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Extraction procedures for the liquid chromatographic determination of thiamin, riboflavin and vitamin B_6 in foodstuffs

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Abstract

Treatment with a mixture of enzymes (α-amylase, papain and acid phosphatase) led to the release in a single step of the different forms (phosphorylated and protein-bound) of the vitamins B_1, B_2 and B_6 present in various food samples, not supplemented with vitamins. Analysis by means of HPLC/fluorimetry of the extracts obtained showed that the vitamin contents were at least as high as those found when the enzymatic treatment was performed with the aid of the most efficient diastase. Hydrochloric acid hydrolysis, very often combined with the enzymatic treatment and the role of which is to denature the proteins, proved to be superfluous owing to the presence of a protease in the mixture of enzymes used. When reference materials were analysed, the protocol of extraction proposed always made it possible to obtain vitamin contents in agreement with the certified values.

1. Introduction

In foodstuffs, the vitamins B_1, B_2 and B_6 may be present in free (thiamin, riboflavin, pyridoxol, pyridoxal and pyridoxamine) and phosphorylated forms [essentially thiamin pyrophosphate, riboflavin-5′-phosphate (FMN), riboflavin-5′-adenosyldiphosphate (FAD) and pyridoxal phosphate]. Furthermore, they may be bound tightly but non-covalently to proteins. Only a very small portion of the FAD is thought to be bound covalently to amino acids in mammalian tissues (Ball, 1998). In plant tissues, an often considerable percentage of pyridoxol may also be linked to sugars to form conjugate glucosides. Pyridoxol glucosides, just like covalently bound FAD, are vitamin forms of very low bioavailability in man (Ball, 1998).

The use of high performance liquid chromatography coupled with fluorimetric detection has enabled specific and sensitive methods to be developed for the determination of these vitamins in foodstuffs. Among the very many methods suggested (Ball, 1994, 1998), vitamins are most often determined in the free form, which involves hydrolysis of the phosphorylated forms and/or those bound to proteins (and optionally glycosylated) during the extraction step performed prior to the chromatographic isolation.

In the determination of the vitamins B_1 and B_2, this extraction usually consists of an acid hydrolysis and an enzymatic treatment. The purpose of the hydrochloric acid treatment usually performed (0.1 M HCl in a water bath at 100°C or in an autoclave at 121°C) is essentially to denature the proteins and to release the vitamins from their association with the proteins and, secondarily, if the foodstuff under study contains a lot of starch, to convert this polysaccharide into soluble sugars. As for the enzymatic treatment, it must permit the dephosphorylation of the vitamins. For economic reasons, the enzymes selected are almost always diastases possessing a phosphatase activity. Now all of these diastases also have a protease activity. It is thus possible to ask whether it is essential to carry out an acid hydrolysis under these conditions.

The extraction protocols suggested for the determination of vitamin B_6 in the free form are more varied. Some authors (Bognar, 1985; Brubacher, Müller-Mulot & Southgate, 1985) have simply recommended dephosphorylation by means of sulfuric acid. Other authors have

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preferred to perform this dephosphorylation by means of an acid phosphatase (Bognar & Ollilainen, 1997; Morrison & Driskell, 1985; Reitzer-Bergaentzlé, Marchioni & Hasselmann, 1993; Sierra & Vidal-Valverde, 1997) or by means of a takadiastase (van Schoonhoven, Schriver, van den Berg & Haenen, 1994; van Schaik, Finglas & Froidmont-Görtz, 1996), sometimes combined it with a β-glucosidase treatment (Bognar & Ollilainen, 1997; Sierra & Vidal-Valverde, 1997; van den Berg et al., 1996) in order to determine the glycosylated forms of vitamin B_6 as well. In some cases, the enzymatic treatments were preceded by mineral acid hydrolysis (Bognar & Ollilainen, 1997; van den Berg et al., 1996).

During preliminary studies for the development of a French method of reference for the determination of vitamin B_6 in foodstuffs (Bergaentzlé, Arella, Bourguignon & Hasselmann, 1995), it had, however, been shown that sulfuric acid hydrolysis alone (0.1 M H_2SO_4, autoclave 121°C, 1 h) only led to partial dephosphorylation of the phosphorylated forms of vitamin B_6 and that the combination of mineral acid hydrolysis with a phosphatase (or diastase) treatment was justified only in the case in which the foodstuff to be analysed contained a lot of starch and where the hydrolysis of this polymer proved necessary to eliminate filtration problems when the analytical protocol was used.

The primary objective of this study, performed on a range of foodstuffs (cereals, vegetables, milk, meats, fish, yeast) not supplemented with vitamins, was thus to verify during the determination of the vitamins B_1, B_2 and B_6 whether it was really necessary to perform an acid hydrolysis when it is accompanied by a treatment with a diastase (vitamins B_1 and B_2) or a phosphatase (vitamin B_6).

The second objective was to try to show that it was possible to develop a standard mixture consisting of several enzymes (protease, phosphatase, amylase) which is at least as efficient as the most efficient diastase, but which has more reproducible enzymatic activities and hence is better adapted than a diastase to use in a reference analytical protocol.

2. Materials and methods

2.1. Reagents

The vitamin standards (thiamin-HCl, thiamin pyrophosphate, thiamin monophosphate, riboflavin, riboflavin-5′-adenosyldiphosphate, riboflavin-5′-phosphate, pyridoxol-HCl, pyridoxal phosphate and pyridoxamine phosphate) were obtained from Sigma Chemicals (Saint-Quentin Fallavier, France).

The following enzymes were used: takadiastase from *Aspergillus oryzae* (Fluka, catalogue No.86247), takadiastase (Pfaltz and Bauer, catalogue No.T00040), clara-diastase (Fluka, catalogue No.27540), papain (Prolabo, catalogue No.26.146.180), α-amylase from *Aspergillus oryzae* (Sigma, catalogue No.A0273), acid phosphatase from potato, grade II (Boehringer, catalogue No.108227) and β-glucosidase from almonds (Sigma, catalogue No.G0395). Their enzymatic activities were measured at pH 4.5 (50 mM sodium acetate solution) and 37°C (oven) according to the quality control protocols provided by Sigma–Aldrich (amylase activity: 1 unit releases 1 mg of maltose from starch in 3 min; protease activity: 1 unit releases 1 μmol of tyrosine from casein in 1 min; phosphatase activity: 1 unit releases 1 μmol of 4-nitrophenol from 4-nitrophenyl phosphate in 1 min; β-glucosidase activity: 1 unit releases 1 μmol of D-glucose from salicin in 1 min). The relative phosphatase activities of several enzymes towards the phosphorylated forms of the vitamins were measured after 18 h incubation under the same conditions as above (pH 4.5 and 37°C). Impurities of vitamins B_1, B_2 and B_6 were found in relatively high concentrations in Pfaltz and Bauer takadiastase and clara-diastase. On the other hand, Fluka takadiastase contained only traces of vitamins B_2 and B_6. Low concentrations of vitamin B_2 were also found in α-amylase and acid phosphatase. All the results given in this study were systematically enzyme blank corrected.

Acetonitrile and methanol (for HPLC) were obtained from Carlo Erba (Milan, Italy). All other chemicals used were of the highest purity available.

2.2. Sample preparation

Foods studied [yeast, powdered milk, pork (escalope), veal (escalope), mackerel (fillet), wheat flour, porridge oats, rice, peas, orange juice and carrots] were purchased at local sources. They were not supplemented with vitamins. The certified reference materials [pig's liver (CRM 487) and milk powder (CRM 421)] were obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). The various samples (1 g of yeast, 2.5 g of certified reference materials, 5 g of other foodstuffs) were finely ground.

2.2.1. Extraction protocols for thiamin and riboflavin determinations

(a) Fifty millilitres of 0.1 M hydrochloric acid were added to the sample in a 250 ml conical flask. The solution was placed in a water bath at 100°C or in an autoclave at 121°C for 30 min. After being allowed to cool it was adjusted to pH 4.5 with 2.5 M sodium acetate. Takadiastase or clara-diastase (500 mg) was added. The solution was incubated for 18 h in an oven at 37°C, then diluted to 100 ml with distilled water in a volumetric flask and filtered through a filter paper. The filtrate obtained after a second filtration through a cellulose

acetate filter (0.2 μm) was used for the chromatographic determination of riboflavin. An aliquot of the first filtrate (1 ml) was used for the pre-column derivatization to thiochrome (Arella, Lahély, Bourguignon & Hasselmann, 1996).

(b) Same protocol as in (a), but no diastase was added.
(c) Fifty millilitres of 0.05 M sodium acetate (pH 4.5), followed by 500 mg of takadiastase or claradiastase, were added to the sample in a 250 ml conical flask. The solution was incubated for 18 h in an oven at 37°C and then treated as in (a).
(d) Same protocol as in (c), but no diastase was added.
(e) Same protocol as in (c), but the diastase was replaced by a mixture of papain (100 mg, or less), 1% glutathione (500 μl), acid phosphatase (20 mg, or less) and α-amylase (10 mg, or less).
(f) Same protocol as in (c), but the diastase was replaced by a mixture of papain (100 mg), 1% glutathione (500 μl) and α-amylase (10 mg).

2.2.2. Extraction protocols for vitamin B_6 determination

(a) Fifty millilitres of 0.1 M hydrochloric acid were added to the sample in a 250 ml conical flask. The solution was placed in a water bath at 100°C or in an autoclave at 121°C for 30 min. After being allowed to cool it was adjusted to pH 4.5 with 2.5 M sodium acetate. 1 M glyoxylic acid (2.5 ml), 2% ferrous sulfate solution (400 μl) and acid phosphatase (20 mg) were added. The solution was incubated for 18 h in an oven at 37°C, then diluted to 100 ml with distilled water in a volumetric flask and filtered through a filter paper. A 5 ml aliquot was added to 4.5 ml of a solution of 0.2 M sodium hydroxide and 0.1 M sodium borohydride. After being shaken, this solution was left to stand for 5 min. Glacial acetic acid (0.5 ml) was added. The solution was then filtered through a cellulose acetate filter (0.5 μm).
(b) Fifty millilitres of 0.05 M sodium acetate (pH 4.5), followed by 2.5 ml of 1 M glyoxylic acid, 400 μl of 2% ferrous sulfate solution and 20 mg (or less) of acid phosphatase (or 500 mg of diastase) were added to the sample in a conical flask. The solution was incubated for 18 h in an oven at 37°C and then treated as in (a).
(c) Same protocol as in (b), but the acid phosphatase was replaced by a mixture of papain (100 mg), 1% glutathione (500 μl), acid phosphatase (20 mg, or less) and α-amylase (10 mg).
(d) Same protocol as in (b), but without enzymes.
(e) Same protocol as in (b), but the acid phosphatase was replaced by 20 mg of β-glucosidase.
(f) Same protocol as in (e), but 20 mg of acid phosphatase or 500 mg of Pfaltz and Bauer takadiastase were added to the β-glucosidase.

2.3. Chromatographic determination

2.3.1. Apparatus

The HPLC system consisted of a 3012 multisolvent delivery system (Varian, Les Ulis, France), a 9300 injection system (Varian) and a 9075 fluorescence detector (Varian). Chromatographic peaks were quantified using a Star Chromatographic integrator (Varian).

A Lichrospher 100RP 18 endcapped (4 mm i.d. x 250 mm; octadecylsilyl; 5 μm particle size; Merck) and a guard column RP 18 (4 mm i.d. x 4 mm; octadecylsilyl; 5 μm particle size; Merck) were used for all analyses.

2.3.2. Chromatographic conditions

The conditions used were those described by Arella et al. (1996) for the determination of thiamin and riboflavin, and by Bergaentzlé et al. (1995) for the determination of vitamin B_6.

3. Results

Table 1 presents the percentages of nonphosphorylated (free and bound to proteins) and phosphorylated forms of thiamin, riboflavin and vitamin B_6 for five of the foodstuffs studied. These results were obtained by application of the reference analytical methods (Arella, et al., 1996; Bergaentzlé et al., 1995) with or without the steps of acid and/or enzymatic hydrolysis, then by performing enzymatic hydrolysis with the aid of a mixture of papain (100 mg) and α-amylase (10 mg) to which potato acid phosphatase (20 mg) was or was not added. Phosphorylated thiamin always appears in small proportions, especially in wheat flour, peas and powdered milk. In this last, riboflavin is essentially present in the free form, as was expected. On the other hand, this form is almost absent in pork meat and peas. As for vitamin B_6, nonphosphorylated forms bound to proteins have never been found, irrespective of the foodstuffs studied.

The activities of the different enzymes used in the course of this study are given in Table 2. Their phosphatase activities were determined by using 4-nitrophenyl phosphate as substrate and there is, of course, nothing to suggest that the phosphatases of these enzymes will hydrolyse this reference substrate and the different phosphorylated forms of the vitamins with the same efficiency. A comparative study of the phosphatase activity of three of these enzymes towards the principal phosphorylated vitamin forms is summarized in Table 3. The quantities of substrate selected (in μmol) corresponded approximately to the quantities of vitamins, expressed as μg of free vitamins, contained in 1 g of yeast (500 μg of thiamin, 50 μg of riboflavin and 60 μg of pyridoxol). The results obtained showed that potato acid phosphatase hydrolysed thiamin pyrophosphate much more difficultly than the phosphatases of the two

Table 1
Estimation of the percentage of nonphosphorylated forms [free (1) or protein- bound (2)] and phosphorylated (3) forms of thiamin, riboflavin and vitamin B_6 (pyridoxol, pyridoxal, pyridoxamine) in various foodstuffs

	Yeast			Pork			Wheat flour			Powdered milk			Peas		
Vitamins	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
Thiamin	45	35	20	60	25	15	50	45	5	75	25	0	50	45	5
Riboflavin	30	5	65	10	60	30	30	65	5	80	15	5	5	80	15
Vitamin B_6	5	0	95	35	0	65	85	0	15	75	0	25	70	0	30

Table 2
Activities of the different enzymes used (measured at pH 4.5 and 37°C)

Enzymes	Activity			
	Protease	Phosphatase	Amylase	β-Glucosidase
Clara-diastase (U/500 mg)	3.3	51	13.2×10^4	0
Takadiastase Pfaltz and Bauer (U/500 mg)	4.0	48	9.3×10^4	0
Takadiastase Fluka (U/500 mg)	0.2	2	9.8×10^4	0
Acid phosphatase (U/20 mg)	0	100	0	0
Papain (U/100 mg)	10	0	0	0
α-Amylase (U/10 mg)	0.24	0	1800	0
β-Glucosidase (U/20 mg)	–	8	–	104

Table 3
Estimation of the quantities of enzymes [expressed in mg and activity (U)] necessary for obtaining complete hydrolysis (>95%) of the phosphorylated forms of vitamins B_1, B_2 and B_6

Substrate	Quantity (μmole)	Acid phosphatase from potato		Takadiastase (Fluka)		Takadiastase (Pfaltz and Bauer)	
		mg	U	mg	U	mg	U
Thiamin pyrophosphate	1.8	1–5	5–25	50–100	0.2–0.4	5–10	0.5–1.0
Thiamin monophosphate	1.9	⩽0.5	⩽2.5	5–25	0.02–0.1	⩽1	⩽0.1
FMN	0.1	⩽0.5	⩽2.5	5–10	0.02–0.04	⩽1	⩽0.1
FAD	0.1	⩽0.5	⩽2.5	50–100	0.2–0.4	5–10	0.5–1.0
Pyridoxal phosphate	0.4	⩽0.5	⩽2.5	250–500	1–2	200–300	20–30
Pyridoxamine phosphate	0.4	⩽0.5	⩽2.5	250–500	1–2	200–300	20–30

takadiastases tested, but that it was much more efficient than the phosphatase contained in the Pfaltz and Bauer takadiastase in the hydrolysis of the phosphorylated forms of vitamin B_6. The clara-diastase behaved in a completely identical manner with that of the Pfaltz and Bauer takadiastase. In other respects, the phosphatase activities of papain (100 mg) and α-amylase (10 mg) toward the different phosphorylated vitamin forms were always negligible.

During the thiamin determination, it was observed that the contents obtained on combining hydrochloric acid hydrolysis (in a water bath at 100°C or in an autoclave at 121°C) with an enzymatic hydrolysis with the Fluka takadiastase were identical with those found on performing hydrolysis with this diastase alone (Table 4a). The replacement of the Fluka takadiastase by equal quantities of other diastases (Pfaltz and Bauer takadiastase, clara-diastase) with higher protease and phosphatase activities has never led to an increase of the thiamin contents in the foodstuffs studied (Table 4b). By substituting the diastases by a mixture of 100 mg of papain, 20 mg of potato acid phosphatase (whose activities are approximately equal to twice that of the Pfaltz and Bauer takadiastase) and 10 mg of α-amylase from *Aspergillus oryzae* (a quantity a priori sufficient to solubilize completely the starch present in the different foodstuffs studied), contents of thiamin totally identical with those obtained by using the Pfaltz and Bauer takadiastase were found once again (Table 4c).

When enzymatic hydrolysis was performed with Fluka takadiastase for the determination of riboflavin, hydrochloric acid hydrolysis always proved to be unnecessary,

except in the analysis of porridge oats (Table 5a). In this case, the addition of a protease (100 mg of papain) to the diastase enabled a riboflavin content to be obtained [0.733 (0.002) μg g^{-1}] which was completely identical with that obtained by performing hydrochloric acid hydrolysis and a treatment with Fluka takadiastase successively (see Table 5a). On replacing Fluka takadiastase by equal quantities of Pfaltz and Bauer takadiastase or clara-diastase, higher riboflavin contents were obtained in all of the foodstuffs studied [increase of 6% in yeast and upto 32% in porridge oats (Table 5b)]. These riboflavin contents were not modified by performing a preliminary hydrochloric acid hydrolysis (0.1 M HCl, autoclaving at 121°C, 30 min) (Table 5c). By carrying out the hydrolysis step with the same enzyme mixture as above, riboflavin contents identical with those obtained by using the Pfaltz and Bauer takadiastase were found (Table 5d).

The French method of reference for the determination of vitamin B_6 recommends only a phosphatase treatment (Bergaentzlé et al., 1995). The results obtained during this study confirmed that preliminary hydrochloric acid hydrolysis was not necessary (Table 6a). For two of the foodstuffs studied (samples of pork and veal), it was even observed that the acid treatment (0.1 M HCl,

Table 4
Influence of the extraction protocol on the thiamin concentration (μg g^{-1}) in various foodstuffs[a]

Extraction protocol		Concentration							
		Yeast	Pork (escalope)	Mackerel	Wheat flour	Rice	Porridge oats	Powdered milk	Peas
a	(1)[b]	475 (31)	11.0 (0.4)	1.25 (0.08)	2.84 (0.09)	2.54 (0.07)	4.58 (0.07)	3.15 (0.12)	2.86 (0.06)
	(2)[c]	463 (17)	10.9 (0.4)	1.28 (0.08)	2.97 (0.17)	2.39 (0.06)	4.70 (0.12)	3.13 (0.08)	2.91 (0.07)
	(3)[d]	449 (29)	10.5 (0.3)	–	2.94 (0.27)	–	–	3.01 (0.13)	2.79 (0.27)
b	(1)	499 (27)	9.47 (0.08)	–	2.64 (0.06)	–	–	2.57 (0.11)	2.20 (0.07)
	(4)[e]	525 (15)	9.57 (0.06)	–	2.62 (0.07)	–	–	2.67 (0.12)	2.17 (0.08)
	(5)[f]	512 (24)	9.59 (0.06)	–	2.46 (0.08)	–	–	2.66 (0.06)	2.26 (0.33)
c	(4)	470 (6)	9.07 (0.33)	–	2.56 (0.22)	–	–	2.49 (0.18)	2.08 (0.07)
	(6)[g]	465 (5)	9.17 (0.19)	–	2.61 (0.06)	–	–	2.67 (0.12)	2.17 (0.08)

[a] Average of three determinations (standard deviation in brackets) performed on three different batches (a, b and c) of foodstuffs.
[b] (1) Fluka takadiastase (500 mg per sample, 18 h, 37°C).
[c] (2) HCl 0.1 M (water bath at 100°C, 30 min) and Fluka takadiastase (500 mg per sample, 18h, 37°C).
[d] (3) HCl 0.1 M (autoclaving at 121°C, 30 min) and Fluka takadiastase (500 mg per sample, 18 h, 37°C).
[e] (4) Pfaltz and Bauer takadiastase (500 mg per sample, 18 h, 37°C).
[f] (5) Clara-diastase (500 mg per sample, 18 h, 37°C).
[g] (6) 100 mg of papain, 10 mg of α-amylase and 20 mg of acid phosphatase per sample (18 h, 37°C).

Table 5
Influence of the extraction protocol on the riboflavin concentration (μg g^{-1}) in various foodstuffs[a]

Extraction protocol		Concentration							
		Yeast	Pork (escalope)	Mackerel	Wheat flour	Rice	Porridge oats	Powdered milk	Peas
a	(1)[b]	29.6 (2.6)	1.19 (0.06)	2.92 (0.18)	0.414 (0.019)	0.288 (0.007)	0.563 (0.009)	10.7 (0.2)	0.72 (0.02)
	(2)[c]	30.5 (0.9)	1.07 (0.07)	3.15 (0.13)	0.437 (0.020)	0.271 (0.008)	0.733 (0.007)	10.9 (0.2)	0.77 (0.06)
	(3)[d]	26.5 (1.0)	0.98 (0.09)	–	0.424 (0.008)	–	–	11.4 (0.1)	0.76 (0.03)
b	(1)	47.3 (0.7)	1.18 (0.03)	–	0.445 (0.010)	–	0.53 (0.04)	13.5 (0.1)	0.68 (0.02)
	(4)[e]	50.2 (0.5)	1.55 (0.04)	–	0.526 (0.026)	–	0.78 (0.02)	14.7 (0.2)	0.89 (0.02)
	(5)[f]	50.4 (0.2)	1.57 (0.03)	–	0.533 (0.011)	–	–	14.9 (0.5)	0.89 (0.05)
c	(4)	44.8 (1.0)	1.84 (0.03)	–	0.514 (0.011)	–	–	13.9 (0.4)	1.14 (0.05)
	(6)[g]	42.1 (1.0)	1.77 (0.03)	–	0.530 (0.003)	–	–	14.3 (0.3)	1.11 (0.04)
d	(4)	63.4 (2.5)	1.70 (0.09)	–	0.517 (0.012)	–	–	14.6 (0.1)	0.86 (0.04)
	(7)[h]	63.6 (3.2)	1.73 (0.07)	–	0.524 (0.016)	–	–	14.7 (0.2)	0.90 (0.03)

[a] Average of three determinations (standard deviation in brackets) performed on four different batches (a, b, c and d) of foodstuffs.
[b] (1) Fluka takadiastase (500 mg per sample, 18 h, 37°C).
[c] (2) HCl 0.1 M (water bath at 100°C, 30 min) and Fluka takadiastase (500 mg per sample, 18 h, 37°C).
[d] (3) HCl 0.1 M (autoclaving at 121°C, 30 min) and Fluka takadiastase (500 mg per sample, 18 h, 37°C).
[e] (4) Pfaltz and Bauer takadiastase (500 mg per sample, 18 h, 37°C).
[f] (5) Clara-diastase (500 mg per sample, 18 h, 37°C).
[g] (6) HCl 0.1 M (autoclaving at 121°C, 30 min) and Pfaltz and Bauer takadiastase (500 mg per sample, 18 h, 37°C).
[h] (7) 100 mg of papain, 10 mg of α-amylase and 20 mg of acid phosphatase per sample (18 h, 37°C).

Table 6
Influence of the extraction protocol on the vitamin B_6 concentration (expressed in μg g^{-1} of equivalent pyridoxol) in various foodstuffs[a]

Extraction protocol		Concentration							
		Yeast	Pork (escalope)	Veal	Mackerel	Wheat flour	Porridge oats	Powdered milk	Peas
a	(1)[b]	25.3 (0.8)	6.37 (0.08)	5.35 (0.08)	–	1.12 (0.09)	–	2.74 (0.06)	0.78 (0.03)
	(2)[c]	26.3 (1.8)	5.64 (0.06)	4.72 (0.06)	–	1.20 (0.09)	–	2.50 (0.07)	0.76 (0.02)
b	(3)[d]	57.0 (3.0)	6.42 (0.20)	–	–	1.22 (0.08)	–	2.24 (0.09)	1.17 (0.08)
	(4)[e]	59.3 (1.0)	5.81 (0.55)	–	–	1.20 (0.06)	–	1.98 (0.08)	1.07 (0.09)
c	(1)	63.3 (1.9)	4.23 (0.14)	–	4.75 (0.19)	1.10 (0.16)	1.42 (0.11)	2.15 (0.14)	0.71 (0.04)
	(3)	58.6 (1.2)	4.40 (0.16)	–	4.71 (0.38)	1.06 (0.11)	1.39 (0.07)	2.05 (0.14)	0.78 (0.03)
d	(1)	28.0 (0.2)	6.99 (0.22)	–	–	1.41 (0.05)	–	2.50 (0.08)	1.23 (0.08)
	(5)[f]	27.8 (0.4)	7.22 (0.06)	–	–	1.36 (0.02)	–	2.50 (0.11)	1.12 (0.10)

[a] Average of three determinations (standard deviation in brackets) performed on four different batches (a, b, c and d) of foodstuffs.
[b] (1) Acid phosphatase (20 mg per sample, 18 h, 37°C).
[c] (2) HCl 0.1 M (water bath at 100°C, 30 min) and acid phosphatase (20 mg per sample, 18 h, 37°C).
[d] (3) Fluka takadiastase (500 mg per sample, 18 h, 37°C).
[e] (4) Pfaltz and Bauer takadiastase (500 mg per sample, 18 h, 37°C).
[f] (5) 100 mg of papain, 10 mg of α-amylase and 20 mg of acid phosphatase per sample (18 h, 37°C).

water bath at 100°C, 30 min) led to a statistically significant fall, of the order of 10–15%, in the vitamin B_6 contents. Such a diminution was also noted during the analysis of samples of carrots after hydrochloric acid hydrolysis (0.1 M) in an autoclave at 121°C for 30 min (see Table 7). The replacement of the acid phosphatase by a takadiastase (Fluka or Pfaltz and Bauer) or the addition of papain (100 mg) and α-amylase (10 mg) to this enzyme led to the production of completely identical vitamin B_6 contents (Table 6b, c and d), which confirms, at least in the foodstuffs studied which did not contain any glycosylated forms of vitamin B_6, the unique importance of the phosphatase activity in the diastases used. For the analysis of two food samples (orange juice and carrots) in which the glycosylated forms represent more than 50% of the total vitamin B_6 (Leklem, 1996), different conditions of hydrolysis were proposed (Table 7). The results obtained showed that a preliminary treatment with hydrochloric acid in an autoclave did not hydrolyze the glycosylated forms of the vitamin B_6 and that all the diastases used had a β-glucosidase activity sufficient to release the glycosylated forms of vitamin B_6 present in the two foodstuffs studied [this activity had not been detected by using salicin as substrate (see Table 2)]. Since no phosphorylated vitamin B_6 was present in the carrot samples, the absence of β-glucosidase activity from the various enzymes constituting the enzymatic mixture toward the glycosylated forms of vitamin B_6 is proved. The phosphatase activity of the β-glucosidase enzyme used was largely sufficient to carry out a complete release of the phosphorylated vitamin B_6 present in the orange juice.

The enzymatic mixture proposed for the extraction (100 mg of papain, 20 mg of acid phosphatase and 10 mg of α-amylase) was used for the determination of vitamins B_1, B_2 and B_6 contents in two reference certified materials [pig's liver (CRM 487) and milk powder (CRM 421)] instead of the classical protocol of extraction (treatment with dilute acid with heating, followed

Table 7
Influence of the extraction protocol on the vitamin B_6 concentration (expressed in μg g^{-1} of equivalent pyridoxol) in foodstuffs containing glycosylated forms of this vitamin[a]

Extraction protocol	Concentration	
	Orange juice	Carrots
(1)[b]	0.066 (0.004)	0.405 (0.011)
(2)[c]	0.112 (0.010)	0.328 (0.006)
(3)[d]	0.125 (0.005)	0.402 (0.023)
(4)[e]	–	0.404 (0.008)
(5)[f]	–	0.398 (0.019)
(6)[g]	0.292 (0.011)	1.16 (0.06)
(7)[h]	0.301 (0.009)	1.23 (0.07)
(8)[i]	0.270 (0.010)	1.10 (0.05)
(9)[j]	–	1.10 (0.01)
(10)[k]	–	1.12 (0.02)
(11)[l]	0.273 (0.006)	1.10 (0.05)

[a] Average of three determinations (standard deviation in brackets).
[b] (1) none.
[c] (2) HCl 0.1 M (autoclaving at 121°C, 30 min) and acid phosphatase (20 mg, 18 h, 37°C).
[d] (3) Acid phosphatase (20 mg per sample, 18 h, 37°C).
[e] (4) Papain (100 mg per sample, 18 h, 37°C).
[f] (5) α-amylase (10 mg per sample, 18 h, 37°C).
[g] (6) β-glucosidase (20 mg per sample, 18 h, 37°C).
[h] (7) β-glucosidase (20 mg) and acid phosphatase (20 mg) (18 h, 37°C).
[i] (8) Pfaltz and Bauer takadiastase (500 mg per sample, 18 h, 37°C).
[j] (9) Fluka takadiastase (500 mg per sample, 18 h, 37°C).
[k] (10) Clara-diastase (500 mg per sample, 18 h, 37°C).
[l] (11) β-glucosidase (20 mg) and Pfaltz and Bauer takadiastase (500 mg) (18 h, 37°C).

by dephosphorylation with suitable diastase or phosphatase enzyme) recommended by the Community Bureau of Reference (BCR, Brussels) (van den Berg et al., 1996). The vitamin contents in these two foodstuffs were always in the confidence interval of the certified values (Table 8).

4. Discussion

The extraction protocols used for the determination of the vitamins B_1, B_2 and B_6 almost always comprise a denaturation of proteins by an acid treatment and an hydrolysis of the phosphorylated forms of the vitamins by a diastase or a phosphatase (Ball, 1998). In a preliminary study devoted to the vitamins B_1 and B_2, it had however been noted that an hydrochloric acid hydrolysis in a water bath at 100°C during 30 min, when performed alone, sometimes led to the release of only a part of the vitamins bound to proteins (for example 50% of the bound thiamin in peas and 15% of the bound riboflavin in pork meat) and that the presence of a protease in the diastase used was necessary to obtain a complete release.

4.1. Is it necessary to perform a preliminary hydrochloric acid hydrolysis?

The results obtained during this study, carried out on foodstuffs with very different matrices, showed that the acid hydrolysis step was in fact always superfluous for the determination of the vitamins B_1 and B_2 if the protease activity of the selected diastase was sufficiently high, as in the case of Pfaltz and Bauer takadiastase or clara-diastase (Tables 4 and 5). On the other hand, the protease activity of Fluka takadiastase [apparently sufficient when this diastase is used for the determination of thiamin (Table 4b)] allowed only a partial release of riboflavin bound to proteins (Table 5b). In this case, a preliminary hydrochloric acid hydrolysis never led to higher contents of riboflavin, except in the porridge oats sample (Table 5a), whereas an increase of the protease activity, by the addition of papain (100 mg) to the Fluka takadiastase (see above p.5) or by replacement of this diastase by the Pfaltz and Bauer takadiastase (Table 5b), led to the complete release of riboflavin bound to proteins in the various foodstuffs studied, thus indicating a much higher efficacy of the proteases than hydrochloric acid hydrolysis to carry out this release. In the protocol for the determination of vitamin B_6, the treatment with hydrochloric acid also appeared superfluous, even in the absence of a subsequent protease treatment (Table 6a, c), which was foreseeable since no nonphosphorylated vitamin B_6 bound to proteins was found in any of the analysed foodstuffs (Table 1).

If a hydrochloric acid treatment induced (after autoclaving at 121°C) neither destruction of the vitamin B_1 in the wheat flour (Table 4a), contrary to what had been suggested by Ball (1998), nor even partial hydrolysis of the glycosylated forms of the vitamin B_6 (Table 7), on the other hand it led to a significant fall of the vitamin B_6 contents in the veal and pork meats (after heating in a water bath at 100°C for 30 min) (Table 6a) and carrots (after autoclaving at 121°C for 30 min) (Table 7). If the dephosphorylation of vitamin B_6 is carried out by addition of an acid phosphatase, instead of a diastase (thus in the absence of an amylase), acid hydrolysis can appear useful for the analysis of certain foods (breakfast cereals, food containing modified starches) in which the presence of starch may involve problems of filtration during the application of the analytical protocol, but this treatment is not essential since the addition of an amylase to the acid phosphatase also makes it possible to solve these problems. Thus nothing seems to justify the performance of an hydrochloric acid treatment before enzymatic hydrolysis for the determination of vitamins B_1, B_2 and B_6 in foodstuffs.

4.2. The release of the phosphorylated and protein-bound vitamin forms by a diastase enzyme

As was mentioned above (see Table 5b), the Fluka takadiastase did not allow the complete release of riboflavin bound to proteins because of its low protease activity. It should, however, be pointed out that no increase of the vitamin B_1 contents found in the analysed foodstuffs resulted from the replacement of the Fluka takadiastase by the Pfaltz and Bauer takadiastase (Table 4b) whose protease activity is much higher (Table 2). Most probably, in this case, the protease activity of the Fluka takadiastase led only to a partial, but nevertheless sufficient, denaturation of the proteins, to allow a total release of the bound thiamin. As for its phosphatase activity, it is apparently sufficient to

Table 8
Comparison of the thiamin, riboflavin and vitamin B_6 contents (expressed in μg g^{-1}) of two certified reference materials (pig's liver CRM 487 and milk powder CRM 421) (1) (standard deviation in brackets), with those obtained (in duplicate) by application of the proposed protocol (2)

Vitamin	Concentration			
	Pig's liver (CRM 487)		Milk powder (CRM 421)	
	(1)	(2)	(1)	(2)
Thiamin	7.67 (0.98)	8.43 8.56	5.81 (0.43)	6.03 6.03
Riboflavin	106.8 (5.6)	99.8 103.0	14.5 (0.6)	15.1 15.1
Vitamin B_6	15.9 (2.4)	17.69 16.69	5.5 (0.7)	6.14 6.20

dephosphorylate all of the phosphorylated components present in the various analysed food samples. This result is rather unexpected considering that the phosphatase activity of the Fluka takadiastase is approximately 20 times lower than that of the Pfaltz and Bauer takadiastase or the clara-diastase (Table 2), when these activities are measured by using 4-nitrophenyl phosphate as substrate. In reference to Table 3, however, the phosphatase enzyme of the Fluka takadiastase appears much more efficient than the potato acid phosphatase toward thiamin pyrophosphate and than the phosphatase enzyme of the Pfaltz and Bauer takadiastase towards pyridoxal and pyridoxamine phosphates. Without any doubt, it should also be very effective towards the other phosphorylated components present in the foodstuffs.

Nevertheless, the diastases most often used to bring about the vitamin dephosphorylation were the Pfaltz and Bauer takadiastase, first of all recommended for the determination of thiamin by Defibaugh (1987), then, more recently, for that of thiamin and riboflavin (100 mg per g of sample) by van den Berg et al. (1996), and the Fluka clara-diastase, recommended for the extraction of these two vitamins (300 mg for a 5 g sample) by Hägg (1994). In fact, the two diastases have very similar phosphatase and protease activities (Table 2) and the use of either made it possible to obtain completely identical contents of vitamins B_1 and B_2 in the different foodstuffs studied (Tables 4b and 5b). The Pfaltz and Bauer takadiastase has also often been recommended, but less systematically, for the extraction of vitamin B_6 and in this case, the quantities of diastase recommended were very high (500 mg per g of sample) (van den Berg et al., 1996).

Considering the results presented in Tables 4–6, the Pfaltz and Bauer takadiastase could effectively be proposed for a simultaneous release, in only one step, of the protein-bound and/or phosphorylated vitamins B_1, B_2 and B_6. However, irrespective of vitamins analysed, it does not appear very reasonable to recommended in reference methods the use of an enzyme whose activities are not guaranteed and whose commercial availability is uncertain. Furthermore, and for at least two reasons, the use of Pfaltz and Bauer takadiastase does not seem desirable for the extraction of the vitamin B_6. Firstly, this diastase, which needs to be added in very large quantities to the sample to be analysed, contains a lot of impurities. On that account, serious interferences were detected in the course of routine analysis during the chromatographic isolation of pyridoxol. Moreover, the Pfaltz and Bauer takadiastase, like the other diastases tested, presents a β-glucosidase activity sufficient to release the glycosylated forms of vitamin B_6 possibly present in plant-derived foods (Table 7). Its use would allow only a determination of the total vitamin B_6, without any possibility of distinguishing between the nonglycosylated (bioavailable) forms and the glycosylated forms, whose bioavailability is negligible.

4.3. The release of the phosphorylated and protein-bound vitamin forms by a well-defined mixture of enzymes

The use of a readily available well-defined mixture of enzymes, including a protease (papain), a phosphatase (potato acid phosphatase) and an amylase (α-amylase), led to satisfactory chromatographic isolation of the vitamins (thiamin as thiochrome, riboflavin and pyridoxol). The vitamin contents obtained with this enzyme mixture, without any preliminary acid hydrolysis, were always in good agreement with those obtained by application of the most practiced extraction protocol (acid hydrolysis, then treatment with the Pfaltz and Bauer takadiastase enzyme) to the different foodstuffs studied, provided however, in the case of the determination of vitamin B_6, that these foodstuffs do not contain any glycosylated forms (Tables 4–6). During the analyses of foodstuffs in which part of the vitamin B_6 is glycosylated, it presents the advantage over the use of the Pfaltz and Bauer takadiastase of making it possible the exclusive determination of the nonglycosylated vitamin B_6, since none of the enzymes constituting the mixture has a β-glucosidase activity (Table 7). If required, the total vitamin B_6 concentration can easily be obtained by addition of β-glucosidase (20 mg) to the other enzymes. For all these reasons, this enzymatic mixture was preferred to the Pfaltz and Bauer takadiastase (or Fluka clara-diastase) for the simultaneous extraction of the vitamins B_1, B_2 and B_6 from foodstuffs.

The quantity of α-amylase (10 mg) had been initially selected to completely solubilize the starch present in the various foodstuffs analyzed, and thus to avoid possible problems of filtration during the application of the protocol. Actually, filtration never presented a problem during the various analyses carried out, even when the samples possess considerable starch contents (yeast, wheat flour, peas). Nevertheless, and very curiously, a certain quantity of this enzyme, moderate for the determination of riboflavin (< 5 mg), larger for the thiamin determination (10 mg), was required to obtain maximal vitamin contents during the analysis of yeast samples. The presence of α-amylase (10 mg) led to an increase of 10% in the riboflavin content and of 30% in that of thiamin. Most probably, interactions occurred between these vitamins and the starch present in high concentrations in this complex food matrix, but no experiment was however undertaken to check this assumption.

Although the presence of a protease in the enzyme mixture was never necessary for the determination of vitamin B_6 (see Table 6), it was indeed shown to be essential for the determination of thiamin and riboflavin. During the study of a foodstuff rich in proteins, such as pork meat (0.20 g g^{-1}), the quantity of papain required in the enzyme mixture to obtain total release of the thiamin bound to proteins was moderate, comprised between 5 and 10 mg for a 5 g weighed sample (Fig. 1).

This makes it possible to understand why the quantity of protease present in 500 mg of Fluka takadiastase also proved sufficient to carry out this release [in this experiment α-amylase was lacking since this enzyme contained protease impurities (see Table 2)]. As was predictable in the light of the results of Table 5, the quantity of papain required to obtain a total release of protein-bound riboflavin, present in a high proportion in this foodstuff (Table 1), was markedly higher, between 35 and 50 mg for a same weighed sample. This quantity corresponds to a protease activity of 3.5–5.0 U, of the same order as that provided by 500 mg of Pfaltz and Bauer takadiastase (4.0 U). Finally, the quantity of papain recommended in the enzyme mixture (100 mg) is thus approximately twice as high as that necessary to release riboflavin bound to proteins in pork meat samples and ought always to be more than enough for the total release of the thiamin and riboflavin bound to proteins, irrespective of the protein nature of the foodstuffs analysed.

The results obtained concerning the influence of the quantity of acid phosphatase in the enzyme mixture have shown that the latter needed to be between 5 and 10 mg (5–10 U g^{-1} of sample weight) to obtain maximal vitamin contents in a pork meat sample (Fig. 2a). For the analysis of the yeast samples, about 30 times richer in phosphorylated vitamins B_1, B_2 and B_6 than the pork meat samples, the quantity of acid phosphatase required to obtain maximal vitamin contents was about 10 mg (50 U g^{-1} of sample weight, i.e. half of the phosphatase activity in the enzyme mixture) (Fig. 2b), clearly higher than that necessary to dephosphorylate the pork meat samples, but less than would have been suspected a priori from the respective quantities of phosphorylated vitamins in these two foodstuffs.

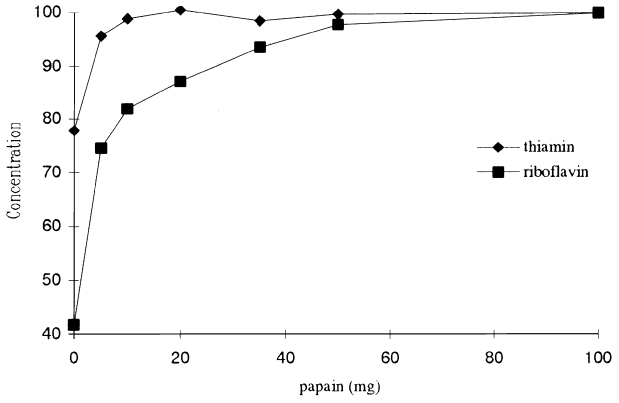

Fig. 1. Concentrations of thiamin and riboflavin (expressed as percentage of the maximum concentration of vitamin extracted) in pork samples in relation to the quantity per sample of papain (in mg) in the reference enzymatic mixture.

In fact, the acid phosphatase present in the enzymatic mixture acts not only on the phosphorylated vitamins, but also on all the other phosphorylated components present in the foodstuffs, generally present in much higher concentrations than those of the phosphorylated vitamins. For example, when the dephosphorylation of a yeast sample (1 g) required the use of 10 mg of acid phosphatase according to the estimates resulting from Tables 1 and 3–6, less than 2 mg of this enzyme would be necessary for total dephosphorylation of the vitamins B_1, B_2 and B_6, still present in very considerable concentrations in this foodstuff. Accordingly, the quantity of acid phosphatase necessary in the enzymatic mixture will primarily depend on the nature of the foodstuffs to be analysed and only to a slight extent on the concentration in phosphorylated vitamins. Considering the different results obtained, 20 mg of acid phosphatase seems, however, largely sufficient to guarantee complete dephosphorylation of the different vitamins in the foodstuffs studied.

The enzymatic mixture proposed for the extraction of vitamins B_1, B_2 and B_6 (10 mg of α-amylase, 100 mg of papain, 20 mg of acid phosphatase, and optionally 20 mg of β-glucosidase), which made it possible to obtain vitamin contents in agreement with the certified values in reference materials (Table 8), thus constitutes an excellent enzymatic system of reference for the analysis of these three vitamins in foodstuffs.

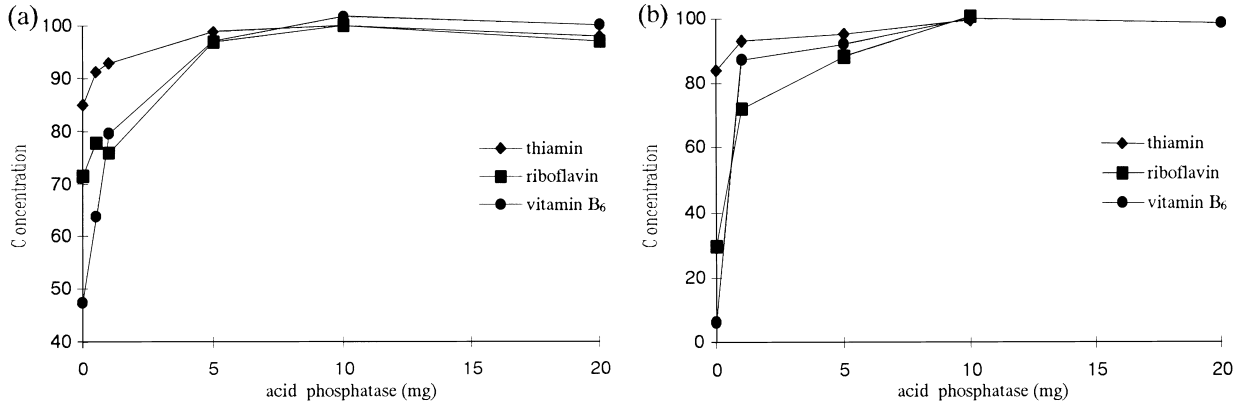

Fig. 2. Concentrations of thiamin, riboflavin and vitamin B_6 (expressed as percentage of the maximum concentration of vitamin extracted) in pork (a) and yeast (b) samples in relation to the quantity per sample of acid phosphatase (in mg) in the reference enzymatic mixture.

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