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Analytical Methods Section

HPLC determination of vitamin B_6 in foods after pre-column derivatization of free and phosphorylated vitamers into pyridoxol

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A method to determine the content of vitamin B_6 in foods by ion pair high performance liquid chromatography is proposed and includes pre-column transformation of phosphorylated and free vitamers into pyridoxol followed by fluorescence detection. Dephosphorylation of vitamers is first achieved by enzymatic hydrolysis (acid phosphatase). By reaction with glyoxylic acid in presence of Fe^{2+} as catalyst, pyridoxamine is transformed into pyridoxal, which is then reduced to pyridoxol by the action of sodium borohydride in alkaline medium. The proposed method has a good recovery rate (90–95%), a satisfactory repeatability (coefficient of variation less than 8%) and a very low detection limit (0·02 $\mu g\ g^{-1}$).

INTRODUCTION

Quantitative methods for vitamin B_6 other than chromatographic ones, have several disadvantages such as time-consuming procedures and variability in growth response for the microbiological method (AOAC, 1984) or interfering impurities affecting the accuracy of the fluorometric method (Gregory & Kirk, 1977).

In the last fifteen years, high performance liquid chromatography, first with UV detection (Wong, 1978; Lim *et al.*, 1980), and later with fluorometric detection (Brubacher *et al.*, 1985; Morrison & Driskell, 1985; Bitsch & Moller, 1989) has been widely used for quantitative determination of vitamin B_6 components—pyridoxal (PL), pyridoxol (PN) and pyridoxamine (PM)—in foods. Moreover, preliminary hydrolysis by sulfosalicylic acid, instead of hydrochloric or sulphuric acid, permits the chromatographic separation of the free PN, PL, PM components and the phosphorylated PNP, PLP, PMP forms of vitamin B_6 (Vanderslice *et al.*, 1980, 1981; Gregory & Feldstein, 1985).

In these publications, the samples analyzed were usually either supplemented or simple matrix foods, which do not present serious problems of chromatographic interference. In complex foods, the various vitamin forms which may be naturally present have never been correctly isolated and quantified by the chromatographic methods proposed by Brubacher *et al.* (1985) or Morrison and Driskell (1985) (unpublished results of an interlaboratory analysis organized by the Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes (France)).

Since the free and phosphorylated forms of vitamin B_6 have the same activity (Driskell, 1984), the proposed solution for quantifying the total vitamin B_6 in these complex foods consisted first in a pre-column transformation of the different vitamers into pyridoxol. The latter compound is subsequently separated by ion pair reversed liquid chromatography and quantified by fluorometry.

MATERIALS AND METHODS

Sample preparation

Foods studied (yeast (samples A, B and C from three different manufacturers), wheat germ (samples A and B from two different manufacturers), breakfast cereals (with bran) and muesli) were randomly selected at local sources. They contained guaranteed amounts of vitamin B_6 and were not supplemented with vitamins.

Finely ground sample (2·5 g) was weighed into a conical flask. 0·05 M sodium acetate (pH 4·5) (25 ml) 1 M glyoxylic acid (adjusted to pH 4·5 with 2·5 M sodium acetate (2·5 ml)), ferrous sulphate, $7H_2O$ (2 g $litre^{-1}$) (400 μl) acid phosphatase (from potatoes, Boehringer Grad II lyophilized, 2U mg^{-1} at 37°C) (20 mg) were

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added. The solution was continuously shaken and incubated in an oven at 37°C overnight and then completed to 50 ml with distilled water. This solution was shaken and filtered. A 5-ml aliquot was added to 5 ml of a solution containing 0·2 M sodium hydroxide and 0·1 M sodium borohydride. After shaking, the solution was filtered first through a paper and then through cellulose acetate filter (0·2 μm). This filtrate was used for chromatographic investigation.

Chromatographic determination

Apparatus

The HPLC system consisted of a Model 600E multisolvent delivery system (Waters), a Model 700 WISP injection system (Waters) and a Model 470 scanning fluorescence detector (Waters). Chromatographic peaks were quantified using a Model 991 PDA integrator (Waters).

A Lichrospher 60 RP Select B column (5 mm i.d. × 250 mm; octylsilyl, 5 μm particle size; Merck) and a guard column RP 18 (4 m i.d. × 4 mm); octadecylsilyl, 10 μm particle size; Merck) were used for all analyses.

Chromatographic conditions

Separation by ion pair chromatography was accomplished isocratically with a mobile phase consisting of acetonitrile/0·05 M potassium dihydrogen phosphate (4:96, v/v) containing $0{\cdot}5 \times 10^{-3}$ M sodium heptane sulfonate. The mobile phase was then adjusted to pH 2·50 with phosphoric acid and filtered through cellulose acetate filter (0·45 μm).

The separation was performed at ambient temperature at a flow rate of 1 ml min^{-1}.

The fluorometric detector operated at an excitation wavelength of 290 nm and at an emission wavelength of 395 nm.

The injection volume was 20 μl. Data were quantified using external calibration.

For the recovery tests, known volumes of the standard solutions were added to the sample solution before the enzymatic hydrolysis step.

RESULTS AND DISCUSSION

The dephosphorylation of vitamin B_6 may be achieved either by acid or enzymatic hydrolysis. In the method proposed by Brubacher *et al.* (1985), the sample is hydrolysed by sulphuric acid (0·1 M) at 121°C for 30 min. With this procedure, it has been shown that the PMP → PM conversion rate is rather low (46·5%). Autoclaving for two hours, as mentioned in the AOAC microbiological method (0·44 M HCl, 2 h, 121°C) (AOAC, 1984), is necessary to obtain a conversion rate above 90%. However, such drastic conditions may lead to thermal degradation of B_6 vitamers (Gregory *et al.*, 1986) and induce the formation of chromato-graphic interferences. The mild (enzymatic) conditions of hydrolysis used by Morrison and Driskell (1985) (acid phosphatase, 1 h at 37°C) do not present this drawback. However, according to these authors, the dephosphorylation is not complete (dephosphorylation rate of 70% for PMP into PM). In fact, is has been shown that prolonged incubation (12 h at 37°C), associated with an increased amount of acid phosphatase (40 U in the sample instead of 1·2 U as proposed by Morrison and Driskell (1985)), leads to complete dephosphorylation of each phosphorylated vitamin.

Whatever the procedure used, a specific and rapid conversion of pyridoxamine and pyridoxal into pyridoxol cannot readily be carried out owing to the presence of various reactive chemical groups in these molecules.

For the conversion of pyridoxamine into pyridoxol, none of the possible reactions (i.e. diazotation or enzymatic transamination) was satisfactory. The conversion rate was always below 40%. Therefore, this conversion was performed in two steps: first PM → PL conversion by action of glyoxylic acid (in the presence of ferrous ions), then reduction of pyridoxal into pyridoxol by sodium borohydride in alkaline medium.

The transamination equilibrium:

pyridoxamine + α-ketoglutarate ⇌ pyridoxal + glutarate

has been studied in detail by Metzler and Snell (1952). These authors have specified the optimum conditions for PM → PL conversion as pH 4·5, 100°C for 30 min and the use of Cu^{2+} (0·1 mg $litre^{-1}$) as catalyst. Unfortunately, under such conditions and with equimolar reactant concentration, the equilibrium is only of the order of 24%. In order to increase this conversion rate, a large excess of keto acid (glyoxylic acid instead of α-ketoglutaric acid, as recommended by Gregory and Kirk (1977)) was used. This led to an effective shift of the equilibrium and the PM → PL conversion rate exceeded 95%. Unexpectedly, however, this conversion rate was strongly reduced when the Cu^{2+} concentration was higher than 1 mg $litre^{-1}$ (only 43% for a Cu^{2+} concentration of 20 mg $litre^{-1}$). Since a large excess of copper ion catalyst must be added to a food sample (at the risk of reaction with various food components), this result is a severe drawback. Fortunately, ferrous ions at all concentrations exceeding 1 mg $litre^{-1}$ may be substituted for cupric ions without modifying the reaction rate (usually higher than 95%). For this reason, Fe^{2+} has been used as catalyst (at a concentration of 10 mg $litre^{-1}$) in the proposed method. In order to avoid a possible thermal modification of the matrix, the sample was moderately heated (water bath at 37°C for 12 h instead of 100°C for 30 min). This modification did not affect the PM → PL conversion rate (still above 95%) and allowed simultaneous progress of the desamination and dephosphorylation reactions.

For total conversion of pyridoxal into pyridoxol, Chaikin and Brown (1949) have used sodium borohydride (0·01 M) in alkaline medium (0·2 M sodium hydroxide) as reducing agent, and recommended a reaction time of 2 h at room temperature. Under these experimental conditions, only standard solutions of

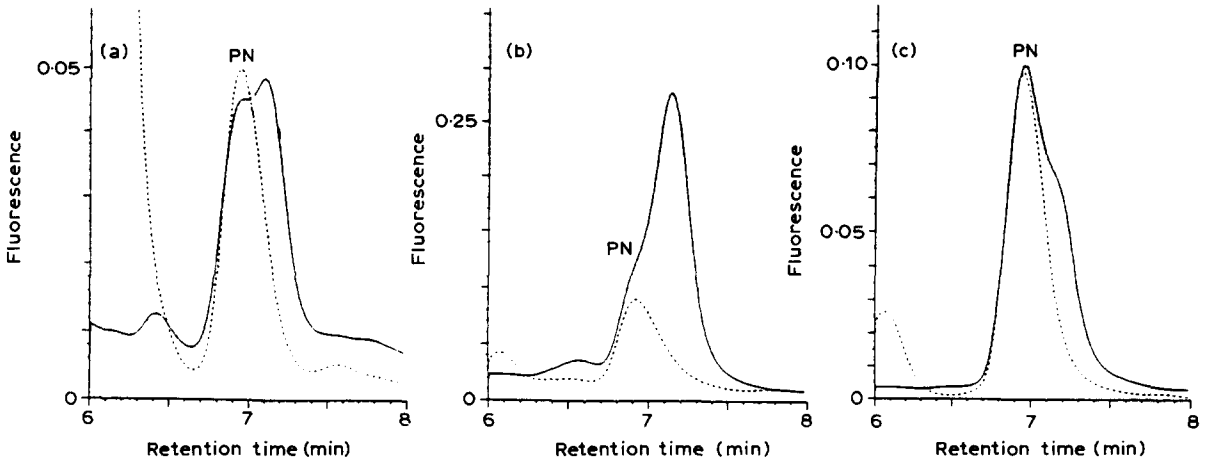

Fig. 1. Chromatographic separation of pyridoxol in various foods : (a) yeast B, (b) breakfast cereal and (c) muesli, according to the proposed method: after hydrolysis with HCl (continuous line); after acid phosphate hydrolysis (dotted line).

pyridoxal were totally reduced into pyridoxol. When food samples were analyzed the conversion rate was variable from one sample to another and was always less than 100%. On the other hand, in all foods studied, the choice of a more concentrated solution of sodium borohydride (0·1 M) led to a complete and instantaneous PL → PN conversion at room temperature.

The three reactions described above (dephosphorylation, either by hydrochloric or acid phosphatase hydrolysis, desamination of pyridoxamine into pyridoxal by glyoxalate in the presence of Fe^{2+} and reduction of pyridoxal into pyridoxol by sodium borohydride in alkaline medium) thus permit a complete conversion of the various forms of vitamin B_6 into pyridoxol.

The separation of pyridoxol has been realized by ion pair reversed liquid chromatography, as already used by Morrison and Driskell (1985), Gregory and Feldstein (1985) and Bitsch and Moller (1989). The retention time of pyridoxol is 6·95 min under the selected experimental conditions (those of pyridoxamine and pyridoxal are respectively 2·7 min and 5·4 min).

In most of the foods studied, interfering substances prevented satisfactory separation and quantification of pyridoxol when dephosphorylation of the sample was achieved with hydrochloric acid hydrolysis. On the other hand, acid phosphatase hydrolysis led to a very good resolution of the pyridoxol peak (Fig. 1) and is to be preferred to hydrolysis using HCl. In wheat germ, however, the separation of pyridoxol was not entirely satisfactory (Fig. 2(a)) but could be markedly improved by a slight increase of the counter ion concentration in the mobile phase (Fig. 2(b)).

The successive conversion steps of pyridoxamine first into pyridoxal and then into pyridoxol were followed by chromatography (Fig. 3). The chromatogram obtained for the determination of total vitamin B_6 as pyridoxol indicated satisfactory separation of this compound (Fig. 3(c)). No traces of residual pyridoxamine or pyridoxal were observed.

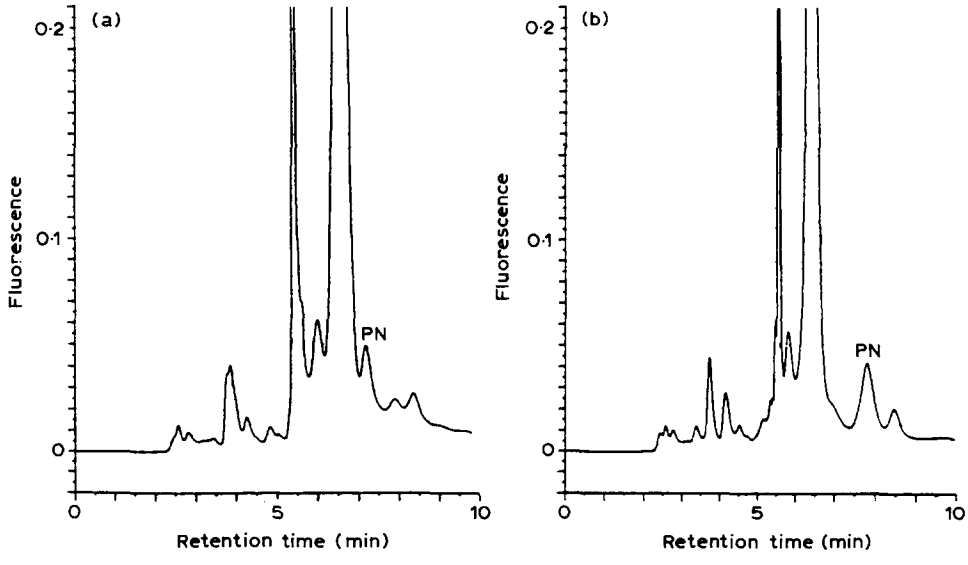

Fig. 2. Chromatographic separation of pyridoxol in wheat germ A with a mobile phase acetonitrile/0·05 M potassium dihydrogen phosphate (4 : 96, v/v) containing either (a) $0{\cdot}5 \times 10^{-3}$ M or (b) $0{\cdot}7 \times 10^{-3}$ M sodium heptane sulfonate and adjusted to pH 2·5 with phosphoric acid.

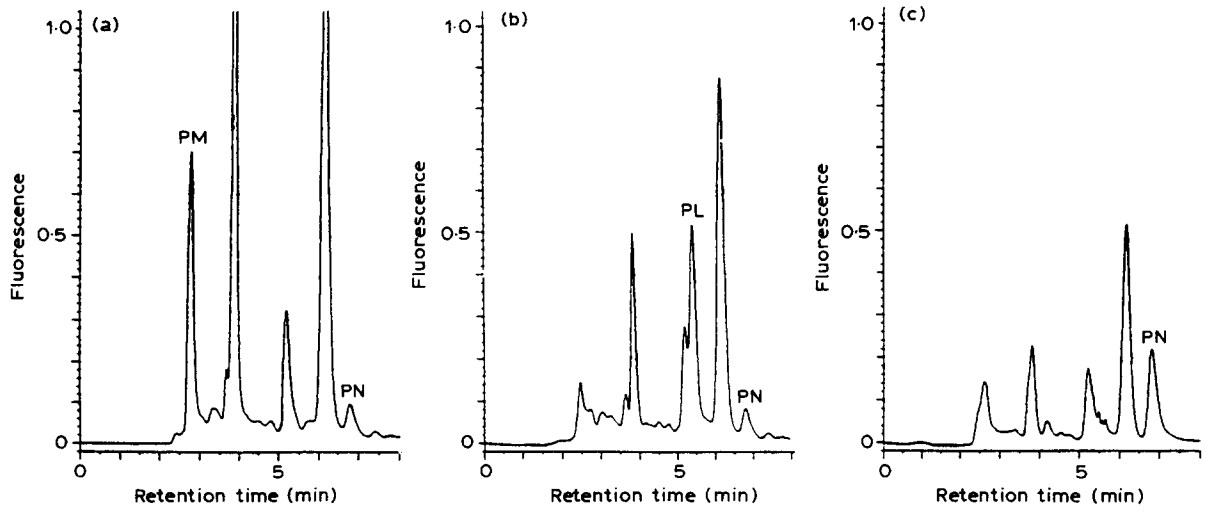

Fig. 3. Chromatographic separation of B_6 vitamers in yeast C, according to the proposed method. (a) with deletion of the desamination and reduction steps; (b) with deletion of the reduction step; (c) without deletion.

The pyridoxol recovery rates, obtained by successive additions of known quantities of standard solutions of pyridoxamine, pyridoxal and pyridoxol in the sample before the enzymatic hydrolysis step were closely similar and always achieved between 90 and 95% for all foods studied (a control of the recovery rate was carried out for each measurement), indicating that the PM → PL and PL → PN conversion rates are as high with food samples as with standard solutions.

The quantification of pyridoxol (taking into account a mean recovery rate of 90%) was obtained by external calibration (linearity range 0–405 $\mu g\ g^{-1}$). The repeatability of the proposed method is satisfactory (Table 1) and the detection limit (0·02 $\mu g\ g^{-1}$) appears largely sufficient when one considers the relatively high amounts of vitamins that are found in the foods studied.

Table 1. Vitamin B_6 contents (expressed as pyridoxol, $\mu g\ g^{-1}$) of various foods (mean of six different determinations)

Food	Vitamin B_6 content ($\mu g\ g^{-1}$)	Standard deviation ($\mu g\ g^{-1}$)	Coefficient of variation (%)
Yeast (A)	18·8	1·0	5·3
Yeast (B)	12·3	0·4	3·3
Yeast (C)	37·7	3·0	7·9
Wheat germ (A)	8·6	0·4	4·6
Wheat germ (B)	4·5	0·1	2·2
Breakfast cereal	21·0	1·2	5·7
Muesli	28·0	1·4	5·0

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