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Determination of total vitamin B_6 in foods by isocratic HPLC: a comparison with microbiological analysis

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Abstract

This paper describes a rapid and sensitive, high performance liquid chromatography (HPLC) method for analysis of vitamin B_6 in various foods. The method is based on isocratic elution and it provides complete separation of the three major B_6-vitamers: pyridoxine, pyridoxal and pyridoxamine within 12 min. Samples of vegetable origin were extracted with mild acid hydrolysis prior to enzymatic digestion with acid phosphatase and β-glucosidase and by analysis of the two digests separately it was possible to distinguish between free pyridoxine and β-glucosylic forms of pyridoxine. Results for several food samples analysed by this method were compared to the results of a microbiological analytical method using *Saccharomyces uvarum*. The comparison showed a systematic difference in results obtained with the two methods. Vitamin B_6 data from the HPLC method were approximately 70% higher for animal foodstuffs, 20% higher for fruit and vegetables, but approximately 20% lower for grain products than for the microbiological method. Models explaining these differences are discussed.

Keywords: Vitamin B6; Pyridoxine; Pyridoxal; Pyridoxamine; HPLC; Food; Foodstuffs; Acid hydrolysis; Enzymatic hydrolysis; Microbiological analysis

1. Introduction

Vitamin B_6 consists of three closely related derivatives of 2-methyl-hydroxypyridine, i.e. pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM) and their 5′-phosphate forms. In addition, foods of vegetable origin may contain β-glycoside and oligoglucosides of pyridoxine.

Yasumoto, Tsuji, Iwami, and Mitsuda (1977) isolated and identified 5′-*O*-(β-D-glucopyranosyl) pyridoxine from rice bran, later referred to as pyridoxine-β-D-glucoside (PNG). This compound was easily converted to PN by treatment with β-glucosidase or takadiastase. Tadera, Kaneko, and Yagi (1986) described a more persistent glucosylic pyridoxine form in rice brand, B_6X, a compound that only released PN when treated alkali prior to β-glucosidase digestion. B_6X was later identified as an indolo β-cellobiosylic pyridoxine (Tadera & Orite, 1991) and was found to liberate PN on heating with 0.44 M HCl for 2 h at 121 °C. Further three oligoglucosides of pyridoxine were identified in rice bran, all liberating PN on mild acid hydrolyse or direct β-glucosidase treatment (Tadera, Kaneko, & Yagi, 1988).

The overall bioavailability of vitamin B_6 is reduced in foodstuff of vegetable origin compared to foodstuff of animal origin and fortified foodstuff (Kabir, Leklem, & Miller, 1983a, 1983b; Leklem, Miller, Perera, & Peters, 1980). This may be due to the presence of considerable amounts of β-glucosidic forms of pyridoxine in most vegetables (Kabir et al., 1983b). Recent studies have indicated that the bioavailability of PNG in humans is reduced compared to PN when administrated orally (Hansen, Leklem, & Miller, 1996; Kabir et al., 1983a, 1983b). The bioavailability of PNG in humans has been estimated to approximately 50% of the PN bioavailability (Gregory et al., 1991; Nakano, McMahon, & Gregory, 1997), ranging from 40 to 100% (Gregory,

Abbreviations: PN, pyridoxine; PL, pyridoxal; PM, pyridoxamine; PMP, pyridoxamine-5′-phosphate; PNG, 5′-*O*-(β-D-glucopyranosyl) pyridoxine; B_6X, 5′-*O*-[6-*O*-((+)-5-hydroxy-dioxindole-3-acetyl)-β-cellobiosyl] pyridoxine; PN-glu, easily digested oligo β-glucosidic forms of pyridoxine; HPLC, high performance liquid chromatography; MA, microbiological analysis

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1997). No data on the human bioavailability of B_6X and the oligoglucosides of pyridoxine are available. The utilisation of PNG varies within the rodent group, thus the rat displays approximately 20% capacity to hydrolyse PNG to PN (Ink, Gregory, & Sartain, 1986), mice and hamster display approximately 70% hydrolyses capacity, and guinea pig displays approximately 90% hydrolyse capacity (Banks & Gregory, 1994). The presence of a broad-specificity β-glucosidase has been shown in mammalian tissue (Banks, Porter, Martin, & Gregory, 1994; Trumbo, Banks, & Gregory, 1990), but the β-glucosidase present in human intestinal mucosa showed significantly higher activity compared to rat and guinea pig intestinal mucosa (Trumbo et al., 1990) and this may explain the higher bioavailability of PNG found in humans compared to rodents. The PNG contribution to the total vitamin B_6 content in a custom mixed non-vegetarian diet was in 1990 estimated to 10–15% (Andon, Reynolds, Moder-Veillon, & Howard, 1989; Gregory et al., 1991). The extraction of vitamin B_6 from foodstuff by the microbiological vitamin B_6 analysis (MA) depends on the matrices; vegetable samples are usually extracted by treatment with 0.22–0.44 M mineral acid for 2–4 h at 121 °C and samples of animal origin are treated with 0.055 M mineral acid for 4–5 h (Toepfer & Polansky, 1970). Optimization of food extraction (Berg, Schaik, Finglas, & Froidmont-Gortz, 1996; Bognar & Ollilainen, 1997) have shown that mineral acid hydrolysis at 121 °C for 30 min followed by a combined enzymatic treatment with acid phosphatase and β-glucosidase liberates all major bound forms of vitamin B_6. In this way, it is possible to distinguish between forms with alternating bioavailability. Principally, this may also be applicable for MA (Kabir et al., 1983b).

The variety and number of B_6 vitamers complicate the analysis of vitamin B_6: the growth rates of the microorganism are reduced to approximately 80 and 50% response of PL and PM relative to PN, respectively (Gregory, 1982; Guilarte, McIntyre, & Tsan, 1980; Schoonhoven, Schrijver, Berg, & Haenen, 1994), and furthermore dependent on the concentration in the test tube. On the other hand, PL and PM are considered as equal to PN as vitamin B_6 equivalents to humans (Gregory, 1997). In cases where the external standard for vitamin B_6 growth is based on PN only, underestimation of the vitamin B_6 content in foodstuffs should be expected, thus Schoonhoven et al. (1994) showed that an HPLC method in average found approximately 40% higher vitamin B_6 content than a MA method with PN as standard.

One objective of this study was to develop a HPLC based analytical method capable of fulfilling requirements from a routine lab performing control analysis as well as collecting valid data to a food database. Another objective was to explain the quantitative differences between the HPLC and microbiological methods and to be able to extrapolate new HPLC data to previously obtained microbiological data. The significance of the reduced bioavailability of PNG and other glycosides is not clear and an objective was to be able to distinguish between free and glycoside bonded pyridoxine. Therefore, we developed an extraction procedure without unintended and uncontrolled degradation of β-glucoside bound forms of vitamin B_6 providing the possibility of collecting data on the free and bound vitamin B_6 forms, separately.

2. Materials and methods

2.1. Reagents

Pyridoxine hydrochloride (Merck 7523), pyridoxal hydrochloride (Merck 7527), pyridoxamine dihydrochloride, monohydride (Merck 7527), acid phosphatase (Type IV-S from potato, Sigma P-1146), β-glucosidase (from almond, Sigma G-0395), triethylamine (99%, Sigma T-0886), potassium dihydrogen phosphate (Merck), di-potassium-hydrogen-phosphate–tri-hydrate (Merck), ortho-phosphoric acid(85%, Merck) 1-octan-sulfonic acid, sodium monohydrate (99%, Fluka 74884), acetonitrile, HPLC grade (Fisher Certified A/0627/17). Stock solutions of pyridoxine hydrochloride, pyridoxal hydrochloride, pyridoxamine dihydrochloride were prepared in concentrations of 100 μg/ml 0.1 M HCl and stored up to 2 months at −18 °C. The concentration of stock solutions were checked by UV absorption and the absorption should be (corresponding to 10.00 μg/ml): 0.430 ± 0.012 at 291 nm for PN, HCl, 0.443 ± 0.006 at 288 nm for PL, HCl. 0.337 ± 0.011 at 293 nm for PM, 2×HCl. Stock solutions of acid phosphatase (25 Units/ml) and β-glucosidase (45 Units/ml) were prepared every day. The activity of a new batch acid phosphatase was checked by analysis of certified reference material CMR 487, and by CMR 487 spiked with PMP corresponding to 2 mg PMP pr 1 g CMR 487. Accept criteria for the spiked samples were a recovery of 90–110%.

The HPLC buffer was 2.2 mM 1-octan sulfonic acid in 81 mM potassium dihydrogen phosphate and 19 mM 85% phosphoric acid and 4.0 mM triethylamine, adjusted to pH 2.75 with 3.5 M KOH or 85% phosphoric acid.

2.2. Apparatus

The HPLC system was operated on a Waters 2670 alliance separation module (Waters Corporation, Milford, MA, USA) equipped with a Waters 474 fluorescent detector controlled by Millennium 32 chromatography manager data acquisition system. The

post column pump was a Dionex RP-1 (Dionex, USA) and the HPLC column temperature was kept constant at 22 °C by an Iglo-sil column cooler (Cluzeau Info Lab, Sainte-Foy-La-Grande, France).

The autoclave used in this study was connected to a central, closed steam system, allowing rapid alternations in the autoclave pressure. In that way, it was possible to autoclave samples of vegetable origin at 121 °C in short periods of 5 min. An alternative heat treatment for non-animal samples should be 15 min on a water bath at 100 °C.

2.3. Microbiological analysis

The microbiological analysis compared with the HPLC method was an in-house accredited method with *S. uvarum* as test organism. Food samples of vegetable origin were extracted with 0.22 M H_2SO_4 for 4 h at 121 °C and samples of animal origin were extracted for 4 h with 0.0275 M H_2SO_4 at 121 °C. Dose–response curves of vitamin B_6 dependent yeast growth were obtained in concentrations correspondent to 0.3–2.4 ng/ml pyridoxine hydrochloride. The method participated in the BCR study (Berg et al., 1996) and produced valid data.

2.4. Analytical procedure

Prior to weighing out, food samples were treated in a food processor or otherwise processed to ensure homogeneity. About 5 g food was transferred to a 250 ml conical flask.

2.5. Foodstuffs of animal origin

Fifty milliliters 0.1 M HCl was added to the sample and the conical flask was closed and autoclaved for 30 min at 121±2 °C. The sample was cooled to room temperature and pH was adjusted to 4.5±0.1 with 2 M sodium acetate and transferred quantitatively to a 100 ml measurement flask and filled up with water. The flask was shaken carefully and an aliquot of approximately 80 ml was centrifuged for 10 min at 8500 *g* and 5 °C followed by filtration on filter paper. Precisely 15 ml of the filtrate was transferred to a 30 mL measurement flask, added 1 ml of the 25 Unit/ml acid phosphatase solution and incubated over night (18 h) at 45 °C. To stop the incubation, the sample was cooled to room temperature, added 5 ml cold 1 M HCl and the flask was filled up with 0.01 M HCl. An aliquot was filtered though a 0.45 μm PP filter. Turbid samples were centrifuged at 14,000 *g* for 10 min at 5 °C prior to filtration, and transferred to an HPLC vial.

2.6. Foodstuffs of vegetable origin

Fifty milliliters 0.1 M HCl was added to the sample and the conical flask was closed and autoclaved in 5 min at 121 °C±2 °C. The sample was cooled to room temperature and pH was adjusted to 4.5±0.1 with 2 M sodium acetate and transferred quantitatively to a 100 ml measurement flask and filled up with water. The flask was shaken carefully and an aliquot of approximately 80 ml was centrifuged for 10 min at 8500 *g* and 5 °C followed by filtration. Aliquots of precisely 15 ml of the supernatants were transferred to two 30 ml measurement flasks, A and B. Flask A was added 1 ml of the 25 Units/ml acid phosphatase solution and flask B was added 1 ml of the 25 Units/ml acid phosphatase solution plus 3 ml of 45 Units/ml of the β-glucosidase stock solution. Samples were incubated over night (18 h) at 45 °C.

To stop the incubation, samples were cooled to room temperature, added 5 ml cold 1 M HCl and the flasks were filled up with 0.01 M HCl. An aliquot was filtered though a 0.45 μm PP filter. Turbid samples were centrifuged at 14,000 *g* for 10 min at 5 °C prior to filtration and transferred to an HPLC vial.

2.7. Isolation of glycoside isomers

Graham flour was extracted like foodstuffs of vegetable origin, however, without the β-glucosidase treatment step. The analytical system was connected to a Waters fraction collector II, and fractions were collected for every 20 s. The injection volumes were 100 μl. Fractions from approximately 100 injections were pooled and evaporated to dryness at room temperature and at reduced pressure by a Maxi dry lyo rotary evaporator (Heto-Holten, Allerød, Denmark). The dry fractions were re-suspended in 0.1 M HCl and analysed.

2.8. HPLC-analysis

The HPLC column was a Phenomenex Hypersil 3μ C18 150x4.6 (Phenomenex Inc., Torrance, USA) equipped with a Phenomenex Security Guard C18.

Samples were injected in 50 μl volumes and were kept at 5 °C in the dark during analyses in the autosampler. The column was applied isocraticaly at 1 ml/min with 93% HPLC buffer and 7% acetonitrile with a runtime of 15 min for standards and 18 min for samples. To improve the detector specificity, the mobile phase pH was adjusted to 7.5 by a post column infusion of a 0.5 M phosphate buffer adjusted to pH 7.5, at 0.3 ml/min. Thus, the B_6 vitermers were detected by fluorescence detection: excitated at 333 nm and the emission was detected at 375 nm.

2.9. Expression of results

Data expressed as vitamin B6 were calculated as pyridoxine hydrochloride (PN, HCl), unless other is stated, and were calculated according to: PN, HCl = PN + (1.01PL) + (0.79PM).

The certified reference materials analysed in this study (BCR) was declared as PN, HCl and in one out of the two proficiency tests participated in, the vitamin B6 was declared as PN, HCl.

2.10. Sample collection

Food samples analysed in this study were collected in order to test and validate the method, and to compare the method with a microbiological method. The food samples were chosen at random or samples originated from other studies. The intention of the study was not to contribute to food composition tables and the food composition data should only be used with that reservation.

2.11. Statistics

The methods were compared by a two-tailed paired *t*-test for different mean values. *P*-values below 0.05 were considered significant. Statistically analyses, linear regression and test for intercept different from (0.0) were performed by The SAS system, ver. 6.12 (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. HPLC

The described HPLC method provides separation of the three un-phosphorylated B_6 vitamers and a compound probably consisting of pyridoxine-5′-β-D-glucoside. Fig. 1 shows a typical chromatogram of a 50 ng/ml standard solution. Fig. 2 shows chromatograms of lyophilised cabbage reference material (a) treated with acid phosphatase and (b) treated with acid phosphatase and β-glucosidase. In Fig. 2A one major unknown peak (peak 5) with a retention time of approximately 6 min disappeared after treatment with β-glucosidase (Fig. 2B). In Fig. 2B the pyridoxine peak increased with an area corresponding to the area of peak 5 in Fig. 2A. The molar absorption (Andon et al., 1989) and fluorescence (Gregory & Ink, 1987) of PNG have been reported to be equivalent to that of PN. This is in agreement with the data shown in Table 1 and thus, peak 5 was assumed to be pyridoxine-5′-β-D-glucoside, regretfully, no LC–MS identification was performed.

Fig. 3 shows chromatograms of graham flour reference material (a) treated with acid phosphatase and (b) treated with acid phosphatase and β-glucosidase. Further four unknown peaks (peaks 1–4) disappeared from Fig. 3A after incubation with β-glucosidase (Fig. 3B). The sum of the areas of peaks 1–5 in Fig. 3A corresponded (99%) to the increase in PN peak area in Fig. 3B. Collection of fractions from the extracted graham flour resulted in isolation of five fractions corresponding to peaks 1–5 in regard to retention time. All five fractions liberated pyridoxine when treated with β-glucosidase or with 0.5 M HCl for 4 h at 121 °C. During extraction of some foodstuffs, e.g. rolled oats and graham flour, increasing peak areas for PM were observed after incubation with β-glucosidase. In order to rule out lacking de-phosphorylation, additional amounts of acid phosphatase were used, however, no increase in PM areas were observed. In Fig. 3A,B the PM peak increased 32% after β-glucosidase treatment and this corresponded (86%) to the decrease in peak 6.

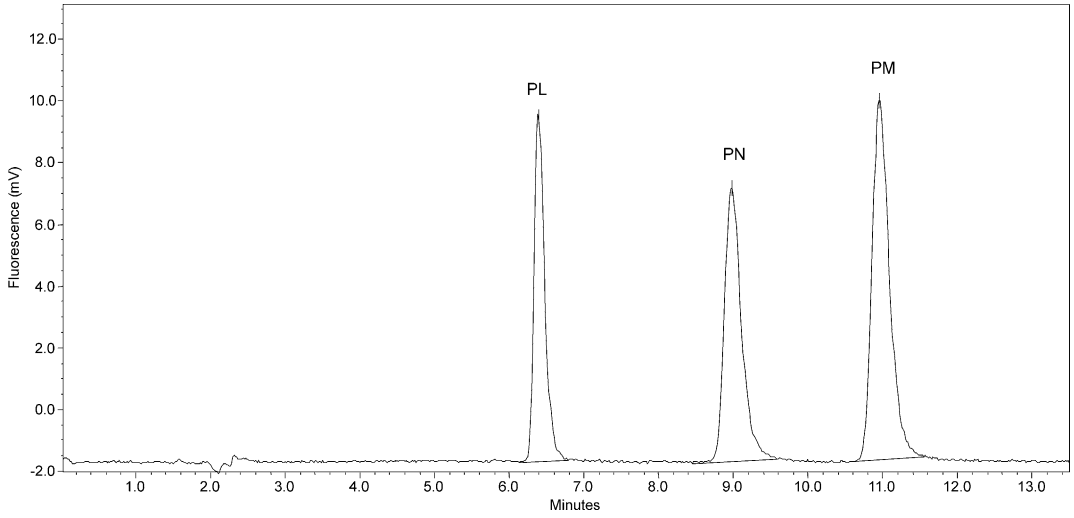

Fig. 1. Chromatogram of 50 ng/ml standards analysed on a Phenomenex Hypersil 3μ, C18, 150×4 mm HPLC column. The effluent-buffer was based on 2.2 mM 1-octan sulfonic acid in 81 mM potassium dihydrogen phosphate and 19 mM 85% phosphoric acid and 4.0 mM triethylamine, adjusted to pH 2.75. The column was applied isocraticaly at 1 mL/min with 93% eluent-buffer and 7% acetonitrille with a runtime of 15 min for standards and 18 min for samples. To improve detector specificity, pH was adjusted to 7.5 by a post-column infusion with 0.5 M phosphate buffer and the B_6 compounds were excitated at 333 nm and the emission was detected at 375 nm.

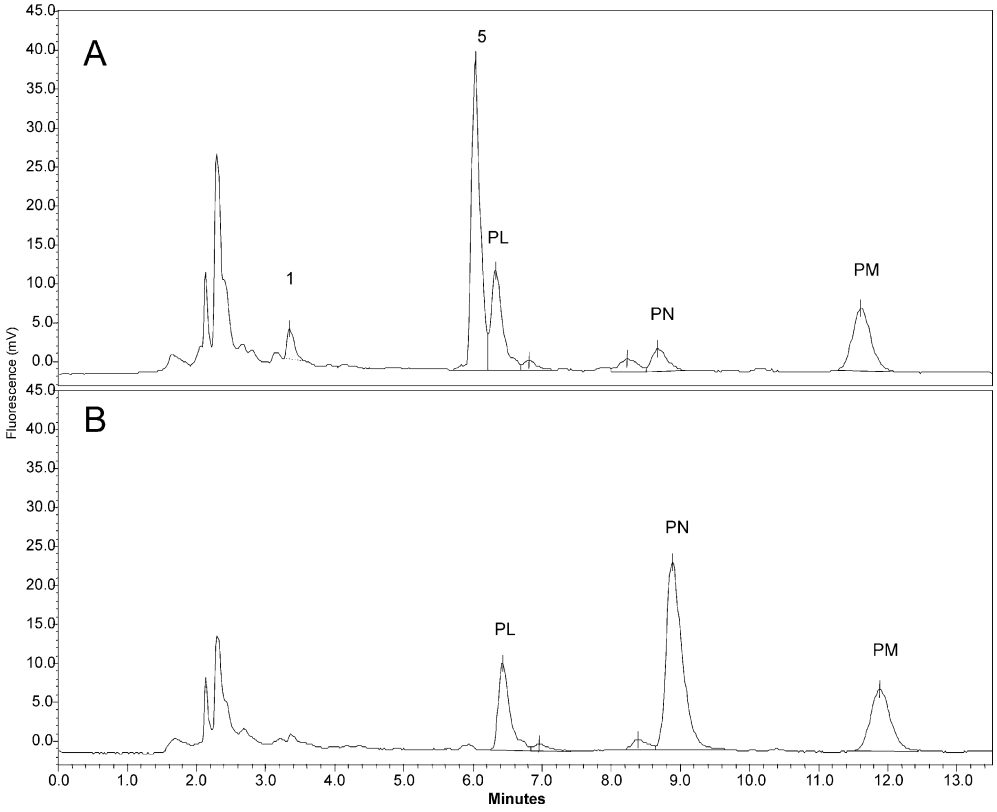

Fig. 2. Chromatogram of lyophilised cabbage reference material. (A) Fraction treated with acid phosphatase only, (B) fraction treated with acid phosphatase and β-glucosidase.

Table 1
Occurrence of easily digested pyridoxine β-glucosylic forms in vegetables and grains

Foodstuff	Peak area				Ratio[e] (%)
	PNG[a]	PNG[b]	PN-free[c]	PN-total[d]	
	Determined	Calculated	Determined	Calculated	
Brussels sprouts	101,037	93,756	17,281	111,037	108
Onion	146,765	137,544	11,896	149,440	107
Spring cabbage	41,743	39,540	59,309	98,849	106
Broccoli	27,203	25,858	51,848	77,706	105
Banana	17,748	17,223	82,582	99,805	103
Leek	56,862	56,935	110,410	167,345	100
Cabbage	47,547	47,910	30,438	78,348	99
Potato	90,077	91,838	23,361	115,199	98
Green beans	14,143	14,486	5026	19,512	98
Brown rice	41,887	46,743	31,553	78,296	90
Graham bread	6542	8500	18,758	27,258	77
Polished rice	9632	13,788	25,361	39,149	70
Wholemeal bread	34,229	53,734	16,426	70,160	64
Rolled oats	8960	15,868	19,889	35,757	56
Graham flour	84,251	155,460	30,448	185,908	54

[a] Area of peak 5 in Fig. 2.
[b] The difference between the PN peak area in the fraction treated with acid phosphatase and the fraction treated with acid phosphatase and β-glucosidase (Fig. 2A,B).
[c] The peak area of PN in the fraction only treated with acid phosphatase (Fig. 2A).
[d] The peak area of PN in the fraction treated with acid phosphatase and β-glucosidase (Fig. 2B).
[e] The ratio was calculated as $(PNG_{determined}/PNG_{calculated})\times 100$ and reflects the PNG contribution to the total amount of easily digestive pyridoxine glycosides.

Similar phenomenons were observed in rolled oats, leeks and green beans. This may indicate the presence of a pyridoxamine β-glucoside in graham flour, rolled oats, leeks and green beans.

3.2. Extraction

The extraction procedure was based on a study of Bognar and Ollilainen (1997), but modified as regards choice of enzyme and extraction time. The acid phosphatase used in the study by Bognar had inadequate activity to hydrolyse pyridoxamine-5′-phosphate (PMP) in some samples, however, acid phosphatase No. 108 from Boehringer had adequate activity (Bognar, personal communication). We found that an acid phosphatase Type IV-S from potato (Sigma P-1146), had similar phosphatase activity as the Boehringer acid phosphatase No. 108. In contrast to Boehringer No. 108, the Sigma P-1146 showed no β-glucosidase activity (results not shown) and therefore we chose this enzyme.

Table 1 shows that in a series of common vegetables analysed, the sum of PN and PNG represents the major part (93–102%) of total pyridoxine contents, with only minor content of other glycosides, probably corresponding to peak 1 in Fig. 2A, in brussels sprouts, spring cabbage, broccoli, potato and green beans. In the series of grains analysed, there were pronounced

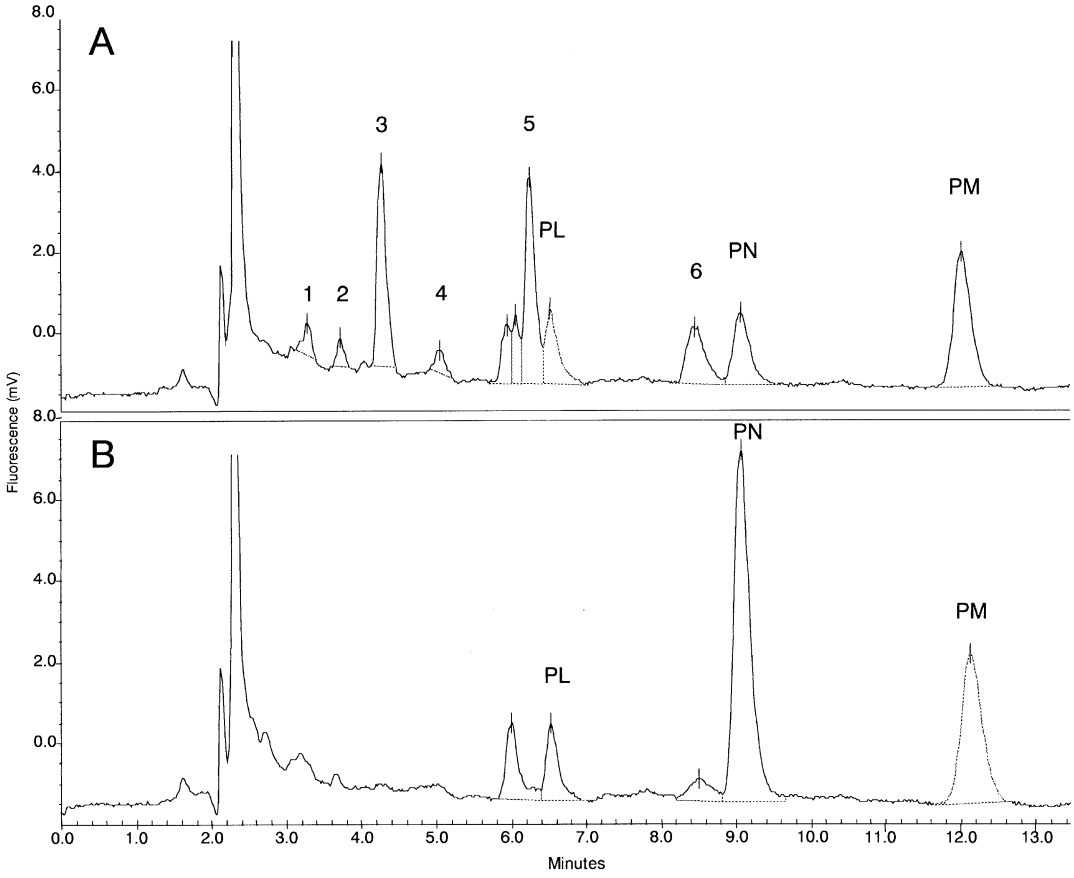

Fig. 3. Chromatogram of graham flour. (A) Fraction treated with acid phosphatase only, (B) fraction treated with acid phosphatase and β-glucosidase.

differences between the sum of PN and PNG and the total pyridoxine determined. This may be explained by occurrence of a number of easily digested oligo β-glycosides (here denoted as PN-glu), previously described in grains (Tadera et al., 1988). The peak conversion, expressed as areas, of the peaks 1–4 showed in Fig. 3A,B supports this assumption. However, this does not explain the difference between the HPLC and MA results for grains. Tededa et al. (1986; Tadera & Orite, 1991) described B_6X as a very stable compound that only liberates pyridoxine after alkaline treatment prior to enzymatic hydrolyse or after boiling in diluted mineral acid for several hours. The main difference between the extraction procedures used in the MA method and in the HPLC method compared in this study, was the acid hydrolyses. For MA vegetable samples were treated with 0.22 M H_2SO_4 at 121 °C for 4 h while vegetable samples for HPLC analysis were treated with 0.1 M HCl at 121 °C for 5 min prior to enzymatic digestion. The difference between results obtained from the two methods was approximately 90% for brown rice and 14% for cabbage (Table 2). Figs. 5 and 6 show the effect of increasing autoclave treatment periods from 5 min to 4 h on cabbage reference material and milled brown rice, (a) hydrolysed in 0.1 M HCl and (b) hydrolysed in 0.22 M H_2SO_4. Sample extracts were analysed by MA without further treatment, but treated with enzymes as described in materials and methods prior to HPLC analyses. Independent of applied acids, increase in autoclave periods resulted in liberation of an increasing number of co-eluting compounds, complicating correct integration of the vitamer peaks (results not shown). This could explain the fluctuation of the PL and PM levels determined in both samples. The total vitamin B_6 content determined in cabbage by means of HPLC was unaffected by heat treatment and type of acid, but the free PN and PNG levels were highly affected and alternations in concentrations were almost superimposed. The PNG was quantitatively converted to PN after 4 h H_2SO_4 treatment and it seems that the H_2SO_4 treatment was more aggressive in destruction of β-glucoside links than the HCl, however, this may also be due to the higher H^+ concentration. The total vitamin B_6 content determined by MA in cabbage was almost identical to the free PN determined by HPLC on the same fractions, indicating that the utilisation of PNG for *S. uvarum* is negligible—in agreement with (Andon et al., 1989). As shown in Fig. 5 (cabbage), the

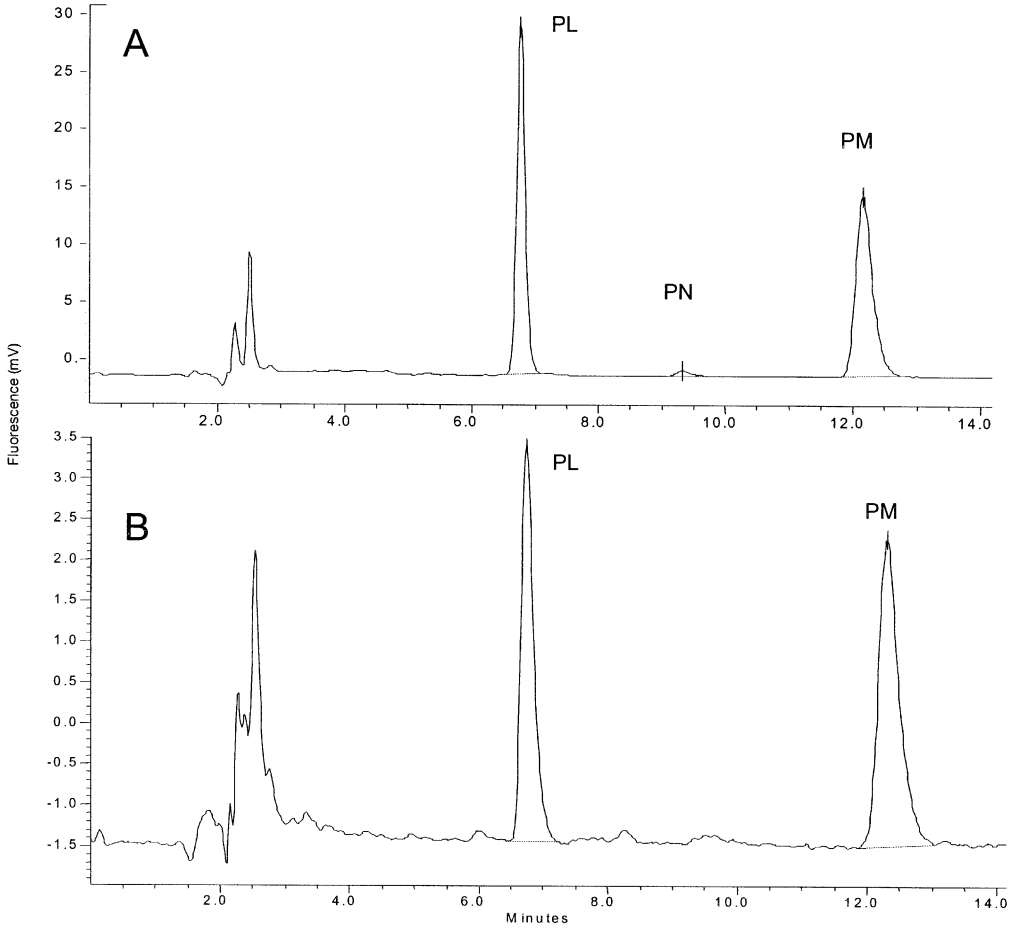

Fig. 4. Chromatogram of samples with animal origin. (A) Beef treated with acid hydrolysis and acid phosphatase, (B) yoghurt treated with acid hydrolysis and acid phosphatase.

PNG liberation continuously decreased as PN liberation increased along with the increasing heat treatment. Opposite, in Fig. 6 (brown rice), the liberation of PNG increased to a maximum after 1 h autoclave with H_2SO_4 and 2 h with HCl, and then decreased to almost zero (H_2SO_4) after 4 h. At the same time, free PN continuously increased, indicating the presence of compounds liberating PNG during degradation simultaneously with degradation of PNG to PN. The total vitamin B_6 determined by MA in the H_2SO_4 extract reached the same level as total vitamin B_6 determined by HPLC, indicating that the increase of PN peak area was due to liberated PN and not caused by some co-eluted compound.

The question is how this information should be evaluated from a nutritional point of view. The bioavailability of β-glucosidic forms of pyridoxine has been proposed to be dependent on the occurrence of a broad spectrum β-glucosidase localised in the human intestinal mocusa (Nakano et al., 1997), and an estimation of the bioavailable PN could be based on the amount liberated after treatment with mild acid hydrolysis followed by β-glucosidase treatment. In Fig. 6A, the total vitamin B_6 determined by HPLC in brown rice was 0.21 mg/100 g. Of this amount, 0.06 mg/100g was PNG. However, if the sample was treated for 4 h with 0.1 M HCl the total vitamin B_6 was 0.43 mg/100 g, e.g. an overestimation of more than 100%. The brown rice analysed in this study had a B6 vitermer distribution of 24% non-bonded, presumable easily digestible, B6 vitamers and 76% bounded vitermer forms liberating PN after extensive acid and heat treatment. In a study published recently (Roth-Maier, Ketter, & Kirchgessner, 2002), it was shown that the precaecal digestibility of boiled brown rice was as low as 16%.

3.3. Comparison of HPLC and microbiological analysis

In order to compare the HPLC method with the MA method, a number of food samples were analysed simultaneously and in doublets (Table 2). The MA data were calculated as PN, HCl and the total vitamin B_6 data from the HPLC analyses were calculated according to Eq. (1):

$$\text{PN, HCl} = \text{PN} + (1.01\text{PL}) + (0.79\text{PM}) + \text{PN-glu} \quad (1)$$

Table 2
Comparison of HPLC and MA exemplified on a range of foodstuffs

	HPLC (mg/100 g)					MA		Adjusted HPLC	
	PN	PL	PM	PNG	B6[a]	B6[b] (mg/100 g)	d[d] (%)	B6[c] (mg/100 g)	D[e] (%)
Pork meat raw	0.007	0.314	0.181	nd[f]	0.47	0.25	59	0.27	6
Pork cooked	0.038	0.094	0.454	nd	0.49	0.30	48	0.23	−28
Pork-1	0.011	0.506	0.151	nd	0.64	0.39	49	0.39	2
Pork-2	0.054	0.143	0.336	nd	0.46	0.35	28	0.24	−36
Pork-3	0.039	0.120	0.282	nd	0.38	0.29	27	0.20	−38
Skim milk	nd	0.039	0.010	nd	0.05	0.03	53	0.03	7
Sausages	0.021	0.045	0.073	nd	0.12	0.08	44	0.07	−12
Feta cheese	nd	0.008	0.026	nd	0.03	0.02	59	0.01	−21
Yoghurt	nd	0.034	0.023	nd	0.05	0.03	50	0.03	−7
Cured saddle of pork	0.018	0.084	0.314	nd	0.35	0.24	40	0.16	−37
Liver (Calf)	0.040	0.105	0.745	nd	0.73	0.47	43	0.32	−40
Beef	0.013	0.224	0.147	nd	0.35	0.17	70	0.20	18
Turkey	0.006	0.557	0.382	nd	0.87	0.43	67	0.49	12
Ham	0.017	0.584	0.127	nd	0.71	0.32	75	0.45	33
Salmon	[g]	0.602	0.081	nd	0.67	0.30	78	0.43	37
Cod	0.099	nd	0.036	nd	0.13	0.06	69	0.11	55
Potato	0.026	0.040	0.025	0.10	0.19	0.16	16	0.16	1
Apple	0.023	0.008	0.005	[g]	0.04	0.05	−25	0.03	−40
Pear	0.008	0.020	[g]	[g]	0.03	0.02	59	0.02	31
Banana	0.104	0.042	0.243	0.022	0.36	0.25	38	0.22	−10
Leek	0.124	0.083	0.017	0.063	0.28	0.24	18	0.25	4
Onion	0.011	0.025	0.013	0.135	0.18	0.21	−15	0.17	−24
Green beans	0.009	0.076	0.027	0.018	0.13	0.11	14	0.09	−24
Spring cabbage	0.065	0.084	0.025	0.046	0.22	0.15	36	0.17	15
Brussels sprouts	0.018	0.097	0.066	0.131	0.30	0.26	13	0.23	−12
Cabbage	0.038	0.044	0.022	0.065	0.16	0.14	14	0.14	−3
Cauliflower	0.052	0.121	0.020	[g]	0.19	0.12	44	0.14	14
Broccoli	0.068	0.118	0.028	0.029	0.24	0.21	14	0.18	−12
White bread	0.008	0.017	0.042	0.006	0.06	0.07	−2	0.04	−56
Wholemeal bread	0.027	0.051	0.065	0.076	0.21	0.25	−21	0.16	−48
Rye bread	0.062	0.034	0.034	0.028	0.15	0.15	1	0.12	−20
Graham bread	0.028	0.017	0.039	0.023	0.10	0.11	−10	0.07	−39
Wheat flour	0.027	0.013	0.015	0.022	0.07	0.09	−23	0.06	−40
Graham flour	0.033	0.061	0.041	0.162	0.29	0.36	−23	0.25	−38
Rye flour	0.039	0.074	0.055	0.075	0.23	0.22	5	0.18	−21
Rolled oats	0.023	0.039	0.112	0.022	0.17	0.14	23	0.10	−29
Polished rice	0.030	0.017	0.034	0.016	0.09	0.11	−17	0.07	−46
Brown rice	0.040	0.039	0.064	0.072	0.20	0.54	−92	0.16	−111

[a] PN, HCl calculated as: PN + (1.01 × PL) + (0.79 × PM) + PN-glu.
[b] PN, HCl.
[c] HPLC data adjusted to MA data based on MA growth curves of PL and PM relative to PN. PN, HCl (transformed to MA) = PN_{HPLC} + ((1.01PL_{HPLC})0.67) + ((0.79PM_{HPLC})0.35) + PN-glu_{HPLC}.
[d] Difference (d) between the HPLC and MA data calculated as difference, divided by the mean value.
[e] Adjusted difference (D) between the HPLC and MA data calculated as difference between the MA and the adjusted HPLC data, divided by the mean value.
[f] Not detected.
[g] Below quantification limit.

The factors in Eq. (1) are due to different molar weight of PL, HCl and PM, 2HCl (mono-hydride) and PN, HCl. Table 3A shows that the two methods differ in results and that the degree of difference is related to the nature of the foodstuff. Thus, the HPLC method measured in average 70% higher ($P < 0.0005$) vitamin B_6 content in 16 food samples with animal origin (Fig. 4) than the MA method did. In 12 samples analysed with vegetable origin, the HPLC data were in average 21% ($P < 0.05$) higher than the MA data. However, in the group of grain products analysed ($n = 10$), the MA data were in average 23% higher than the HPLC data (not significant). These observations are in agreement with data published in a similar study (Schoonhoven et al., 1994), comparing HPLC data with MA data based on PN as external standard. The study estimated that growth response of PL and PM for *S. uvarum* was 80 and 50% of PN, respectively. The response was previously shown

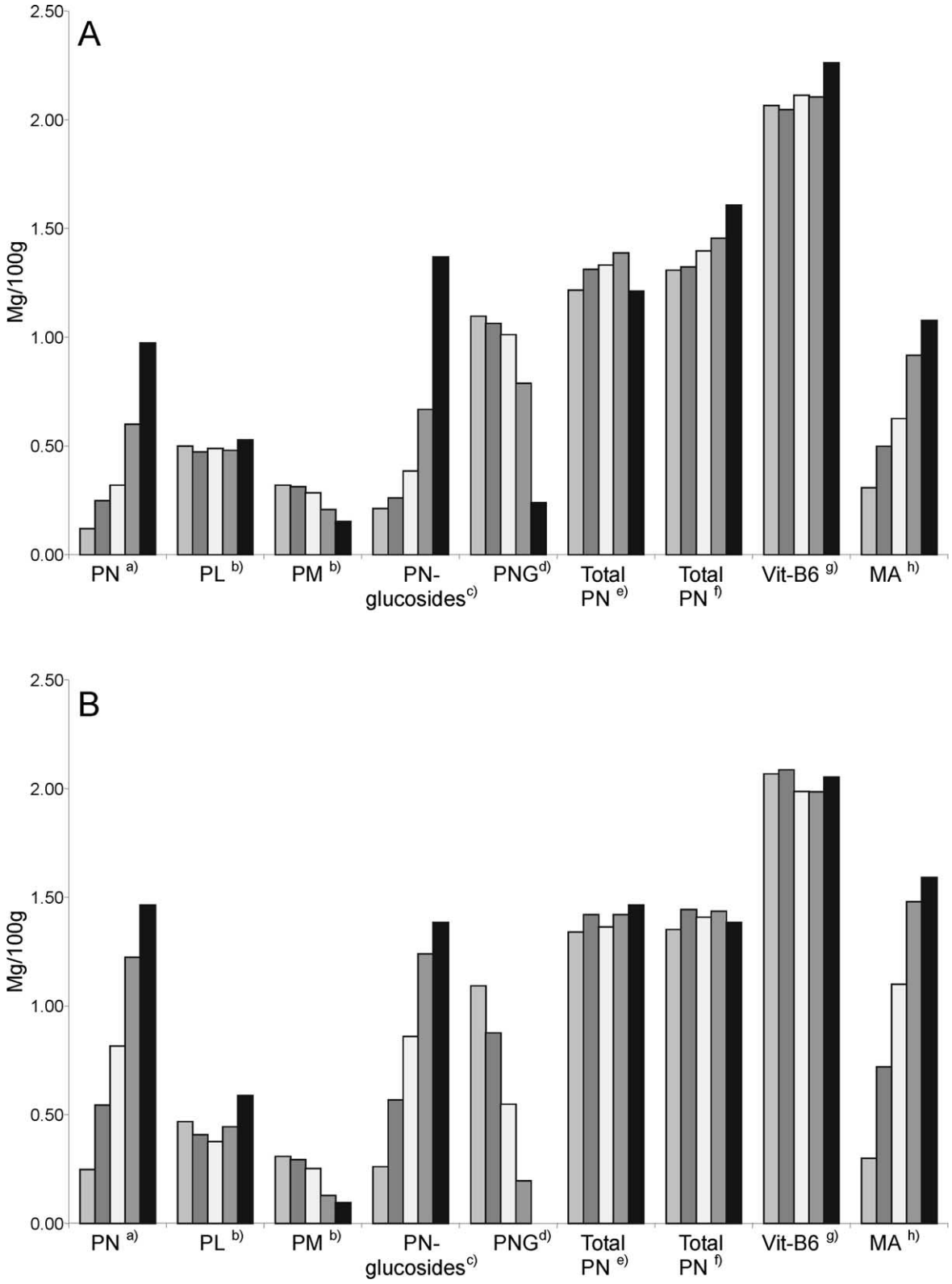

Fig. 5. Distribution of vitamers in lyophilised cabbage dependent on extraction acid and treatment time in autoclave at 121 ± 2 °C, (A) in 0.1 M HCl and (B) in 0.22 M H_2SO_4. Bar codes: 5 min, 30 min, 60 min, 120 min, 240 min. (a) Free PN was determined in the fraction only treated with acid phosphatase. (b) PL and PM were determined in the fraction treated with acid phosphatase and β-glucosidase. (c) PN-glucosides were calculated as the difference between PN in the fraction treated with and without β-glucosidase. (d) PNG was quantified by multiplication of the PN-standard response factor with peak 5 area, see Fig. 2A. (e) Total PN was calculated as PNG + PN-free, i.e. (a) + (d). (f) Total PN was determined in the fraction treated with β-glucosidase. (g) Vitamin B6 was calculated as in Eq. (1). (h) Microbiological analyses were performed on the fraction treated with acid glucosidase only.

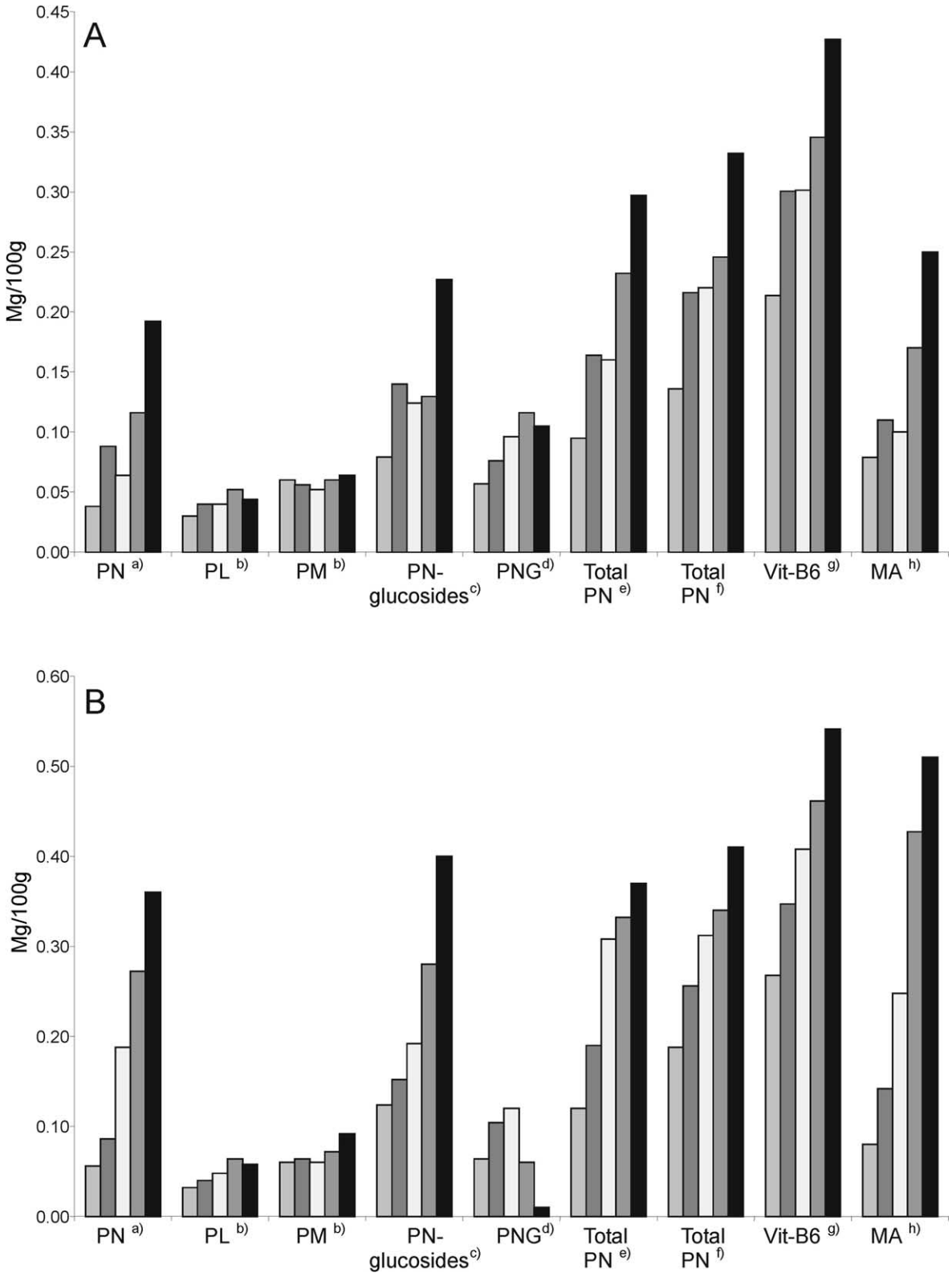

Fig. 6. Distribution of vitamers in graham flour dependent on extraction acid and treatment time in autoclave at 121 ± 2 °C, (A) in 0.1 M HCl and (B) in 0.22 M H_2SO_4. Bar codes: 5 min, 30 min, 60 min, 120 min, 240 min. (a) Free PN was determined in the fraction only treated with acid phosphatase. (b) PL and PM were determined in the fraction treated with acid phosphatase and β-glucosidase. (c) PN-glucosides were calculated as the difference between PN in the fraction treated with and without β-glucosidase. (d) PNG was quantified by multiplication of the PN-standard response factor with peak 5 area, see Fig. 2A. (e) Total PN was calculated as PNG + PN-free, i.e. (a) + (d). (f) Total PN was determined in the fraction treated with β-glucosidase. (g) Vitamin B6 was calculated by Eq. (1). (h) Microbiological analyses were performed on the fraction treated with acid phosphatase only.

Table 3
Comparison of analytical methods distributed on foodstuff groups

	n[a]	A		B	
		Raw data		Adjusted HPLC data[d]	
		d[b] (%)	P[c]	D[d,e] (%)	P[c,d]
Animal products	16	70	$P<0.0005$	0.7	$P=0.75$
Fruit and vegetables	12	21	$P<0.05$	−5.3	$P=0.20$
Grain products	10	−23	$P=0.20$	−41	$P<0.05$

[a] Number of foodstuffs in the group.
[b] Difference (d) between HPLC and MA results calculated as: 100−((HPLC/MA)×100).
[c] Paired t-test.
[d] PN,HCl (transformed to MA): $PN_{HPLC}+((1.01PL_{HPLC})0.67)+((0.79PM_{HPLC})0.35)+PN\text{-}glu_{HPLC}$.
[e] Difference (D) between adjusted HPLC and MA results calculated as: 100−((Adjusted HPLC/MA)×100.

to depend on the concentration of the vitamers (Gregory, 1982; Guilarte et al., 1980). In the present study, the response was determined in the concentration range from 0.3 and 2.4 ng/ml and the growth response of PL and PM for *S. uvarum* were found to be 67% (50–80%) (mean, range) and 35% (25–45%) of PN, respectively. The systematic difference between the two methods may be explained by alternating amounts of the three vitamers in the analysed samples. In order to evaluate this, the HPLC data was transformed to the MA data, based on the knowledge of the MA growth rates of PL and PM relative to PN.

$$\text{Vitamin } B_{6\ (\text{HPLC transformed to MA})} = PN_{HPLC} + ((1.01PL_{HPLC})0.67) + ((0.79PM_{HPLC})0.35) + PN\text{-}glu_{HPLC} \quad (2)$$

In practise, this equation could be useful to explain differences in analytical results in situations where a laboratory changes analytical technique e.g. from MA to HPLC.

Eq. (2) is to some extent in agreement with Schoonhoven et al. (1994), however, the relative growth rate for PL and PM was less in this study despite the fact that the microbiological methods were comparable. Yet, the difference illustrates that concentration of the vitamers in the test tube may have significant impact on the result of the analyses (Gregory, 1982; Guilarte et al., 1980). From Table 3B it is obvious that Eq. (2) may be true for animal and some vegetable samples, however, the model does not explain the difference between the data in the group of grain products, probably due to high amounts of very stable β-glucoside forms of pyridoxine in grains.

The model in Eq. (2) should only be used to test whether differences between HPLC and MA data are caused by the analytical differences or are caused by an actual change in vitamin B_6 contents in a particular foodstuff, e.g. when updating a database. This is important if all old data are based on MA analysis and new or additional data are based on HPLC analysis.

3.4. Internal validation

The performance of the HPLC method was examined by an internal validation. The resolutions were higher than six and five between PL and PN, and PN and PM, respectively. The typical number of theoretical plates was higher than 15,000 for PL and higher than 20,000 for PN and PM.

Limit of detection was 1 ng/ml of PL, PM and PM corresponding to 5 μg B6 vitamin/100 g sample with a sample size of 1–5 g. In the concentration range from 10 to 500 ng/ml we found excellent linearity, $R^2=0.999$, including the intercept (0.0) $P>0.05$.

The precision was determined as an internal reproducibility, i.e. as the mean difference of double determinations on different days of authentic samples, or as the variation between repeatable single determinations of in-house control materials (Table 4). The accuracy was measured as recovery in samples added PN or PMP prior to extraction and calculated as total vitamin B_6, i.e. PN, HCl (Table 5). The accuracy was further evaluated by participation in the FAPAS and BIPEA profi-

Table 4
Internal reproducibility

	Number (n)	RSD (%)
Reference material[a]		
Cabbage	20	3.8
Graham flour	6	5.6
Milk powder	6	6.3
Multivitamin tablet	6	2.6
Sample[b]		
Foodstuffs	40	3.6
Multivitamin tablets	6	3.0
Feeds	6	2.7

[a] The internal reproducibility was determined by repeatable analysis of homogeneous reference materials (x-chart).
[b] The internal reproducibility was determined by by calculation of the middle difference of double determinations on different days of authentic food samples (R-chart).

Table 5
Accuracy, recovery

Number	Statistic	
	Mean (%)	SD (%)
6	96	3.0

The recovery was calculated as percent PN, HCl found after PN or PMP were added to the sample prior to extraction.

Table 6
Accuracy, participation in proficiency testing

FAPAS			Vitamin B_6		z-score[a]	n[b]
			Result	Reference		
			mg/100 g[c]			
Serie XXI	Round 3	Milk powder	0.31	0.263	0.53	18
	Round 5	Oatmeal	0.09	0.0725	1.39	21
	Round 6	Milk powder	3.61	3.97	−0.7	32

BIPEA		Result	Reference	Tolerance[d]	n
		mg/100 g[e]			
	Musli	2.16	2.20	0.77	21
	Baby food	0.07	0.07	0.05	21

[a] z-scores ±2 are valid.
[b] Number of participating laboratories.
[c] Calculated as PN, HCl.
[d] The tolerance was calculated as 2×SD of the mean of the reported data after examination for outliers.
[e] Calculated as PN.

Table 7
Accuracy, analysis of Certified reference materials (BCR)

Matrices		Result	Reference	Uncertainty[a]
		mg B6-vitamin/100 g[b]		
CRM487	Pig liver	1.91	1.93	0.29
CRM421	Milk powder	0.68	0.67	0.085
CRM121	Wholemeal flour	0.31	0.41	0.102
CRM485	Mixed vegetables	0.66	0.48	0.08

[a] The uncertainty is defined as the half-width of the 95% confidence interval of the mean of the data set averages.
[b] Calculated as PN, HCl.

ciency test programs (Table 6) and by analyses of certified materials (Table 7).

The results of the proficiency test and analysis of certified reference materials were satisfying, however, analyses of CRM 485 (lyophilised mixed vegetables), gave results significantly higher than the certified value. According to the certification report (Finglas, Scott, van den Berg, & de Froidmont-Görtz, 1998), 10 laboratories participated in the certification process. Two of those found values of 0.68 and 0.65 mg/100 g, in concordance with our results. In the seven laboratories using MA, all used PL or PN as external standard and three laboratories used HPLC analysis determining all three vitamers. According to our analyses, approximately 80% of vitamin B_6 in CRM 485 surprisingly were PM and therefore it is tempting to suggest that the certified level of vitamin B_6 in CRM 485 has been underestimated.

In conclusion, a simple and fast HPLC method is available for analysis of vitamin B_6 in foods. This study suggests the presence of additional β-glucosylic forms of pyridoxine than previously described and in addition presence of a pyridoxamine β-glycoside in some foodstuffs.

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