ELSEVIER

Food Chemistry **52** (1995) 81–86

0308-8146/95/$9.50

Analytical Food, Nutritional and Clinical Methods Section

Determination of vitamin B6 in foods by HPLC — a collaborative study

M. Bergaentzlé,[a] F. Arella,[b] J. B. Bourguignon[c] & C. Hasselmann[a,b]*

[a]*Département des Sciences de l'Aliment, Faculté de Pharmacie 74, route du Rhin, 67400 Illkirch, France*

[b]*Laboratoire Interrégional de la Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes, Chemin du Routoir, 67400 Illkirch, France*

[c]*Commission Générale d'Unification des Méthodes d'Analyse, DGCCRF Ministère de l'Economie et des Finances, 59, boulevard Vincent Auriol, 75703 Paris Cedex 13, France*

(Received 5 January 1994; revised version received and accepted 28 February 1994)

A collaborative study was conducted to evaluate the chromatographic method for determination of vitamin B6 in food, recently proposed by Reitzer-Bergaentzlé *et al.* (*Food Chem.* **48** (1993) 321–4). Twelve participants analysed eight samples containing various amounts of vitamin B6 (from 0·6 to 32·8 $\mu g\ g^{-1}$ of pyridoxol). Repeatability relative standard deviations (RSD_r) ranged from 3 to 18%, depending on the vitamin B6 concentration. Reproducibility relative standard deviations (RSD_R) were around 30% for samples containing lowest concentrations of vitamin B6 (< 3 $\mu g\ g^{-1}$ of pyridoxol) and 12–13% when concentration exceeded 5 $\mu g\ g^{-1}$ of pyridoxol, except for the sample of yeast for which the RSD_R was 26%. These values were satisfactory.

Four of the laboratories taking part in this collaborative study have also used the microbiological method recommended by the AOAC. The average results obtained for the two methods were in agreement. The chromatographic method, which is simple and rapid to perform, has been chosen as the official French method for vitamin B6 determination in foodstuff for nutritional purposes.

INTRODUCTION

In recent years, high performance liquid chromatography with fluorometric detection has been recommended often for quantitative determination of vitamin B6 in foods (Vanderslice *et al.*, 1980, 1981; Gregory & Feldstein, 1985; Brubacher *et al.*, 1985; Morrison & Driskell, 1985; Bitsch & Möller, 1989; Olds *et al.*, 1993).

The procedures put forward always envisage a separate determination of the different forms of vitamin B6 (pyridoxol, pyridoxal, pyridoxamine and possibly their phosphorylated forms). For this reason, they are usually inapplicable to foodstuffs with a complex matrix for which an accurate chromatographic isolation of the different vitamers, often present in small amounts, is very difficult. As a result, vitamin B6 determination is still performed by the microbiological method (AOAC, 1990) in the French food control laboratories, even though this method has several disadvantages such as being a complicated, time-consuming procedure and causing variability in growth response of *Saccharomyces uvarum*. This greatly limits the number of laboratories capable of dosing this vitamin, at least in France.

A simple and rapid chromatographic method has recently been proposed (Reitzer-Bergaentzlé *et al.*, 1993). Firstly, this method involves transformation of the different vitamers, which all have the same vitamin activity (Driskell, 1984), into pyridoxol. This compound is then isolated by ion pair chromatography and dosed by fluorometry. 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 $\mu g\ g^{-1}$).

The aim of this collaborative study was to validate this method (repeatability and reproducibility) using judiciously chosen foodstuffs, with a view to recommending it as the official French method for controlling vitamin B6 levels in foodstuffs for nutritional purposes.

MATERIALS AND METHODS

Participants

Participants (12) were analysts in food industries, commercial laboratories, universities and government

* To whom correspondence should be addressed.

laboratories. They received eight different test samples: chocolate powder (obtained from CPC-France, Ludres, France), powdered milk, baby food (vegetable and veal liver) and cereal A (obtained from Nestlé, Vevey, Switzerland), yeast, biscuit, cereal B and solution for tube-feeding (obtained from Créalis — BSN, Brive, France). The participants in this collaborative study firstly familiarized themselves with the method during a pretrial test, for which foodstuffs of a very similar nature were proposed. The results obtained, allowed the final writing of the procedure described below.

For each foodstuff tested, all the laboratories taking part were asked to perform the determination in triplicate. Likewise, the laboratories which had the capability were asked to carry out vitamin B6 determination by the microbiological method (in duplicate) following the AOAC procedure (1990). Unfortunately, only four participants (out of 12) were able to carry out this determination. Therefore, a statistical analysis of the results obtained was not possible.

Reagents

Stock standard solutions. Individual stock standards of pyridoxamine-2HCl, pyridoxal-HCl and pyridoxol-HCl (all from Sigma Chemical Co.) (1 g litre^{-1} as free base) were prepared in distilled water (the stock standards were stable for 4 weeks under refrigeration).

Acid phosphatase from potatoes. Boehringer Grad II lyophilized, 2 U mg^{-1} at 37°C.

Sodium acetate solution. To produce a 2·5 M solution, 170·10 g of sodium acetate, trihydrate (Prolabo, normapur, or equivalent) was dissolved in 500 ml distilled water.

Glacial acetic acid. 100% Merck, pro analysi, or equivalent.

Sodium acetate solution. To produce a 0·05 M solution, 6·80 g of sodium acetate, trihydrate (Prolabo, normapur, or equivalent) was dissolved in *c.* 950 ml distilled water. This was adjusted to pH 4·5 with glacial acetic acid and diluted to 1000 ml with distilled water in a volumetric flask.

Glyoxilic acid solution. For a 1 M solution, 4·70 g glyoxilic acid, monohydrate (Merck, pro analysi, or equivalent) was dissolved in *c.* 30 ml 0·05 M sodium acetate. This was adjusted to pH 4·5 with 2·5 M sodium acetate and diluted to 50 ml with distilled water in a volumetric flask.

Ferrous sulphate solution. For a concentration of 2 g litre^{-1}, 36·56 g of ferrous sulphate, heptahydrate (Merck, pro analysi, or equivalent) was dissolved in 10 ml 0·05 M sodium acetate.

Sodium hydroxide solution. For a 0·2 M solution, 800 mg of sodium hydroxide (Carlo Erba, RPE ACS, or equivalent) was dissolved in 100 ml distilled water.

Sodium borohydride solution. For a 0·1 M solution, 378 mg of sodium borohydride (Merck, pro analysi, or equivalent) was dissolved in 100 ml 0·2 M sodium hydroxide.

Ion-pairing reagents. 1-Heptanesulfonic acid sodium salt and 1-octanesulfonic acid sodium salt (Aldrich).

Acetonitrile. Prolabo, chromanorm for HPLC, or equivalent.

Potassium dihydrogen phosphate solution. For a 0·05 M solution, 6·80 g of potassium dihydrogen phosphate (Prolabo, rectapur, or equivalent) was dissolved in 1000 ml distilled water.

Orthophosphoric acid. (Min. 85%) Merck, pro analysi, or equivalent.

Sample preparation

Finely ground sample (2·5 g if the vitamin B6 content exceeded 2·0 μg g^{-1}; 5 g if the vitamin content was less than 2·0 μg g^{-1}) was weighed into a conical flask. 0·05 M sodium acetate (25 ml), 1 M glyoxilic acid (2·5 ml), ferrous sulphate solution (400 μl), acid phosphatase (20 mg) were added. The solution was continuously shaken and incubated in an oven at 37°C overnight, then, after cooling, made up to 50 ml with distilled water. This solution was shaken and filtered, after centrifugation if necessary. A 5-ml aliquot was added to 4·5 ml of 0·1 M sodium borohydride. Then, after shaking, 0·5 ml of glacial acetic acid was added. The solution was filtered first through a paper and then through cellulose acetate filter (0·45 μm). This filtrate was used for chromatographic investigation.

Chromatographic determination

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 mm i.d. × 4 mm; octadecylsilyl, 10 μm particle size, Merck) were used for all analyses.

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 0·5 × 10^{-3} M sodium heptane sulfonate (or 0·3 × 10^{-3} M sodium octane sulfonate). The mobile phase was then adjusted to pH 2·5 with orthophosphoric acid and filtered through a 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.

Pyridoxol standard solutions for external calibration

Stock standard solution of pyridoxol was firstly diluted to 1/100, then again to 1/10,1/20 and 1/100 with distilled water in order to obtain calibrated solutions containing respectively 1·0 μg ml^{-1}, 0·5 μg ml^{-1} and 0·1 μg ml^{-1} of pyridoxol. These solutions were filtered through a cellulose acetate filter (0·45 μm) and the filtrates were used for external calibration.

Recovery rates

For each foodstuff, four series of three samples were weighed exactly. The three samples of the series 0 were analysed as described above. Concerning the samples of the three other series, an addition of vitamin B6 was

carried out before the incubation stage at 37°C (see Sample preparation):

— 50 μl of pyridoxamine stock standard solution in the series 1 samples
— 50 μl of pyridoxal stock standard solution in the series 2 samples,
— 50 μl of pyridoxol stock standard solution in the series 3 samples.

The recovery rate τ_i obtained for each sample of the same series is given by:

$$\tau_i = \frac{y_i - \bar{k}m_i}{z_i}$$

in which:

i represents the number of the series: 1 (addition of pyridoxamine), 2 (addition of pyridoxal), 3 (addition of pyridoxol),
$\bar{k}$ the average value, for the three samples of the series 0, of the relationship between the amount of pyridoxol (in μg ml^{-1}) present in the solution injected in the chromatograph and the weight of the sample (in g),
y_i the amount of pyridoxol (in μg ml^{-1}) in the solution injected of a sample from the series i,
m_i the weight (in g) of the same sample,
and z_i the amount of pyridoxol (in μg ml^{-1}) in the solution injected, resulting from the transformation of the different forms of the vitamin B6 added, assuming the transformation yield is equal to 100% (z_1 = 0·503 μg ml^{-1}, z_2 = 0·506 μg ml^{-1} and z_3 = 0·500 μg ml^{-1}).

Calculations

The amount X of vitamin B6 in a sample analysed (expressed as μg g^{-1} of pyridoxol) is given by the formula:

$$X = \frac{100x}{m \cdot \bar{\tau}}$$

in which:

x represents the amount of pyridoxol (μg ml^{-1}) in the solution injected for the sample studied,
m the weight (in g) of the sample,
and $\bar{\tau}$ the recovery rate for the method (corresponding to the average of different recovery rates determined in the preceding paragraph).

Statistical methods

Statistical interpretation of the results was carried out according the ISO 5725 standard. Cochran test was performed to remove data showing significantly greater variability among replicate (within laboratory) analyses than other laboratories for a given sample. Dixon test was performed to remove laboratories with extreme averages.

Precision of the method was estimated by calculating the following parameters: repeatability and reproducibility standard deviations (S_r and S_R) and relative standard deviations (RSD = 100 SD/X) for repeatability (RSD_r) and for reproducibility (RSD_R). S_r represents the standard deviation (SD) within laboratory and S_R the standard deviation among laboratories.

RESULTS AND DISCUSSION

The method proposed in the collaborative study for vitamin B6 determination was the method described by Reitzer-Bergaentzlé *et al.* (1993) with minor modifications introduced at the end of the pretrial test, thus: centrifugation before filtration, if necessary, of the solution obtained after overnight incubation at 37°C; use of cellulose acetate filters of 0·45 μm porosity (instead of 0·2 μm) for the filtration and acidification by glacial acetic acid of the solution thus obtained, in order to assure the complete destruction of the excess of sodium borohydride. Concerning the mobile phase used, its composition (proportion of acetonitrile and of potassium dihydrogen phosphate, nature and concentration of counter ion) can of course be slightly modified in order to allow a satisfactory isolation of pyridoxol, whatever the foodstuff analysed.

Four of the chosen foodstuffs had guaranteed levels of vitamin B6 (tube-feeding solution, chocolate powder, cereal A and powdered milk). The tube-feeding solution only contained pyridoxol chlorhydrate. This same vitamer was added in varying quantities to the three other foodstuffs which also contained low quantities of natural vitamin B6. Biscuit, baby food, cereal B and yeast did not have guaranteed levels of vitamin B6 and only contained this vitamin in its natural form. All, with the exception of the tube-feeding solution, were complex matrix foodstuffs. The amounts of vitamin B6 measured in the different foodstuffs during this collaborative study are indicated in Table 1.

Certain laboratory results were rejected, either because they showed significantly greater variability among replicate (within laboratory) analyses than did other laboratories for a given sample (Cochran test, three cases) or because they showed extreme averages (Dixon test, three cases) or for both reasons (one case). The rejection rate (7 out of 96) is not excessive.

Inter-laboratory mean values obtained showed, not surprisingly, that the amounts of vitamin B6 were higher in the foodstuffs which had pyridoxol chlorhydrate added, but that these amounts were quite significant in the other foodstuffs. A statistical analysis of the results is presented in Table 2. The relative standard deviation for repeatability (RSD_r) varied from 18% for the foodstuff with the lowest vitamin B6 content (baby food) to 3% for the foodstuff with the highest vitamin B6 content (powdered milk). The decrease in this parameter was definitely linked to the increase in vitamin B6 levels in the foodstuffs. However the repeatability of the measurement seemed less satisfactory with the cereal A and yeast samples than with the other samples.

Table 1. Collaborative results (in triplicate) on LC determination of vitamin B6 ($\mu g\ g^{-1}$ of pyridoxol) in various foodstuffs

Laboratory	Baby food	Biscuit	Cereal B	Yeast	Tube-feeding solution	Chocolate powder	Cereal A	Powdered milk
	0·6	1·0	2·5	3·7	5·7	7·7	14·7	24·6
1	0·6	1·3	2·4	4·1	5·9	8·2	14·0	24·6
	0·6	1·1	2·3	4·0	6·0	8·0	14·3	24·6
	0·3	0·2[a]	1·6	8·1	5·8	5·9	15·5	35·9
2	0·3	0·0[a]	1·3	8·6	6·1	6·5	15·7	34·5
	0·3	0·2[a]	1·3	8·0	5·7	5·8	16·0	33·7
	0·5	1·7	3·1	4·4	5·8	6·4	13·2	21·4[b]
3	0·4	2·3	2·4	4·8	5·7	6·5	13·6	27·9[b]
	0·4	1·9	2·4	5·1	6·1	6·6	13·9	29·3[b]
	0·7	2·1	3·6	4·7	5·6	7·7	18·6	37·4
4	0·7	1·9	3·3	4·6	5·4	7·1	16·9	35·5
	0·8	2·0	3·3	4·6	5·5	7·5	18·2	39·2
	0·8	2·4	1·9	6·1	6·1	6·5	14·3	32·4
5	0·6	1·9	2·2	4·8	5·9	6·9	15·3	33·5
	0·5	1·9	2·2	4·9	6·2	7·0	15·2	32·9
	0·7	0·8	1·5	6·6	5·9	10·8[a]	16·2	35·2
6	0·7	0·9	1·4	6·7	5·5	11·6[a]	15·7	35·0
	—	—	—	7·0	—	11·8[a]	16·0	35·7
	0·7	1·0	3·1	6·9	4·7	6·3	19·7	35·6
7	0·5	0·9	3·1	7·1	5·3	6·5	16·1	37·1
	0·7	0·9	—	6·7	4·9	6·4	16·1	37·3
	0·8	1·1	1·8	4·3	4·9	5·0	14·0	23·0
8	0·7	1·2	1·6	4·2	5·6	5·5	16·0	27·0
	0·7	1·1	1·5	4·4	5·1	5·2	15·0	28·0
	1·6[b]	2·0	2·3	5·8	6·7	7·4	15·2	36·7
9	2·6[b]	1·8	2·4	4·9	6·6	7·2	15·9	38·5
	1·8[b]	1·8	2·9	4·7	6·6	7·5	16·3	38·2
	0·5	1·2	1·5	5·4	4·5[b]	5·7	13·9	33·3
10	0·5	1·2	1·6	4·7	5·2[b]	5·8	13·8	32·5
	0·5	1·0	1·5	6·4	3·2[b]	6·7	15·8	34·5
	0·5	0·9	2·2	4·4	4·6	7·1	13·9	30·9
11	0·5	0·9	2·4	4·4	4·8	7·3	13·4	30·3
	0·5	0·9	2·3	4·6	4·4	7·3	13·0	31·1
	0·4	5·1[a,b]	5·3[a]	3·9	4·3	6·9	12·6	28·7
12	0·8	4·8[a,b]	4·9[a]	3·7	4·4	6·7	12·4	29·3
	0·4	6·1[a,b]	5·1[a]	3·9	4·4	6·5	11·7	29·3
mean ($\bar{X}$)	0·6	1·4	2·2	5·3	5·5	6·7	15·0	32·8

[a]Dixon test outlier.
[b]Cochran test outlier.

Table 2. Summary statistics[a] for collaborative data on LC determination of vitamin B6 ($\mu g\ g^{-1}$ of pyridoxol) in various foodstuffs

Material	Baby food	Biscuit	Cereal B	Yeast	Tube-feeding solution	Chocolate powder	Cereal A	Powdered milk
n	12	12	12	12	12	12	12	12
n'	11	10	11	12	11	11	12	11
N	32	29	31	36	33	33	35	33
$\bar{X}$	0·6	1·4	2·2	5·3	5·5	6·7	15·0	32·8
S_r	0·1	0·2	0·2	0·4	0·2	0·3	1·0	0·9
RSD_r	18	13	10	8	4	4	6	3
S_R	0·2	0·5	0·7	1·4	0·7	0·8	1·8	4·3
RSD_R	30	35	30	26	13	12	12	13

[a]Symbols used: n = number of participants; n' = number of participants retained; N = number of results; $\bar{X}$ ($\mu g\ g^{-1}$ of pyridoxol) = material mean; for others symbols, see section on statistical methods.

Table 3. Collaborative results (in duplicate) on microbiological determination of vitamin B6 ($\mu g\ g^{-1}$ of pyridoxol) in various foodstuffs

Laboratory	Baby food	Biscuit	Cereal B	Yeast	Tube-feeding solution	Chocolate powder	Cereal A	Powdered milk
5	1·4	2·2	2·3	7·0	5·9	6·5	14·5	33·2
	0·7	2·0	2·3	6·8	4·9	6·2	18·4	—
6	1·1	2·0	3·0	5·9	5·2	4·2	13·8	31·4
	1·1	2·0	2·9	6·0	5·2	5·2	16·9	33·1
8	0·9	1·6	2·2	4·3	5·1	5·0	14·9	31·0
	0·8	1·4	2·2	5·3	4·9	5·6	14·0	30·0
10	1·2	2·2	2·1	4·7	5·9	7·1	20·6	44·3
	1·1	2·2	2·2	4·4	5·2	7·2	15·7	45·2
mean ($\bar{X}$)	1·0	1·9	2·4	5·4	5·3	6·0	16·1	35·5

As far as the reproducibility of the measurements is concerned, the relative standard deviations for reproducibility (RSD_R) were in the order of 12–13% for the foodstuffs with guaranteed vitamin B6 contents and from 30 to 35% for foodstuffs with low levels of vitamin B6, noticeably two to four times greater than the RSD_r. These values were satisfactory considering the magnitude of the concentration measured. In the case of the yeast sample, the RSD_R was relatively high (26%) for a foodstuff rich in vitamin B6 (5·3 $\mu g\ g^{-1}$) This was probably due to the complex composition of the substrate and to the difficulties encountered in bringing about total liberation of the vitamers by enzymatic hydrolysis. Similar problems have already been observed for the determination of vitamins B1 and B2 in this foodstuff (Hasselmann *et al.*, 1989).

It would have been interesting to present a comparative statistical study of the results obtained by the chromatographic method studied above and of those obtained from the microbiological method (AOAC, 1990), the latter being the only method used at the present time in France in the food control laboratories. Unfortunately, out of the 12 laboratories taking part in this interlaboratory study, only three laboratories (6,8 and 10) practised the microbiological method and only one other (laboratory 5) agreed to become acquainted with this method. Consequently the results obtained (Table 3) only concern four laboratories and as a result have not been treated statistically. However, it is important to note that the average values of the vitamin B6 levels resulting from the two methods are very similar and that neither method gives rise systematically to higher or lower levels.

Following this collaborative study, the Commission Générale d'Unification des Méthodes d'Analyse (Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes (France)) has proposed that the chromatographic method studied becomes the official French method for vitamin B6 determination in foodstuffs for nutritional purposes. Indeed in comparison with the microbiological method, it appears to be a rapid method, simple to perform and also provides a reproducibility and detection limit which are entirely satisfactory.

ACKNOWLEDGEMENT

The President of the Commission Générale d'Unification des Méthodes d'Analyse (Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes (France)) thanks the following participants in the collaborative study:

J. Bayle, Laboratoire Interrégional de la DGCCRF, Rennes, France;

F. Chicot Scercat, Ministère de la Défense, Saint Cloud, France;

J. L. Deborde, Laboratoire Interrégional de la DGCCRF, Villeneuve d'Asq., France;

J. M. Dordonnat, Laboratoire Interrégional de la DGCCRF, Marseille, France;

M. F. Gagnepain, Société Scientifique d'Hygiène Alimentaire (ISHA), Longjumeau, France;

D. Guillonneau, Laboratoire AQUANAL, Bordeaux, France;

A. Laschi-Loquerie, Institut Pasteur, Lyon, France;

P. Ruth, CPC France, Ludres, France;

M. H. Salagoity, Laboratoire Interrégional de la DGCCRF, Bordeaux, France;

C. T. Ta, Créalis BSN, Brive, France;

E. Tagliaferri, Nestec Ltd Research Centre, Lausanne, Switzerland.

REFERENCES

AOAC (1990). *Official Methods of Analysis*, 15th Edn, W. Horwitz. Association of Official Analytical Chemists, Washington.

Bitsch, R. & Moller, J. (1989). Analysis of B6 vitamers in foods using a modified high performance liquid chromatographic method. *J. Chromatogr.*, **463**, 207–11.

Brubacher, G., Muller-Mulot, W. & Southgate, D. A. T. (eds) (1985). Vitamin B6 in foodstuffs. In *Methods for the Determination of Vitamins in Food.* Elsevier Appl. Sci., London & New York, pp. 129–40.

Driskell, J. A. (1984). Vitamin B6. In *Handbook of Vitamins—Nutritional, Biochemical and Clinical Aspects*, ed. H. J. Machlin. M. Dekker Inc., New York & Basel, pp. 379–401.

Gregory, J. F. & Feldstein, D. (1985). Determination of vitamin B6 in foods and other biological materials by paired ion high performance liquid chromatography. *J. Agric. Food Chem.*, **33**, 359–63.

Hasselmann, C., Franck, P., Grimm, P., Diop, P. & Soulés, C. (1989). High performance liquid chromatographic analysis of thiamin and riboflavin in dietetic foods. *J. Micronutr. Anal.*, **5**, 269–79.

Morrison, L. A. & Driskell, J. A. (1985). Quantities of B6 vitamers in human milk by high performance liquid chromatography. *J. Chromatogr.*, **337**, 249–58.

Olds, S. J., Vanderslice, J. T. & Brochetti, D. (1993). Vitamin B6 in raw and fried chicken by HPLC. *J. Food Sci.*, **58**, 505–7, 561.

Reitzer-Bergaentzlé, M., Marchioni, E. & Hasselmann, C. (1993). HPLC determination of vitamin B6 in foods after pre-column derivatization of free and phosphorylated vitamers into pyridoxol. *Food Chem.*, **48**, 321–4.

Vanderslice, J. T., Maire, C. E., Doherty, R. F. & Beecher, G. R. (1980). Sulfosalicylic acid as an extraction agent for vitamin B6 in food. *J. Agric. Food Chem.*, **28**, 1145–9.

Vanderslice, J. T., Maire, C. E. & Yakupkovic, J. E. (1981). Vitamin B6 in ready to eat cereals: analysis by high performance liquid chromatography. *J. Food Sci.*, **46**, 943–6.