rather than combined, in meat products has been interesting and difficult due to the low concentrations of some forms. The final method described in this study uses metaphosphoric acid as optimum protein denaturant with separation on a reverse-phase column. This technique is an efficient and favorable alternative to the anion-exchange method because separations are achieved in half the time, so the application of the present method results in a rapid, sensitive, precise, and accurate procedure adequate for a routine assay.

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