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Third EU MAT intercomparison on methods for the determination of vitamins B-1, B-2 and B-6 in food

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An intercomparison study on the determination of vitamin B-1, B-2 and B-6 was performed as part of the EU MAT project involving 16 laboratories. Each laboratory was requested to analyse three different food samples (lyophilized pig's liver, mixed vegetables and wholemeal flour, respectively) using their 'in-house' method as well as an 'optimal extraction protocol' and using a common batch of takadiastase enzyme. High-performance liquid chromatography (both normal-phase and reversed-phase HPLC), microbiological assay and chemical methods were used.

For vitamins B-1 and B-2 the agreement between laboratories (using their 'in-house' methods) was generally good (vitamin B-1: $CV_w < 5\%$, $CV_b = 16–28\%$; vitamin B-2: $CV_w < 8\%$; $CV_b = 12–40\%$, respectively, where CV_w is the within-laboratory coefficient of variation and CV_b is the between-laboratory coefficient of variation) with no apparent differences between the 'in-house' and 'optimal' extraction protocols. For vitamin B-1 microbiological results tended to be higher (e.g. *ca* 20–50%) than HPLC results. Some further improvement could result from optimalization (i.e. lowering) of the sample–enzyme ratio.

Enzyme efficiency needs to be checked with each new batch of enzyme.

For vitamin B-6 results were much more at variance ($CV_w = 6–13\%$, $CV_b = 36–63\%$), especially with the pig's liver. Some discrepancy was found between laboratories using dilute mineralic acid for extraction without enzyme treatment and those using trichloroacetic acid (TCA) and/or included takadiastase treatment. It is concluded that for vitamin B-6 further standardization and method improvement is needed in order to obtain acceptable variation between laboratories. In particular, B-6 vitamer interconversion during extraction and peak interference needs further study. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Accurate methods for the determination of vitamins in foods are an essential requirement to meet EU food labelling directives, as well as for nutritional purposes such as the production of food composition tables, studies on the effect of food processing on vitamin stability and for bioavailability studies. The EU MAT project on 'Improvements in Vitamin Analysis in Food' aims to improve the measurement of vitamins in food through organizing intercomparison and method improvement studies, as well as the preparation of suitable food reference materials (RMs). In an earlier European intercomparison study on the determination of vitamins in foods, relatively poor reproducibilities were obtained for the determination of vitamin B-2 (between-laboratory CV = 28–74%) and B-6 (CV = 18–51%), while for vitamin B-1 agreement was generally better (CV = 11–18%) (Hollman *et al.*, 1993). It was concluded that the observed variability was most likely explained by differences in extraction and hydrolysis procedures, and in the case of vitamin B-6, to problems in the identification of B-6-vitamers by HPLC. In a follow-up study by a small number of laboratories, the effect of using different enzyme preparations and extraction conditions (pH, incubation time and temperature; sample-enzyme ratios) were studied in a series of selected foods and an 'optimum' extraction and hydrolysis protocol was suggested (Finglas & Hollman, 1992).

In this report, the results of a second intercomparison study are presented for vitamins B-1, B-2 and B-6. Participants were asked to analyse three different samples using both their 'in house' extraction procedure as well as a common 'optimized' extraction and hydrolysis protocol.

MATERIALS AND METHODS

Design of the study

All participants received three samples with a request to measure vitamin B-1, vitamin B-2 and vitamin B-6 contents in each of the samples using both their in-house extraction procedure and an 'optimized' extraction and hydrolysis protocol but using the same (usual) analytical assay principle. The 'optimized' extraction and hydrolysis protocol and an aliquot of takadiastase enzyme, checked beforehand for phosphatase activity (see below), was provided with the samples.

Samples

The following samples were circulated among the participants: (1) a lyophilized pig's liver (PL) preparation packaged in glass-capped bottles (*ca* 15 g material per bottle); (2) a lyophilized mixed vegetables (MV) preparation containing a mixture of sweetcorn, carrot and tinned tomatoes in the ratio 10:1:1 (w:w:w) packaged in aluminium–plastic laminate sachets (sealed under nitrogen; *ca* 25 g material per sachet); and (3) a commercially obtained wheat flour containing no additives packaged in aluminium–plastic laminate sachets (sealed under nitrogen; *ca* 50 g material per sachet). All samples were tested for homogeneity and both short- and long-term stability during storage. For each sample one sachet or bottle was provided for use for all analyses. The samples were stored at −18°C upon receipt and prior to use. Participants were asked to allow samples to equilibrate to room temperature before opening and sub-sampling.

Enzyme preparation

All participants received an aliquot (10 g) from one common batch of takadiastase enzyme (Pfalz & Bauer; Lot No. 042392; Rijnland Chemische Produkten, Capelle a/d Ijssel, The Netherlands). Conversion efficiencies of this batch of enzyme had been established before hand and were >95% as tested with thiamine diphosphate (TDP), flavin mononucleotide (FMN), pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP).

Methods

'In-house methods'
A short description of the 'in-house' extraction procedure and analytical assay principle is summarized in Tables 1–3.

'Optimal' extraction protocol for vitamins B-1 and B-2
A 0.2–5-g sample was autoclaved (30 min at 121°C) with 0.1 N hydrochloric acid (HCl) (the exact sample size and extractant volume was decided by the laboratory). The pH of an aliquot of the extract was adjusted to pH 4.0 with 4.0 M sodium acetate buffer (pH 6.1) and 100 mg takadiastase/g sample was added. The sample was incubated at 37–45°C for 4 (vitamin B-1) or 18 (vitamin B-2) h. After cooling and appropriate dilution, the solution was filtered (e.g. Whatman 42 filter paper or 0.45-μm pore size membrane) and/or centrifuged. An appropriate aliquot of the clear extract was used for quantitation of the vitamin content with the 'in-house' analytical method (HPLC or microbiological assay).

Vitamin B-6
A 0.2–5-g sample was extracted with 5% trichloroacetic acid (TCA) with shaking for 30 min. The pH of an aliquot of the extract was adjusted to 4.8 with 4.0 M sodium acetate buffer (pH 6.1) and 500 mg takadiastase per g of sample added. The sample was incubated at 37–45°C for 18 h. After cooling conc. TCA (e.g. 1.0 ml 25% per 5 ml of incubation mixture) was added to precipitate the enzyme protein. The extract was further filtered/centrifuged and an appropriate aliquot of the clear extract used for quantitation of the vitamin B-6 content using the 'in-house' analytical method (HPLC or microbiological assay).

For laboratories performing a microbiological method for vitamin B-6, the alternative use of HCl or H_2SO_4 in the extraction step was suggested in case the TCA caused problems in the assay. All participants were requested to supply methodological information and copies of standard and sample chromatograms (HPLC), or standard curves (microbiological assay).

RESULTS AND DISCUSSION

In total 16 laboratories participated in the intercomparison for vitamins B-1 and B-2, with 12 laboratories providing results for vitamin B-6. Individual results for each laboratory are graphically shown in Fig. 1(a)–(c). In this figure 'overall' mean values and the within-laboratory ($\%CV_w$) as well as the between-laboratory variability ($\%CV_B$) are presented. In the case of vitamin B-1, results are expressed as thiamin chloride hydrochloride (molecular weight = 337), for vitamin B-2 as riboflavin (molecular weight = 376) and for vitamin B-6 as pyridoxol (molecular weight = 170). The results for the separate vitamins are discussed below.

Vitamin B-1

Details of the analytical 'in-house methods are summarized in Table 1. All laboratories used a heating (autoclaving) step followed by enzymatic treatment (with takadiastase or papain/amylase) for extraction of the vitamin. Most laboratories used HPLC with fluorometric detection for quantification of the thiamin

Table 1. Short description of 'in-house' methods for vitamin B-1

Lab. No.	Extraction and hydrolysis	Analytical principle	Detection
01	15 min/121°C with 0.15 M HCl; 1 h/45°C with takadiastase (40 mg/g sample) at pH 4.5	NPLC, Radial Pak Cartridge 10 μm silica; 125×4.6 mm. El: 0.05 M P-buffer–EtOH (1000:365), pH 7.4. Post-column derivatization	Fluor (367/430 nm)
02	30 min/121°C with 0.1 M HCl; 18 h/37°C with takadiastase (500 mg) and β-amylase (50 mg) at pH 4.5. Pre-column derivatization. Clean-up with Baker C18 cartridge	RPLC, Novapak C18 cartridge, 4 μm; 150×3.9 mm. El: 0.05 M P-buffer–MeOH, pH 7.0 (25:75)	Fluor (366/435 nm)
03	30 min/121°C with 0.1 M HCl; 18 h/45°C with takadiastase (100 mg) at pH 4.0	RPLC, Supelco C18, DB. El: 0.1% tetraethyl-NH_4Cl–5 mM Na-heptansulphonate–9 mM KH_2PO_4, pH 3.5. Post-column derivatization	Fluor (368/420 nm)
04	0.2 M HCl + 70% $HClO_4$; 16 h/45°C with takadiastase at pH 4.5. Pre-column derivatization. Clean-up by isobutanol extraction	RPLC, Hypersyl NH_1, 250×4.6 mm. El: Cl_2CH_2–MeOH (95:5)	Fluor (365/440 nm)
05	30 min/100°C with 0.1 M HCl; 18 h/37°C with takadiastase (500 mg) + β-amylase (50 mg) at pH 4.5. Pre-column derivatization. Clean-up with Sep Pak C18 cartridge	RPLC, RP 60 select B, 5 μm; 25 cm. El: MeOH–0.05 *M* NaAc (40:60)	Fluor (366/435 nm)
06	30 min/121°C with 0.1 M HCl; 4 h/37°C with takadiastase (100 mg) at pH 4.0. Pre-column derivatization. Clean-up by isobutanol extraction	NPLC, Lichrosorb Li60, 5 μm; 250×4.0 mm. El: chloroform–MeOH (80:20)	Fluor (375/430 nm)
07	15 min/121°C with 0.1 M HCl; 3 h/50°C with Clara-diastase (6%) at pH 4.0–4.5. Clean-up with 50% TCA, 15 min/90°C, C18 Sep-Pak. Pre-column derivatization	RPLC, Bondapak C18 radialpak cartridge, 100×8 mm. El: MeOH–0.005 M P-buffer, pH = 7 (35:65)	Fluor (360/425 nm)
08	15 min/110°C with 0.1 M H_2SO_4. Sonication 5 min at 50°C; 20 min/45°C with takadiastase (10%). Oxidation with BrCN in NaOH. Clean-up by isobutanol extraction	Direct fluorimetric determination of thiochrome	Fluor (378/430 nm)
09	30 min/100°C with 0.1 M HCl; 16–18 h/45°C with Clara-diastase (0.2 g). Filtrate (0.22 μm). Pre-column derivatization	RPLC, Spherisorb ODS1 5 μm; 250×4.6 mm. El: MeOH–P-buffer, pH = 6.2 (70:30)	Fluor (375/435 nm)
10	1–3 min/121°C with 0.1 M HCl; 3 h/50°C with papain (40 mg/g) and amylase (40 mg/g) at pH = 4.5	Microbiological assay with *L. fermentum* ATCC 9338; 15 h/37°C, pH = 6.5	Absorbance at 540 nm
11	30 min/102°C with 0.1 N HCl; 18 h/37°C with papain (100 mg) + takadiastase (100 mg). Pre-column derivatization. Clean-up with C18 Sep-Pak	RPLC, Waters μ Bondapak C18; 8×100 mm. El: MeOH–0.05 M NaAc, pH = 4.5 (40:60)	Fluor (366/435 nm)
12	30 min/121°C with 0.1 M HCl; 18 h/45°C with takadiastase (MV–WMF, 200 mg/g; PL, 1000 mg/g) at pH 4.0; adjust pH 3.6	RPLC, Li-Chrospher RP 18, 5 μm; 25×4 and 125×4 mm. El: MeOH–0.025% NH_4OH (+ 1 g hexanesulphonic acid) (250:500), pH 3.6. Post-column derivatization	Fluor (370/425 nm)
13	30 min/121°C with 0.1 M H_2SO_4; 2 h/45°C with Clara-diastase (100 mg/g)	RPLC, C-18, 7 μm; 250×4 mm. El: 0.01 M NaH_2PO_4–0.15 M $NaClO_4$ (1:1), pH 2.2. Post-column derivatization	Fluor (375/435 nm)
14	1 h/100°C with 0.1 M HCl; 16 h/37°C with Clara-diastase (0.2 g) at pH 4.5. Clean-up with 2 ml 50% TCA, 10 min/100°C. Filtrate. Pre-column derivatization	RPLC, Spherisorb-ODS, 5 μm; 250×4.6 mm. El: MeOH–P-buffer (70:30)	Fluor (365/435 nm)
15	30 min/121°C with 0.1 M HCl; takadiastase (0.3 g/g) + papain (0.06 g/g). Pre-column derivatization	RPLC, Hamilton PRP-1, 5 μm; 150×4.6 mm. El: MeOH–H_2O, pH 4.5 (HAc) (40:60)	Fluor (366/435 nm)
16	30 min/121°C with 0.1 M HCl; 16 h/30°C with takadiastase (250 mg/g) + acid phosphatase (20 mg/g) at pH 4.5	Microbiological assay with *L. viridescens* AL, ATCC 12706 (NCIMB 8965); 24 h/30°C at pH 6.0	

Abbreviations: NPLC, normal-phase HPLC; RPLC, reversed-phase HPLC; F, fluorometry; MA, microbiological assay; PL, pig's liver; MV, mixed vegetables; WMF, wholemeal flour; MeOH, methanol; EtOH, ethanol; NaAc, sodium acetate; P-buffer: phosphate buffer.

Table 2. Short description of 'in-house methods for vitamin B-2

Lab. No.	Extraction and hydrolysis	Analytical principle	Detection
01	see vitamin B-1	RPLC, ODS-Hypersyl, 5 μm; 125×4.6 mm. El: MeOH–H_2O (20:80)	Fluor (462/520 nm)
02	see vitamin B-1	RPLC, Waters Novapak C18 cartridge, 4 μm; 150×3.9 mm. El: MeOH–50 mM P-buffer, pH 7.0 (25:75)	Fluor (445/522 nm)
03	see vitamin B-1	RPLC, Supelco C18, DB. El: 0.1% tetraethyl NH_4Cl–5 mM Na-heptansulphonate–9 mM KH_2PO_4, pH 3.5	Fluor (468/520 nm)
04	see vitamin B-1	RPLC, μBondapak C18, 250×3.9 mm. El: MeOH–H_2O + pic® B-6, pH = 4.05	Fluor (445/525 nm)
05	see vitamin B-1	RPLC, RP 60 select B, 5 μm; 250 mm. El: MeOH–0.05 M NaAc (40:60)	Fluor (422/522 nm)
06	see vitamin B-1	RPLC, Apex C18, 3 μm; 250×4.0 mm. El: H_2O–MeOH (1:1)	Fluor (450/510 nm)
07	see vitamin B-1	RPLC, Bondapak C18 radial-pak cartridge; 100×8 mm; El: MeOH-0.005 M P-buffer, pH = 7 (35:65)	Fluor (440/520 nm)
08	see vitamin B-1	RPLC, RP-18 (OD-MP Brownlee Labs), 5 μm. El: MeOH–aq. citric acid (200 mg/litre) (30:70)	Fluor (453/521 nm)
09	see vitamin B-1	RPLC, Spherisorb ODS1, 5 μm; 250×4.6 mm. El: MeOH–H_2O (50:50)	Fluor (450/510 nm)
10	see vitamin B-1	Microbiological assay with *L. rhamnosus* (ATCC7469); 15 h/37°C at pH = 6.8	Absorbance at 540 nm
11	30 min/121°C with 0.1 M HCl; further as for vitamin B-1	RPLC, as vitamin B-1	Fluor (422/522 nm)
12	see vitamin B-1	RPLC, as vitamin B-1	Fluor (467/525 nm)
13	see vitamin B-1	RPLC, C-18, 7 μm; 250×4 mm. El: MeOH–H_2O (50:50)	Fluor (445/530 nm)
14	see vitamin B-1	RPLC, Spherisorb-ODS, 5 μm; 250×4.6 mm. El: MeOH–H_2O (50:50)	Fluor (450/510 nm)
15	see vitamin B-1	RPLC, Kromasil C-18, 5 μm; 250×4.6 mm. El: MeOH–H_2O (40:60)	Fluor (440/520 nm)
16	30 min/121°C with 0.1 M HCl	Microbiological assay with *Enterococcus faecalis* (ATCC 10100); 24 h/30°C at pH 6.0	Absorbance at 590 nm

Abbreviations: NPLC, normal-phase HPLC; RPLC, reversed-phase HPLC; Fluor, fluorometry; MA, microbiological assay; MeOH, methanol; EtOH, ethanol; NaAc, sodium acetate; P-buffer, phosphate buffer.

contents (as thiochroom) with pre- or post-column reaction of thiamin to thiochrome. One laboratory (No. 8) used 'direct' fluorometric determination after thiochrome conversion, while two laboratories (10 and 16) used a microbiological method. The per cent CV_B varied between 17 and 21% with the 'in house' assays, and between 18 and 28% when using the 'optimal extraction' protocol. These variabilities are generally better than those obtained in an earlier intercomparison in which per cent CVs between 24 and 37% were obtained (Hollman *et al.*, 1993). One participant (No. 12) reported the presence of small amounts (< 10%) of 2(-1-hydroxyethyl)-thiamin. This compound is not normally measured although it has been reported to possess biological activity (Ujhe *et al.*, 1990).

Most laboratories generally reached good agreement between results with their 'in house' extraction procedure compared with the 'optimal' extraction protocols. However, some laboratories (Nos 4, 6, 10 and 16) found considerable differences, especially with the pig's liver sample. These differences are not easily explained although, at least in one case, low activity of the ('in-house') enzyme preparation played a role (Lab. No. 6). This may also be the case for the two laboratories using a microbiological assay as they also reported higher values for the pig's liver, but not for the other two samples, when using the 'optimal extraction' procedure. As liver contains most of the thiamin as phosphate ester, treatment with a phosphatase is required. The good comparability between both procedures indicates complete conversion of the thiamin phosphate esters to free thiamin. Inspection of the chromatograms did not indicate the presence of (other) thiamin phosphate peaks.

The sample–enzyme ratio used in the optimal protocol, based upon results from an earlier optimalization study (Finglas & Hollman, 1992), was higher than that used in most in-house procedures and might have been in excess. At least one lab (No. 3) reported a relatively high blank value for the 'common' enzyme preparation, i.e. 0.8 μg/g. Using an enzyme-sample ratio of 100 mg/g the 'blank' thiamin represents at least 10% of the total thiamin content in the extract.

However, most laboratories did not report blank values. Incubation time in the optimal protocol (18 h) was also longer than used in some of the in-house procedures and could probably be shortened (e.g. at least 4 h). Neither type of HPLC column (normal- or reversed-phase) nor type of reaction (pre- or post-column) was found to affect the results.

Table 3. Short description of 'in-house' methods for vitamin B-6

Lab No.	Extraction and hydrolysis	Analytical principle	Detection
01	extract with 5% TCA (30 s Ultra Turrax; shake for 30 min at room temperature (RT); 3 h/45°C with takadiastase (20 mg/g) at pH 4.5; add 1.5 ml of 16.7% TCA; centrifuge for 10 min at 3000 rpm	RPLC, ODS-Hypersyl, 3 μm; 125×4.6 mm. El: 0.1 M P-buffer, pH 2.1–MeOH–pic B-8 (1000:30:0.5). Post-column derivatization with 1 M KH_2PO_4	Fluor (333/375 nm)
02	extract with 0.5 M $HClO_4$ (60 s Ultra Turrax); filtrate, adjust to pH 7.5 (KOH); 30 min at room temperature with alk. phosphatase at pH 3.8 and/or 4 h/37°C with β-glucosidase at pH 5.0	RPLC, Waters Novapak C18 ion-pair RP, 4 μm; 150×3.9 mm. El: gradient elution (33 mM P-buffer, pH 2.2; 8 mM 1-octanesulphonic acid–P-buffer–(2-)propanol. Post-column derivatization with bisulphite in 0.5 M P-buffer, pH 7.5	Fluor (330/400 nm)
03	4 h/121°C with 0.0275 M H_2SO_4; adjust to pH 5.2 (NaOH); filtrate	Microbiological assay with *S. carlsbergensis* (ATCC 9080); 20–24 h at 30°C at pH 5.2	Turbidimetric at 650 nm
04	extraction with acid phosphatase (2 mg/250 mg sample) 16 h/37°C at pH 4.5. Pre-column transformation of PM and PAL in PN	RPLC, Lichrospher 60 RP Select B; 250×4.0 mm. El: 0.05 M KH_2PO_4, pH 2.5 (+ pic B-7)–acetonitrile (96:4)	Fluor (290/395 nm)
05	extract with 0.05 M NaAc (pH 4.5); incubate with 1 M glyoxilic acid + $FeSO_4$ (2 g/litre) + acid phosphatase, 18 h/37°C. Pre-column transformation (PAL→PN) with 0.1 M $NaBH_4$	RPLC, RP 60 select B, 5 μm; 250 mm. El: 0.1 M P-buffer, pH 2.1–MeOH–pic B-8 (1000:30:0.5) (*idem.* lab. 1)	Fluor (290/395 nm)
06	30 min/121°C with 0.1 M H_2SO_4; adjust to pH 4.8 (0.1 M NaOH); 18 h/37°C with takadiastase (500 mg/g) and/or β-glucosidase (60 U/g), 2 h/37°C	RPLC, Apex C18, 3 μm; 250×4.0 mm. El: 30 ml MeOH + 30 ml 0.5 M H_2SO_4 + 10 g NaCl + 220 μl *n*-dibutylamine in 1 litre of H_2O	Fluor (290/395 nm)
10	1–3 min/121°C with 0.1 M HCl; 3 h/50°C with papain (40 mg) and amylase (40 mg) at pH 4.5; filtrate	Microbiological assay with *S. cerevisiae* (ATCC 9080); 15 h/30°C at pH = 5.7	Absorbance at 540 nm
11	1 h/121°C with 0.5 N H_2SO_4; adjust to pH 4.8 (4 M NaOH)	Microbiological assay with *S. uvarum* (ATCC 9080); 20 h/30°C	Absorbance at 660 nm
12	30 min/121°C with 0.1 N HCl; 18 h/37°C with β-glucosidase (24 mg/g MV/WMF; 120 mg/g PL) at pH 4.8; adjust to pH 3.6 (0.7 M H_2SO_4); filtrate (0.2 μm)	RPLC, Nucleosil 120, RP, 18, 5 μm; 125×4 mm. El: 0.04 M H_2SO_4–2.5 M NaAc, pH = 3.0 (97.5:2.5)	Fluor (333/375 nm)
13	30 min/121°C with 0.1 M H_2SO_4	RPLC, C-18, 5 μm; 250×4 mm. El: 0.04 M H_2SO_4	Fluor (295/370 nm)
15	4 h/121°C with 0.055 M HCl (PL); 2 h/121°C with 100 ml 0.44 M HCl (MV/WMF)	Microbiological assay with *S. uvarum* (ATCC 9080); 21 h/30°C at pH 4.5	Turbidimetric at 575 nm
16	5 h/121°C with 150 ml HCl–0.44 M H_2SO_4	Microbiological assay with *S. carlsbergensis* (ATCC 9080); 24 h/30°C at pH 4.5	

Abbreviations: RPLC, reversed-phase HPLC; MA, microbiological assay; PL, pig's liver; MV, mixed vegetables; WMF, wholemeal flour; MeOH, methanol, EtOH, ethanol; NaAc, sodium acetate; P-buffer, phosphate buffer; PL(P), pyridoxal (5'-phosphate); PN, pyridoxine; PM, pyridoxamine.

Results obtained with the microbiological assay tended to be higher compared to those from HPLC methods for all three samples. This was also found in an earlier intercomparison (Hollman *et al.*, 1993). It is not clear whether these higher values result from the presence of additional factors stimulating (non-specific) growth of the organism (i.e. positive drift) or 'bound' thiamin unavailable for HPLC analysis. This apparent difference between methods requires some further comparative study.

Vitamin B-2

Details of the analytical 'in-house' methods are summarized in Table 2. All laboratories used a similar extraction for vitamin B-2 and B-1, i.e. heating (autoclaving) followed by enzymatic treatment (with takadiastase or papain/amylase) for extraction of the vitamin. Most laboratories used HPLC with fluorometric detection for quantification of the riboflavin contents, while two laboratories (Nos 10 and 16) used a microbiological method.

Per cent CV_B varied between 12 and 40% with the 'in house' assays, and between 12 and 34% with the 'optimal extraction' protocol. As for vitamin B-1, these variabilities were much better than those obtained in an earlier intercomparison of between 28 and 74% (Hollman *et al.*, 1993). Laboratory 4 (pig's liver, optimal extraction protocol) and Lab. 10 (mixed vegetables

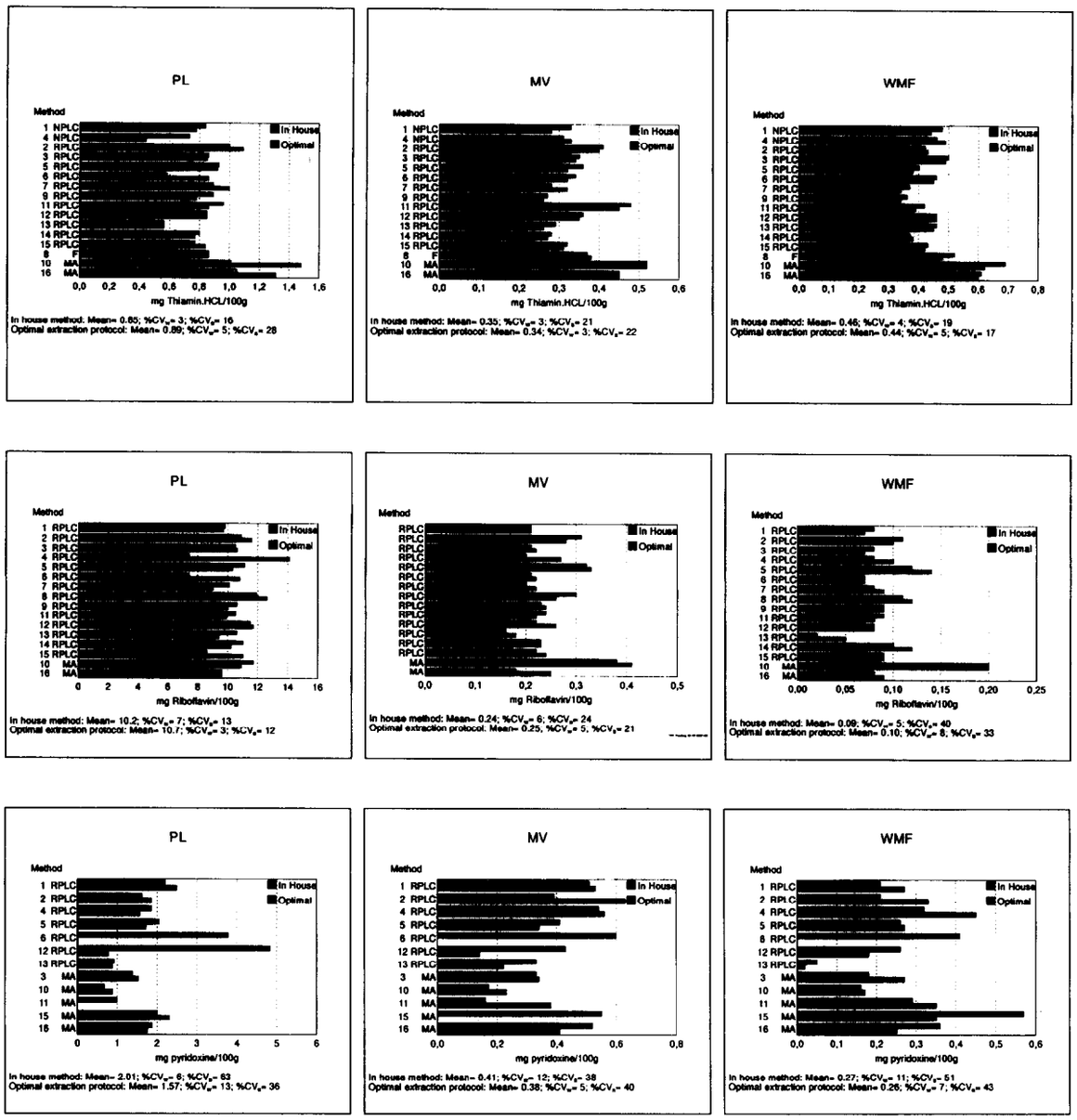

Fig. 1. Bar charts for laboratory means obtained with the 'in house' procedure and 'optimal' extraction protocol. 1A: (upper panel) vitamin B-1; 1B (middle panel: vitamin B-2; 1C (lower panel): vitamin B-6. Abbreviation: PL: lyophilized pig liver; MV: lyophilized mixed vegetables; WMF: whole meal flour). CV_B: between-laboratory coefficient of variation; CV_W: within-laboratory coeficient of variation. Method abbreviations: NPLC: normal phase HPLC; RPLC: reversed phase HPLC; F: Fluorometry; MA: Microbiological assay. Numbers (first column) refer to laboratory codes.

and wholemeal flour) reported the highest values and were identified as outliers (Nalimov test). Laboratory 13 reported apparently lower values for the mixed vegetables and, in particular, for the wholemeal flour sample. This latter sample showed the highest CV and may be considered as a less than suitable sample because of its relatively low vitamin B-2 content.

In general, there was good agreement between results using both the 'in-house' and the 'optimal' extraction procedure. Three laboratories (Nos 4, 6 and 15) reported higher values with the optimal extraction protocol compared to the in-house extraction procedure, especially for the pig's liver sample. This was probably related to incomplete conversion of the riboflavin nucleotides with the ('in house') extraction–hydrolysis procedure. However, inspection of the chromatograms did not indicate the presence of other riboflavin compounds (peaks).

As mentioned for thiamin, the sample–enzyme ratio might have been in excess. Laboratory 3 also reported a relatively high 'blank' value for riboflavin, i.e. *ca* 3 μg/g. Using an enzyme–sample ratio of 100 mg/g such a high 'blank' might, in the case of samples with a low riboflavin content such as the wholemeal flour, be higher than the actual endogeneous amount present. The amount of enzyme might indeed be lower, as most participants used lower enzyme–sample ratios with their in-house procedures, without a significant difference

between the results obtained with both protocols. Incubation time in the optimal protocol (18 h) was also longer than used with some of the in-house procedures and could probably be shortened. However, in the earlier optimalization study higher results were obtained after incubation with takadiastase for 18 h compared to 4 h (unpublished results). No differences between both incubation periods were observed when combined takadiastase–phosphatase treatment was used illustrating that the actual enzyme activity–capacity is an important determinant of the incubation time required for maximal conversion. For riboflavin, no differences between methods were apparent although results for the two laboratories using a microbiological assay showed some discrepancy, especially in case of the mixed vegetable sample. One of these laboratories (No. 10) also found higher levels in the flour. This laboratory included enzymatic treatment during extraction, while the other lab (No. 16) omitted this step assuming that the flavin adenine dinucleotide (FAD) present in the sample becomes completely hydrolysed after boiling with acid and the micro-organism has a similar response for riboflavin and flavin mononucleotide (FMN). Whether the generally higher variability observed for the flour sample is, indeed, due to the relatively low contents, or is also related to specific problems caused by the cereal matrix, remains to be established.

Vitamin B-6

Details of analytical 'in-house' methods are summarized in Table 3. All laboratories used a procedure with dilute acid followed by heating at 121°C (autoclaving). Further extraction and clean-up procedures depended on the type of assay (HPLC or microbiological) and whether separate vitamers were quantitated. Seven laboratories performed HPLC, four of which carried out (enzymatic) dephosphorylation before HPLC, separating the three 'free' vitamers pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM), respectively. One participant (No. 2) performed a mild acid extraction (see Table 3), avoiding dephosphorylation, and separated the individual 'free' vitamers as well as the corresponding phosphate esters using gradient elution (Bitsch *et al.*, 1989). The other two laboratories (Nos 4 and 5) performed a pre-column chemical conversion of PL and PM into PN (with glyoxylic acid–$FeSO_4$ and $NaBH_4$, respectively, as described by Reitzer-Bergaentzle *et al.*, 1993).

Five laboratories performed a microbiological assay after acid extraction–autoclaving and using a similar organism (*Saccharomyces cerevisiae, S. carlsbergensis* and *S. uvarum*). One participant (No. 10) using microbiological assay included enzymatic treatment after a (short) autoclaving step.

Compared to the other two B-vitamins, CV_Bs were considerably greater, varying between 35 and 54% with the 'in house' assays and between 37 and 51% when using the common 'optimal extraction' protocol. Results presented in Fig. 1(c) refer to 'total' vitamin B-6, obtained by summing up the contents for the separate vitamers (in the case of HPLC). Although it is rather common to express results for vitamin B-6 as pyridoxine·HCl, being an easily available, pure and stable standard preparation, 'the advantage' of expressing results as the free base of pyridoxine (PN) is that the 'total' vitamin B-6 content is easily calculated by adding up the amounts of the various free vitamers which have nearly equal molecular weights (for conversion of results expressed as free PN to PN·HCl multiply with a factor 1.22).

Although the 'between-laboratory' variability was considerable, most laboratories reported good agreement between the 'in-house' compared to the common 'optimal' extraction protocol. However, two laboratories (Nos 6 and 12) using an HPLC method including mineral acid extraction, rather than perchloric acid or trichloroacetic (TCA) acid, reported relatively high concentrations for the pig's liver sample, using their 'in-house' extraction procedure. These levels were also higher than those submitted by participants using a microbiological assay and including a similar extraction (autoclaving) with mineral acid. Further inspection of the data submitted for the separate vitamers (results nor shown) indicated that the higher values from Labs 6 and 12 resulted from an apparently higher PL value. Subsequent studies showed that this was due to coelution of an interfering peak with PL under the chromatographic conditions used by laboratory 12 (Bognar & Ollilainen, unpublished results). Remarkably, participant 12 obtained much lower values when using the common 'optimal' protocol. The two laboratories (Nos 4 and 5) performing pre-column chemical conversion of PL and PM into PN reported values comparable to the other laboratories. Laboratory 13 reported relatively lower values, probably due to problems in the HPLC separation and quantification as this group could only identify PM, but not PN and/or PL, in the samples. From the data provided by laboratories (data not shown) on the separate B-6 vitamers, it can be concluded that phosphate esters of PL and PM were the main B6-vitamers present in the liver sample, while PM is the main B-6 vitamer in the mixed vegetables. Pyridoxine-β-glucoside (PNG) is apparently the main B-6 vitamer in the wheat flour sample. Mixed vegetables only contained trace amounts of PNG. Inclusion of an 'extra' β-glucosidase treatment in the measurement of 'total' vitamin B-6 does not seem necessary when takadiastase is used in the extraction procedure. As has already been reported before, this enzyme preparation apparently contains sufficient β-glucosidase activity (Van Schoonhoven *et al.*, 1994). Autoclaving with dilute acid also results in (partial) hydrolysis of the glucosides.

Although definite conclusions cannot be reached due to the considerable variability, results obtained with HPLC methods tended to be slightly higher than those from microbiological methods. This was especially true for the pig's liver and mixed vegetables sample and is most likely explained by the presence of PM in these samples. Yeasts have been demonstrated to have a lower growth response for PM compared to PN which is

commonly used as the standard in these microbiological assays (Gregory, 1988; Van Schoonhoven *et al.*, 1994). However, not all laboratories performing microbiological assay obtained lower values. Results from Labs 15 and 16 compared favourably with HPLC values, and were 2–3 times higher than those from Lab. 10 using the same micro-organism, but a shorter autoclaving time. This may suggest that autoclaving for longer periods results in conversion (deamidation?) of PM into a form better available for the micro-organism.

From these results it is clear that further standardization and method improvement is needed to have better comparability between laboratories. One aspect that needs further study is the occurrence of interconversion due to the extraction and/or analytical procedure, resulting in apparent losses and changes in the actual B-6 vitamer composition.

CONCLUSION

From these data we conclude that for vitamins B-1 and B-2 the agreement between laboratories (using their 'in-house' methods) was generally good and better than in an earlier intercomparison. No apparent differences between the 'in-house' and 'optimal' extraction protocols were obtained. However, for vitamin B-1 microbiological results tended to be higher (e.g. *ca* 20–50%) than HPLC results. Some further improvement could result from optimalization (i.e. lowering) of the sample–enzyme ratio.

For vitamin B-6, results were much more at variance, especially for the pig's liver. Some discrepancy was found between laboratories using dilute mineralic acid for extraction without enzyme treatment and those using trichloroacetic acid (TCA) and/or included taka-diastase treatment. It is concluded that for vitamin B-6further standardization and method improvement is needed in order to obtain acceptable variation between laboratories. In particular, B-6 vitamer interconversion during extraction and peak interference needs further study.

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