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Short communication

Determination of vitamin B_6 (pyridoxamine, pyridoxal and pyridoxine) in pork meat and pork meat products by liquid chromatography

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Abstract

A liquid chromatographic method for determining vitamin B_6 compounds in pork meat and pork meat products is examined. It uses the same extraction procedure as that applied for thiamin and riboflavin determination, followed by a liquid chromatographic separation on a reversed-phase C_{18} column with 0.01 *M* H_2SO_4 as mobile phase at 30°C. 4-Deoxypyridoxine is used as internal standard. The analytical parameters linearity, precision of the method (R.S.D.=7.3 and 6.9% for pyridoxamine and pyridoxal, respectively) and accuracy obtained by recovery assays (99 and 85.1% for pyridoxamine and pyridoxal, respectively) show that the studied method is useful to measure these compounds in pork meat. © 1998 Elsevier Science B.V.

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1. Introduction

Determining vitamin B_6 poses complicated analytical problems because of the occurrence of multiple biologically active forms of the vitamin at low levels in complex matrices. Vitamin B_6 compounds are well suited to ion-exchange or paired-ion reversed-phase liquid chromatography because of their dependent ionic nature.

Vanderslice and co-workers [1–3] and Olds et al. [4] applied ion-exchange chromatography after carrying out an extraction with sulfosalicylic acid. Bognar [5] measured the non-phosphorylated forms of vitamin B_6 by reversed-phase liquid chromatography using sulfuric acid as mobile phase. Toukairin-Oda et al. [6] determined seven vitamin B_6 derivatives by carrying out perchloric acid extraction and using a single reversed-phase ODS column, an isocratic solvent system (acetonitrile, sodium perchlorate, potassium phosphate buffer, pH 2.5) and a fluorescence detector. Tsuge et al. [7] used a similar method, although the mobile phase consisted of acetonitrile, sodium perchlorate and potassium phosphate buffer (pH 3.5). Ang et al. [8] used metaphosphoric acid as extractant and the separation was carried out on a C_{18} reversed-phase column with potassium phosphate buffer solution adjusted to pH 3 as mobile phase. After extracting the samples with perchloric acid Gregory et al. [9] used a reversed-phase liquid chromatographic method for the fluorometric determination of pyridoxal, pyridoxamine and their 5′-phosphates. Schoonhoven et al. [10] carried

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out an extraction with trichloroacetic acid, and dephosphorylated to facilitate the chromatographic separation. The B_6 vitamins were separated by paired-ion reversed-phase liquid chromatography with methanol and octanesulphonic acid in a potassium phosphate buffer (pH 2.15).

The purpose of this study was to set up a liquid chromatographic method to determine pyridoxine, pyridoxal and pyridoxamine in pork meat and pork meat products that is easy to perform and uses the same extraction procedure applied to determine thiamin and riboflavin. This would permit routine determination of vitamins B_1, B_2 and B_6.

2. Experimental

2.1. Instrumentation

The liquid chromatographic (LC) system consisted of a RF-535 fluorescence detector, a SCL-4A chromatopac integrator, and a SCL-6A control system (Shimadzu, Japan). A Spherisorb ODS C_{18} column (5 μm), 250×4 mm I.D. (Teknochroma, Barcelona, Spain) was used.

2.2. Chemicals

Only analytical-grade substances were used: 4-deoxypyridoxine hydrochloride, pyridoxamine dihydrochloride (Sigma, Taufkirchen, Germany); pyridoxal hydrochloride, pyridoxine hydrochloride (Merck, Darmstadt, Germany); takadiastase from *Aspergillus oryzae*, EC 3.2.1.1. (Fluka, Buchs, Switzerland); hydrochloric acid (d=1.18), sodium acetate anhydrous, sulphuric acid (d=1.83), trichloroacetic acid (Panreac Quimica, Barcelona, Spain); deionised water, Milli-Q water system (Millipore, Jafrey, MA, USA).

The 4-deoxypyridoxine, pyridoxamine, pyridoxal and pyridoxine stock solutions (100 μg/ml) were prepared in deionized water and stored at 4°C in glass light-resistant bottle. The working solutions were prepared daily.

2.3. Samples

Pork meat and pork meat products, 'chorizo', 'adobado' and 'embuchado' pork loin were used. Samples were ground and three 5-g portions were weighed.

2.4. Extraction

The procedure described here is based on that proposed by the AOAC [11] for the fluorometric determination of thiamin and riboflavin. It is the routine method used in our laboratory to measure these two vitamins.

A 60-ml volume of 0.1 *M* HCl and 3 ml of 100 μg/ml of 4-deoxypyridoxine (internal standard, I.S.) were added to 5 g of sample. The mixture was then shaken and left for 30 min in a water-bath at (100±5)°C. Upon cooling, the pH was adjusted to 4–4.5 with 2 *M* sodium acetate, and 5 ml of 10% (w/v) takadiastase was added. This mixture was incubated in a water bath for 3 h at (45±5)°C with shaking and cooled. Then, 2 ml of 50% (w/v) trichloroacetic acid were added and it was heated to 95–100°C for 5 min. After cooling the volume was made up to 100 ml with water and filtered. The extract obtained was stored at +4°C (24 h) or −18°C (1 month), until analysis. Before injection into the chromatograph it was filtered through a 0.22-μm pore-size filter.

If the samples under study do not contain the phosphorylated forms of pyridoxamine, pyridoxal and pyridoxine, the extraction can be done merely by the acid hydrolysis.

2.5. Chromatographic conditions

The method used an octadecyl-silica reversed-phase column and 0.01 *M* H_2SO_4 as the mobile phase. The detector's excitation and emission wavelengths were set at 290 nm and 395 nm, respectively. All measurements were done at 30°C with a flow-rate of 1 ml/min. A 20-ml volume of the sample was injected.

Peak areas were used for quantitative analysis. The 4-deoxypyridoxine is used as I.S.

3. Results

After assaying several H_2SO_4 concentrations

(0.04–0.0025 *M*), 0.01 *M* H_2SO_4 was selected as optimum for the mobile phase, because lower concentrations increase the retention time of the four compounds (Table 1).

Addition of small amounts of methanol or acetonitrile induced a slight reduction in retention times. 0.01 *M* H_2SO_4 was therefore selected as a mobile phase to avoid the use of organic solvents.

Different temperatures (20–40°C) were tested and there was no difference in the peak resolution. A temperature of 30°C was chosen, because as an intermediate temperature it favoured the fluidification of the mobile phase, but did not alter the structure of the column.

A typical chromatogram of standard B_6 vitamins is shown in Fig. 1. The retention times for pyridoxamine, pyridoxal, pyridoxine and 4-deoxypyridoxine were 2.52±0.03, 5.25±0.08, 6.87±0.15 and 11.25±0.31 min, respectively (obtained as the mean of 31 analysis).

The chromatograms corresponding to analysis of fresh pork meat, a sample of 'adobado' and 'embuchado' loin and one of 'chorizo', are shown in Fig. 2.

3.1. Analytical parameters

3.1.1. Linearity and sensitivity

The sensitivities, calculated by applying the method proposed by Knoll [12], were 0.0015, 0.0025 and 0.0125 μg/ml for pyridoxamine, pyridoxal and pyridoxine, respectively.

The responses were linear in the following ranges: 0.0015–0.8 μg/ml ($y=18.23x-0.0688$, $r=0.995$, $n=10$), 0.0025–0.8 μg/ml ($y=9.66x-0.0283$, $r=0.999$, $n=10$) and 0.0125–0.8 μg/ml ($y=9.60x-0.0825$, $r=0.994$, $n=10$) for pyridoxamine, pyridoxal and pyridoxine, respectively, where y is the ratio between the peak area of the standard and the peak area of the I.S. and x the standard content (μg/ml).

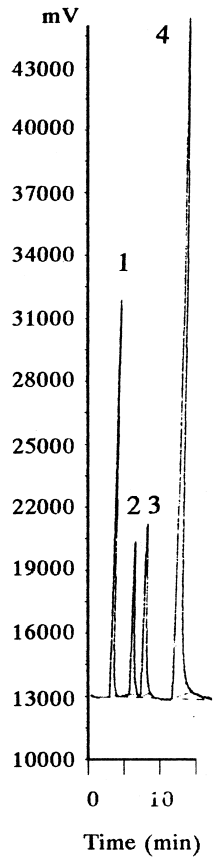

Fig. 1. Chromatogram of standards: (1) pyridoxamine (0.1 μg/ml), t_R=2.6 min; (2) pyridoxal (0.2 μg/ml), t_R=5.4 min; (3) pyridoxine (0.1 μg/ml), t_R=6.9 min; (4) deoxypyridoxine (6 μg/ml), t_R=11.2 min.

Table 1
Retention times (min) of pyridoxamine, pyridoxal, pyridoxine and deopyridoxine at different mobile phase concentration

H_2SO_4 (*M*)	t_R (min)			
	Pyridoxamine	Pyridoxal	Pyridoxine	Deopyridoxine
0.04	2.53	5.22	6.81	11.14
0.02	2.56	5.23	6.73	10.90
0.01	2.62	5.35	6.91	11.18
0.005	2.73	5.61	7.22	11.67
0.0025	2.88	5.90	7.66	12.37

3.1.2. Precision

Instrumental precision was checked from six consecutive injections of a standard solution, the relative standard deviations (R.S.D.s) obtained were 0.92% (0.233±0.0021), 0.37% (0.256±0.0009) and 0.93% (0.274±0.0026) for pyridoxamine, pyridoxal and pyridoxine, respectively. When the standard solution was prepared and measured on alternate days the R.S.D. values were 4.27% (0.227±0.0097, $n=6$), 3.9% (0.261±0.0102, $n=6$) and 3.1%

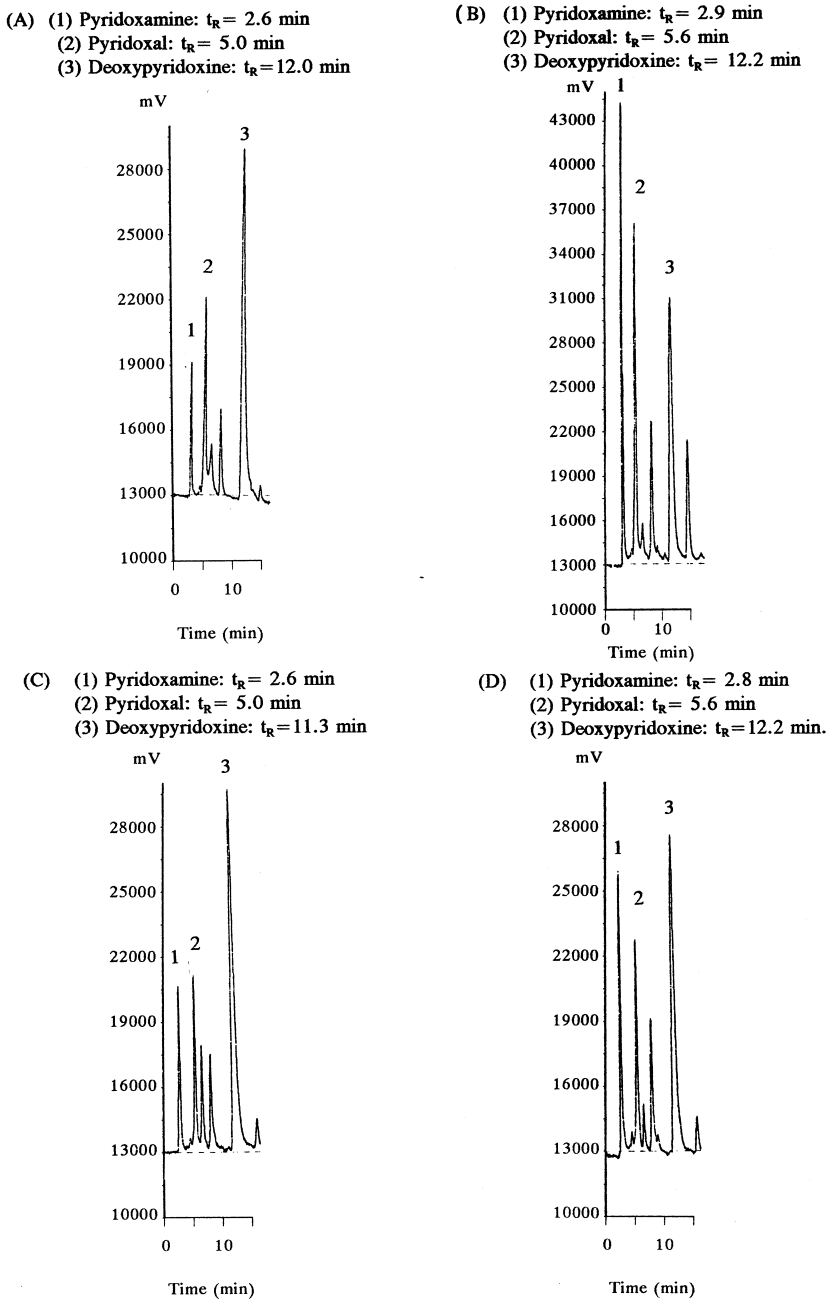

Fig. 2. Chromatograms of (A) fresh pork meat, (B) 'adobado', (C) 'embuchado', and (D) 'chorizo' loin.

(0.263±0.008, n=6) for pyridoxamine, pyridoxal and pyridoxine, respectively.

Instrumental precision was also checked from six consecutive injections of a sample extract. After the first injection it was clear that pyridoxine was not detectable in pork meat. Therefore, from this point on only pyridoxamine and pyridoxal were taken into account. The obtained R.S.D. values were 2.98%

(0.102±0.0030) and 5.25% (0.181±0.0095) for pyridoxamine and pyridoxal, respectively.

The repeatabilities of the method (R.S.D.s) were 7.3% (4.02±0.29 μg/g) and 6.9% (1.27±0.09 μg/g) for pyridoxamine and pyridoxal, respectively.

3.1.3. Accuracy

Accuracy was estimated through recovery assays. The recoveries of standards in reagent blanks subjected to the whole extraction and analytical procedure were 100.9, 86.4 and 72.9% for pyridoxamine, pyridoxal and pyridoxine, respectively. A portion of a meat sample to which a known amount of standard pyridoxamine and pyridoxal (3.7 and 8 μg/g, respectively) was added was subjected to the entire extraction and determination process. The recovery percentages obtained were 99.2 and 85.1% for pyridoxamine and pyridoxal, respectively.

4. Conclusion

The values obtained in the determination of the analytical parameters show that this method is useful to measure vitamin B_6 compounds in pork meat. The method is easy to perform and the fact that the extraction procedure is the same as that used for thiamin and riboflavin determination makes it adequate as the method for routine determinations.

The vitamin B_6 content obtained by applying the proposed method to fresh pork meat is 0.51 (0.46–0.57) mg/100 g, similar to the values reported by Moss et al. [13] (0.35–0.49 mg/100 g), Souci et al. [14] (0.50 mg/100 g) and Moreiras et al. [15] (0.45 mg/100 g).

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