Certification of B-Group Vitamins (B_1, B_2, B_6, and B_{12}) in Four Food Reference Materials

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In 1989, the Community Bureau of Reference started a research program to improve the quality of vitamin analysis in food. To achieve this task, vitamin methodology was evaluated and tested by interlaboratory studies and the preparation of certified reference materials, which will be used for quality control of vitamin measurements. The main improvements in methodology were achieved by testing and standardizing the extraction condition and enzymatic hydrolysis procedures. Results for each individual material are derived from five replicate determinations using at least two independent methods: liquid chromatography (HPLC) and microbiological assay for vitamins B_1, B_2, and B_6; and radioprotein binding and microbiological assays for vitamin B_{12}. The certificate of analysis for four reference materials gives mass fraction values for water-soluble vitamins. These certified values were based on the acceptable statistical agreement of results from collaborating laboratories. Certified values with uncertainties (mg/kg dry matter) for each CRM are as follows: 4.63 (0.20) and 4.10 (0.51) for vitamins B_1 and B_6, respectively, in CRM 121 (wholemeal flour); 6.51 (0.24), 14.54 (0.3), 6.66 (0.43), and 0.034 (0.003) for vitamins B_1, B_2, B_6, and B_{12}, respectively, in CRM 421 (milk powder); 3.07 (0.17) and 4.80 (0.40) for vitamins B_1 and B_6, respectively, in CRM 485 (lyophilized mixed vegetables), and 8.58 (0.55), 106.8 (2.8), 19.3 (1.5), and 1.12 (0.044) for vitamins B_1, B_2, B_6, and B_{12}, respectively, in CRM 487 (lyophilized pig liver).

Keywords: *B-group vitamins; foods; certified reference materials; quality control*

INTRODUCTION

The B-group water-soluble vitamins consist of several chemically and physiologically different organic compounds present in a variety of biological matrices. Improvements in bioanalytical chemistry have expanded the number of methods or techniques available at present. In fact, vitamin analysts are now faced with an extensive array of methods, but the validity of the methodologies is not always self-evident. However, the ruggedness of the method might not be obvious. Reference materials provide an external monitor to evaluate the statistic control of measurements in an analytical work. Reference materials are needed in method validation and evaluation of a laboratory's analytical capability. In general, they play an important role as a link in the traceability chain of the results.

In 1989, the Community Bureau of Reference commenced a program to improve the quality of vitamin analysis in food. This was divided into the following categories: improvements in methodology (extraction, cleanup, and calibration), intercomparison of the methods, and preparation of the reference materials. First intercomparison results dealt with fat-soluble (Hollman et al., 1993a) and water-soluble vitamins (Hollman et al., 1993b). Further methodology produced reports for carotenoids (Scott et al., 1996; Schüep and Schierle, 1997), folates (Finglas et al., 1993; Vahteristo et al., 1996), and vitamins B_1, B_2, and B_6 (van den Berg et al., 1996). Preparation of the reference materials including homogeneity and stability tests has been reviewed extensively elsewhere (Finglas et al., 1997, 1998).

In this paper, we report the certification results for four food reference materials. On the basis of the work of 20 collaborating laboratories, the mass fractions of four B-group vitamins for reference materials CRM 121 (wholemeal flour), CRM 421 (milk powder), CRM 485 (lyophilized mixed vegetables), and CRM 487 (lyophilized pig liver) were determined. Certified values are given for vitamin B_1 (thiamin), vitamin B_2 (riboflavin), vitamin B_6 (pyridoxine), and vitamin B_{12} (cyanocobalamin). The data for folates and carotenoids will be presented elsewhere.

MATERIALS AND METHODS

Calibrants and Enzyme Preparate. Cyanocobalamin (V-2870), thiamin chloride hydrochloride (T-4625), riboflavin (R-4500), pyridoxine hydrochloride (P-9755), pyridoxal hydrochloride (P-9130), and pyridoxamine dihydrochloride (P9130) were purchased from Sigma Chemical Co, (St. Louis, MO) and delivered to the participating laboratories together with the samples and a commercial Takadiastase preparate (lot 04392, Phalz & Bauer). Calibrants, samples, and enzyme preparate

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Table 1. Homogeneity Results for the Certified Materials

	homogeneity results for										
	vitamin B_1			vitamin B_2		vitamin B_{12}		niacin		β-carotene	
material	$\%CV_W$[a]	$\%CV_B$[b]	$\%CV_R$[c]	$\%CV_B$	$\%CV_R$	$\%CV_B$	$\%CV_R$	$\%CV_W$	$\%CV_R$	$\%CV_B$	$\%CV_R$
CRM 121	4.5	9.3						3.0	8.1		
CRM 421				4.0	5.7	2.8	8.5				
CRM 485										6.8	7.4
CRM 487		3.2	10.6	6.5	12.8						

[a] $\%CV_W$ = variation within sachets (bottles). [b] $\%CV_B$ = variation between sachets (bottles). [c] $\%CV_R$ = method reproducibility.

Table 2. Stability Results for the Certified Materials

material	vitamin(s)	short-term stability	long-term stability
CRM 121	vitamins B_1, B_2, and B_6	stable for 8 weeks at 37 °C	stable for 48 months at −20 °C
CRM 421	vitamins B_1, B_2, B_6, and B_{12}[a]	stable for 21 days at 25 °C	stable at −18 °C
CRM 485	vitamins B_1 and B_6[b]	stable for 28 days at 25 °C	stable for 48 months at −40 °C
CRM 487	vitamins B_1, B_2, and B_{12}	stable for 21 days at 25 °C	
	vitamins B_1, B_2, and B_6		stable for 48 months at −18 °C
	vitamins B_1, B_6, and B_{12}		stable for 24 months at 4/18 °C
	vitamin B_2		stable for 18 months at 4/18 °C
	vitamin B_{12}		stable for 24 months at −18 °C

[a] Stability for vitamins B_1, B_2, B_6, and B_{12} is based on the acceptable stability for vitamin C and niacin. [b] Stability for vitamins B_1 and B_6 is based on the acceptable stability for the carotenoids (*trans*-α- and *β*-carotenes, *trans*-lycopene, lutein, and zeaxanthin).

were stored in a refrigerator at −18 °C or lower until analyses were performed.

Calibration and Purity of Calibrants. The concentration levels of an external standard calibration covered the expected vitamin content of the sample extracts, and the standards were treated like samples. This was also the case for each vitamer in vitamin B_6 analysis when individual vitamers were measured. Calibration with at least four concentration levels was established for each vitamin and for each day's analysis. Standard curves should pass through the origin within the limits of 95% confidence limits.

The purity of calibrants was checked, and the results were reported using the sheets provided. Stock standard solutions of vitamins B_2 and B_6 were prepared on a weight basis. The concentration of the stock solutions for vitamins B_1 and B_{12} was measured spectrophotometrically using a specified procedure given in the protocol. Specific (or molar) absorption coefficient values of $E^{1\%}_{1cm} = 421$ ($\epsilon = 14200$ L mol^{-1} cm^{-1}) in 0.1 M hydrochloric acid solution at 248 nm for thiamin (Dawson et al., 1986) and $E^{1\%}_{1cm} = 227.3$ ($\epsilon = 30800$ L mol^{-1} cm^{-1}) in a 0.5 M sodium hydroxide solution containing 0.5% potassium cyanide at 368 nm for cyanocobalamin were used (Lindemans and Abels, 1985).

Certified Reference Materials. Food materials were those described earlier by Finglas et al. (1996). CRM 121 was a wholemeal flour; CRM 421 a vitamin-enriched milk powder; CRM 485, lyophilized mixed vegetables (sweet corn, carrot, and canned tomatoes in the ratio of 10:1:1); and CRM 487, a lyophilized pig liver. Aluminum−plastic laminate sachets of CRMs 121, 421, and 485 were sealed under nitrogen. The sample size was ∼50 g. CRM 487 (∼25 g) was packed into sealed amber bottles. A detailed description of the preparation of the certified reference materials is found elsewhere (Finglas et al., 1998). An approximate range of the vitamin content of each sample was given in the protocol circulated to the participants.

Homogeneity and Stability of the Certified Materials. The prepackaging homogeneity study included the measurement of moisture, water- and fat-soluble vitamins, and carotenoids. No significant difference among sachets (bottles) was found. In the main homogeneity study, the coefficient of variation, $\%CV_w$ (demonstrating the variation within sachets or bottles), was measured by repeated analysis of a single sachet or bottle. The coefficient of variation for between-sachet variation ($\%CV_B$) was measured from a single analysis of 20 sachets taken at regular intervals through the packaging sequence. The method reproducibility ($\%CV_R$) was obtained from the duplicate analyses of different times of several randomly selected sachets used for the long-term stability study. The insignificant difference between $\%CV_B$ and $\%CV_R$ values was concluded to show an absence of significant inhomogeneity.

The homogeneity of the certified materials was measured for two vitamins (vitamin B_1 and niacin) in CRM 121, for two vitamins (vitamins B_1 and B_2) in CRM 421, and for two vitamins (vitamins B_1 and B_2) in CRM 487 as all of the water-soluble vitamins in each of the materials will have a similar behavior and distribution (Association of Vitamin Chemists, 1966). The carotenoid content of CRM 485 was monitored because water-soluble vitamins are likely to be associated with various binding proteins and distributed evenly throughout the material in a similar way to the carotenoids (Association of Vitamin Chemists, 1966). The vitamin or carotenoid measurements were performed using a liquid chromatographic method for vitamins B_1 and B_2 (Weerdhof et al., 1973) and carotenoids (Hart and Scott, 1995) or a microbiological assay for vitamin B_1 and niacin (Bell, 1974). This main homogeneity study confirmed acceptable homogeneity for all four certified reference materials (Table 1). A more detailed information concerning the homogeneity study is reported elsewhere (Finglas et al., 1997, 1998).

Both short- and long-term stabilities of each vitamin in four certified materials were investigated. Short-term study was performed at high ambient temperature (25−37 °C) to estimate the possible vitamin loss during the normal shipment of samples. Long-term stability was assessed by comparing the vitamin contents of the samples stored at different temperatures at the various time points of analyses.

Stability data were evaluated for vitamins B_1, B_2, and B_6 in CRM 121 and for vitamins B_1, B_2, B_6, and B_{12} in CRM 487 (Table 2). Carotenoids (α- and *β*-carotene, lutein, lycopene, and zeaxanthin) showed acceptable stability in CRM 485. As carotenoids are considered to be similarly distributed throughout this material and they are expected to be more labile compounds than vitamin B_1 or B_6, the conclusion on the stability of vitamins $B_1$and B_2 was extrapolated from the carotenoid results. Acceptable stability for vitamins B_1, B_2, B_6, and B_{12} in CRM 421 was based on the stability data evaluated for vitamin C and niacin. Further data on the stability studies will be found in the studies published by Finglas et al. (Finglas et al., 1997, 1998).

Sample Extraction and Enzymatic Hydrolysis. *Vitamins B_1 and B_2.* The extraction procedure for vitamins B_1 and B_2 consisted of an autoclaving process in a dilute hydrochloric acid solution followed by a takadiastase digestion as described by van den Berg et al. (1996). A 1−5 g sample was autoclaved at 121 °C for 30 min in 0.1 M hydrochloric acid solution. An aliquot of the sample extract was adjusted to pH 4.8, taka-

Table 3. Certified Mass Fractions and Collaborative Parameters for Vitamins B_1 and B_6 in CRM 121 Wholemeal Flour[a]

analyte	P[b]	N[c]	units	value (uncertainty)[d]	repeatability SD S_r	repeatability RSD_r	repeatability value r ($2.83S_r$)	reproducibility SD S_R	reproducibility RSD_R	reproducibility value R ($2.83\ S_R$)
vitamin B_1[e]	14	70	mg/kg of DM	4.63 (0.20)	0.15	3.3	0.43	0.67	14.4	1.89
vitamin B_6[f]	10	50	mg/kg of DM	4.10 (0.51)	0.25	6.2	0.71	1.41	34.4	3.99

[a] Methodology: vitamin B_1, normal-phase liquid chromatography−fluorescence detection {3}; reversed-phase liquid chromatography−fluorescence detection {10}, microbiological {1}; vitamin B_6, reversed-phase liquid chromatography−fluorescence detection {3}, microbiological {7}. Number in braces indicates the number of laboratories using a particular method. [b] P = number of accepted sets of results. [c] N = number of accepted replicates. [d] Total uncertainty as the half-width of the 95% confidence interval. [e] Vitamin B_1 as thiamin chloride hydrochloride (M_w 337.3 g mol^{-1}). [f] Vitamin B_6 as a sum of PN, PL, and PM calculated as PN·HCl (M_w 205.6 g mol^{-1}).

diastase solution (100 mg of enzyme/g of sample) was added, and the mixture was incubated at 37−45 °C for 4 h. After cooling and appropriate dilution, the extract was filtered or centrifuged. Sample solutions were stored at 4 °C between extraction and determination phases. This extraction protocol was optimized and validated before the certification work in earlier performed intercomparison studies (Hollman et al., 1993b; van den Berg et al., 1996).

Vitamin B_6. The method applied for vitamin B_6 analysis was refined from previous interlaboratory studies (Hollman et al., 1993b; van den Berg et al., 1996). An acid hydrolysis combined with a takadiastase digestion was performed for extraction of vitamin B_6. Samples (1−5 g) were extracted with a 5% (w/v) trichloroacetic acid (TCA) solution under shaking for 30 min. An aliquot of the mixture was adjusted to pH 4.8 with sodium acetate buffer, takadiastase enzyme (500 mg of enzyme/g of sample, dissolved in water) was added, and the mixture was incubated for 18 h at 37−45 °C. The enzyme protein was precipitated by adding concentrated TCA to the cooled sample extract, and the solution was filtrated before liquid chromatographic or microbiological assay. For those laboratories performing microbiological assay using *Saccharomyces uvarum* ATCC 9080 or *Neurospora sitophila* ATCC 9776 as the test organism, an autoclaving procedure with dilute mineral acid (0.25−0.44 M H_2SO_4 or 0.06−0.1 M HCl) for 4−5 h was mandatory (van den Berg et al., 1996). Sample solutions were stored at 4 °C between extraction and determination.

Vitamin B_{12}. The choice of the sample extraction and cleanup procedures prior to end-method of analysis (competitive protein binding assay or microbiological assay) was left to the participants. Five participants extracted samples with a 0.1 M acetate buffer (pH 4.6−4.7) followed by a potassium cyanide treatment, whereas a phosphate−citrate buffer (pH 4.5), containing sodium sulfite, was used in sample extraction in two laboratories.

Quantitative Measurements. *Vitamin B_1.* A liquid chromatographic method or a microbiological assay was used for vitamin B_1. Most of the laboratories performed the liquid chromatographic analysis using either pre- or postcolumn thiochrome derivatization with potassium hexacyanoferrate in alkaline media preceding the fluorometric detection (Finglas et al., 1998). Thiochrome was separated using either a reversed-phase (11 laboratories) or a normal-phase chromatography (4 laboratories). The microbiological method (using *Lactobacillus fermenti* ATCC9338 or *Lactobacillus viridescens* ATCC12706) was performed by two participants. One laboratory based their determination on direct fluorometry.

Vitamin B_2. Riboflavin was analyzed using a reversed-phase liquid chromatography with a fluorometric detection in most of the measurements (15 laboratories). A microbiological method using *Lactobacillus casei* as a test organism was performed in two laboratories.

Vitamin B_6. The reversed-phase chromatographic method was used in five laboratories to analyze vitamin B_6 content. Three collaborators utilized takadiastase in the enzymatic digestion, whereas β-glucosidase or phosphatase digestion was applied in two laboratories. One laboratory performed a postcolumn derivatization method in which pyridoxal and pyridoxamine were converted to pyridoxine. An extraction procedure using a dilute hydrochloric acid solution was put to use in seven laboratories performing a microbiological assay. Test organisms were then *Saccharomyces uvarum* ATCC 9080 (formerly *S. carlsbergensis*) (six laboratories) or *Neurospora sitophila* ATCC 9776 (one laboratory).

Vitamin B_{12}. Measurement of the total cobalamin was based on the affinity binding analysis in two laboratories, whereas a microbiological method was applied in five laboratories. Laboratories performing microbiological assays used *Lactobacillus leichamannii* ATCC7830 in their analysis.

Determination of Moisture Content. The dry matter content of each sample sachet was determined by drying a 2 g sample in duplicate at 103 ± 2 °C for 4 h.

Number of Determinations and Expression of Results. Five replicate determinations of two separate sachets or bottles were performed on two different days. Three determinations were done on the one sachet on the first day, and the other two analyses were performed on the second sachet on the second day.

Vitamin results were reported as the total vitamin content: total thiamin (total vitamin B_1) as thiamin chloride hydrochloride (M_w 337.3 g mol^{-1}) and vitamin B_2 as total riboflavin. Total vitamin B_6 value was calculated as a sum of pyridoxine, pyridoxal, and pyridoxamine, and the total amount was expressed as pyridoxine hydrochloride (M_w 205.6 g mol^{-1}). Total cobalamin (vitamin B_{12}) was calculated as cyanocobalamin (M_w 1355.4 g mol^{-1}). Certification results were calculated on the basis of dry matter and expressed as milligrams per kilogram of dry matter (DM).

Traceability of the Measurements and Statistics. Laboratory equipment was calibrated to an appropriate accuracy using national or international standards of measurement, where applicable. Calibration and traceability data for balances, spectrophotometers, thermometers, and volumetric glassware were reported to the coordinator together with the results.

Outlying variance, homogeneity of variances, and normality of distribution of mean values were tested using the Cochran test, Bartlett test, and Kolmogorov−Smirnov−Lilliefors test, respectively. A Nalimov test was performed to identify outlying mean values (Finglas et al., 1997, 1998). If the homogeneity of variances did not match Bartlett test with all the vitamin−reference material combinations, a nonparametric Kruskal−Wallis or a two-sample comparison, a Mann−Wilcoxon test, was applied (Sokal and Rohlf, 1995). Reproducibility relative standard deviation RSD_R (relative standard deviation between laboratories) was compared to that value of an empirical Horwitz formula, $RSD = 2^{1-0.5\log C}$, where concentration C is expressed as mass per mass unit (Albert and Horwitz, 1997).

Participating Laboratories. Laboratories involved in this study were experienced in vitamin measurements by taking part in earlier intercalibration and collaborative work (van den Berg et al., 1996). Twenty European laboratories participated this study; from 12 to 18 laboratories took part in the measurement of vitamins B_1 and B_2; vitamin B_6 was determined in 10 laboratories, whereas from 7 to 8 laboratories performed vitamin B_{12} analysis.

RESULTS AND DISCUSSION

Vitamin B_1. Results derived from the measurements of 14−18 laboratories were used for the data evaluation for vitamin B_1 (Tables 3−6). A normal-phase or reversed-phase chromatography combined with a pre- or post-

Table 4. Certified Mass Fractions and Collaborative Parameters for Vitamins B_1, B_2, B_6, and B_{12} in CRM 421 Milk Powder[a]

analyte	P[b]	N[c]	units	value (uncertainty)[d]	repeatability SD S_r	repeatability RSD_r	repeatability value r ($2.83S_r$)	reproducibility SD S_R	reproducibility RSD_R	reproducibility value R ($2.83S_R$)
vitamin B_1[e]	15	75	mg/kg of DM	6.51 (0.24)	0.25	3.8	0.70	0.85	13.1	2.42
vitamin B_2[f]	15	73	mg/kg of DM	14.54 (0.3)	0.60	4.1	1.69	0.88	6.1	2.50
vitamin B_6[g]	11	55	mg/kg of DM	6.66 (0.43)	0.43	6.5	1.22	1.24	18.6	3.51
vitamin B_{12}[h]	7	35	μg/kg of DM	0.034 (0.003)	0.0014	4.2	0.0040	0.0045	13.1	0.013

[a] Methodology: vitamin B_1, normal-phase liquid chromatography−fluorescence detection {4}, reversed-phase liquid chromatography−fluorescence detection {10}, microbiological {1}; vitamin B_2, reversed-phase liquid chromatography−fluorescence detection {13}, microbiological {2}; vitamin B_6, reversed-phase liquid chromatography−fluorescence detection {4}, microbiological {7}; vitamin B_{12}, radioprotein binding assay {2}, microbiological {5}. Number in braces indicates the number of laboratories using a particular method. [b] P = number of accepted sets of results. [c] N = number of accepted replicates. [d] Total uncertainty as the half-width of the 95% confidence interval. [e] Vitamin B_1 as thiamin chloride hydrochloride (M_w 337.3 g mol^{-1}). [f] Vitamin B_2 as riboflavin. [g] Vitamin B_6 as a sum of PN, PL, and PM calculated as PN·HCl (M_w 205.6 g mol^{-1}). [h] Vitamin $_{12}$ as total cobalamine.

Table 5. Certified Mass Fractions and Collaborative Parameters for Vitamins B_1 and B_6 in CRM 485 Lyophilized Mixed Vegetable[a]

analyte	P[b]	N[c]	units	value (uncertainty)[d]	repeatability SD S_r	repeatability RSD_r	repeatability value r ($2.83S_r$)	reproducibility SD S_R	reproducibility RSD_R	reproducibility value R ($2.83S_R$)
vitamin B_1[e]	16	78	mg/kg of DM	3.07 (0.17)	0.18	5.7	0.50	0.63	20.5	1.78
vitamin B_6[f]	10	50	mg/kg of DM	4.80 (0.40)	0.24	5.1	0.69	1.87	22.6	5.28

[a] Methodology: vitamin B_1, mormal-phase liquid chromatography−fluorescence detection {3}, reversed-phase liquid chromatography−fluorescence detection {10}, direct fluorometry {1}, microbiological {2}; vitamin B_6, reversed-phase liquid chromatography−fluorescence detection {3}, microbiological {7}. Number in braces indicates the number of laboratories using a particular method. [b] P = number of accepted sets of results. [c] N = number of accepted replicates. [d] Total uncertainty as the half-width of the 95% confidence interval. [e] Vitamin B_1 as thiamin chloride hydrochloride (M_w 337.3 g mol^{-1}). [f] Vitamin B_6 as a sum of PN, PL, and PM calculated as PN·HCl (M_w 205.6 g mol^{-1}).

Table 6. Certified Mass Fractions and Collaborative Parameters for Vitamins B_1, B_2, B_6, and B_{12} in CRM 487 Lyophilized Pig Liver[a]

analyte	P[b]	N[c]	units	value (uncertainty)[d]	repeatability SD S_r	repeatability RSD_r	repeatability value r ($2.83S_r$)	reproducibility SD S_R	reproducibility RSD_R	reproducibility value R ($2.83S_R$)
vitamin B_1[e]	18	87	mg/kg of DM	8.58 (0.55)	0.31	3.7	0.89	2.20	25.7	6.23
vitamin B_2[f]	12	59	mg/kg of DM	106.8 (2.8)	1.78	1.7	5.04	8.74	8.2	24.74
vitamin B_6[g]	11	55	mg/kg of DM	19.3 (1.5)	1.51	7.8	4.28	4.15	21.5	11.76
vitamin B_{12}[h]	8	40	mg/kg of DM	1.12 (0.044)	0.051	4.6	0.14	0.10	9.1	0.29

[a] Methodology: vitamin B_1, normal-phase liquid chromatography−fluorescence detection {4}, reversed-phase liquid chromatography−fluorescence detection {11}, direct fluorometry {1}, microbiological {2}; vitamin B_2, reversed-phase liquid chromatography−fluorescence detection {11}, microbiological {1}; vitamin B_6, reversed-phase liquid chromatography−fluorescence detection {4}, microbiological {7}; vitamin B_{12}, microbiological {6}, radioimmunoassay {2}. Number in braces indicate the number of laboratories using a particular method. [b] P = number of accepted sets of results. [c] N = number of accepted replicates. [d] Total uncertainty as the half-width of the 95% confidence interval. [e] Vitamin B_1 as thiamin chloride hydrochloride (M_w 337.3 g mol^{-1}). [f] Vitamin B_2 as riboflavin. [g] Vitamin B_6 as a sum of PN, PL, and PM calculated as PN·HCl (M_w 205.6 g mol^{-1}). [h] Vitamin B_{12} as total cobalamine.

column thiochrome oxidation was mainly used; two laboratories performed microbiological assay, and one laboratory used direct fluorometric measurement of thiochrome derivate. There was no significant difference in CRM 485 results derived from the liquid chromatographic methods compared to those of microbiological assays. Mean and median values of liquid chromatographic and microbiological data for CRM 487 seem to differ. Higher values were found with microbiological assay compared to liquid chromatography. Thus, these results did not clearly show that microbiological assay tends to give a somewhat higher result compared to liquid chromatographic procedure as found in an earlier intercomparison study (van den Berg et al., 1996). The presence of some growth-stimulating factors in the liver matrix for microbiological assay or an unavailable "bound" form(s) of thiamin for liquid chromatography has been suggested. Method comparison in the present work can be considered, however, only as a guide because the number of laboratories performing microbiological assay was small. Results from normal-phase chromatographic quantitation were in accordance with those of reversed-phase chromatography even though some heterogeneity of variances, or of medians, was found for some data sets.

Total vitamin B_1 content in four tested reference materials ranged from 3.1 to 8.6 mg/kg of DM (Tables 3−6.) Reproducibility relative standard deviation (RSD_R) and repeatability relative standard deviation (RSD_r) for thiamin ranged from 13.1 to 25.7% and from 3.3 to 5.7%, respectively. Highest RSD_R values were obtained with lyophilized mixed vegetable and lyophilized pig liver samples. However, a noteworthy improvement in variability was achieved compared to the earlier intercalibration studies reported by Hollman et al. (1993b) and van den Berg et al. (1996). Hydrolysis of thiamin phosphate esters using the takadiastase preparation was confirmed in earlier studies, and there was no indication of the presence of TMP or TPP in the reversed-phase chromatograms.

Vitamin B_2. Quantitative measurement of the total vitamin B_2 content was performed either with a liquid chromatographic method or with a microbiological assay. Both methods gave similar results for CRM 421; no difference between data derived from liquid chromatographic and microbiological methods was found.

However, the number of laboratories performing a microbiologic assay was small ($n = 2$). Method comparison was excluded for CRM 487 as only one laboratory based the measurement on microbiological method.

Total riboflavin content was certified in two animal-derived reference materials, CRM 421 and CRM 487, as the vitamin B_2 content of samples of plant origin is typically low compared to those of other food matrices. The total riboflavin contents were 14.5 and 106.8 mg/kg of DM for milk powder and lyophilized pig liver, respectively (Tables 4 and 6.). Variation between or within the laboratories was below 10% in both materials, showing an acceptable variability of the results in the measurement of this vitamin.

Vitamin B_6. Total vitamin B_6 data included four liquid chromatographic and seven microbiological data sets. The non-normal nature of the data sets makes the interpretation of the vitamin B_6 results somewhat difficult; skewness and kurtosis of the mean values found exceeded the expected range in certain measurements. Some variation in the mean values may be present between microbiological assay and liquid chromatography; a higher mean value for CRM 121 was found in the microbiological assay compared to the liquid chromatographic measurement, whereas a contradictory result was found for CRM 485 results. In addition, no disparity of the results was present in the data sets of CRM 421 and CRM 487. The trend for higher results in liquid chromatography compared to microbiological assay was discussed in the earlier interlaboratory study (van den Berg et al., 1996), and the different growth responses of individual vitamers in the microbiological assay were then suggested to cause this disagreement. Unequal growth responses of yeasts have been reported (Guilarte et al., 1984; van Schoonhoven et al., 1994). Strain differences have also been proposed, but standardizing the culture maintenance should overcome this matter. As pyridoxine was the main B_6 vitamer in both CRM 121 and CRM 421, the variation between two methods, if present, seems to be related to the different matrices.

The major vitamer fraction in many plant-derived materials is reported to be glycosylated pyridoxine (Gregory and Ink, 1987; Sampson et al., 1996), which is hydrolyzed into free pyridoxine during enzymatic digestion in our extraction procedure. The efficiency of the present takadiastase enzyme preparation on the hydrolysis of the glucosidic bond was confirmed by the earlier study (van den Berg et al., 1996). Free pyridoxine was then the primary B_6 vitamer found in the wholemeal flour data derived from the liquid chromatographic measurement. The variation in results for CRM 121 is not explained by only the differences in microbial growth response. Transamination reactions with α-keto acids present in the food matrix [i.e., Metzler and Snell (1952)] are involved in the conversion of pyridoxal to pyridoxamine and vice versa, which may change the original vitamer distribution. However, the effect of these reactions on growth responses was rejected as the main vitamer in wholemeal flour was free pyridoxine. Thus, the reason for the variations in vitamin B_6 results remains unclear.

Results for CRM 121, CRM 421, CRM 485, and CRM 487 are presented in Tables 3−6. Total vitamin B_6 amount for these four reference materials varied from 4.8 to 19.3 mg/kg of DM. Repeatability relative standard deviation (RSD_r) fell below 10%, whereas higher variation was noticed between laboratories, as shown by RSD_R ranging from 8.6 to 34.4%. The highest value of reproducibility relative standard deviation (RSD_R) was found in the results of CRM 121. Flour sample matrix may cause difficulty in the interpretation of the chromatogram as good chromatographic peak shape and peak purity were not always achieved. Therefore, fluorescent impurities may give a misidentification of an individual vitamer. However, this does not affect results of the microbiological assay, as the differences in growth response between vitamers in a microbiological method may result in under- or overestimation.

Vitamin B_{12}. Total cyanocobalamin content was measured in CRM 421 and CRM 487. The data sets from eight or seven laboratories were evaluated. Determination based on an affinity binding (competitive protein binding) method was carried out in two laboratories; the other laboratories used a microbiological method. The microbiological assay seemed to produce higher values than the affinity binding method in CRM 421, whereas a statistical agreement between the two methods was found in CRM 487.

The mean of the total vitamin B_{12} content was 34.4 μg/kg of DM for milk powder and 1120 μg/kg of DM for pig liver (Tables 4 and 6). Within-laboratory variation (repeatability relative standard deviation, RSD_r) as well as between laboratories (reproducibility relative standard deviation, RSD_R) was good, 4.2−4.6 and 9.1−13.1%, respectively. Higher variation between laboratories (RSD_R) was earlier reported in an interlaboratory comparison of serum vitamin B_{12} (Mollin et al., 1980); the mean relative standard deviation was >30% in plasma samples with a normal vitamin B_{12} content. Differences in sample matrix and the lower concentration levels compared to those of our study may explain this difference. The test microorganisms were also different in these two studies; the use of *L. leichmannii* has been replaced by that of *Euglena gracilis*.

General Comments. Normally distributed random errors are considered to predominate in chemical measurement, and their magnitude is inversely related to concentration (Horwitz and Albert, 1997). To evaluate this relationship, the reproducibility relative standard deviation (RSD_R) was plotted against concentration. When these data were compared to those calculated using the Horwitz formula, a somewhat higher variation than expected was found with some of the materials (Figure 1). Variation between laboratories (RSD_R) increased in lower analyte concentrations as expected. The $RSD_{found}/RSD_{expected}$ ratio ranged from 0.5 to 2.7; lowest values were found with total riboflavin and total cobalamin determinations, and the highest value was in vitamin B_6 measurements (Figure 2.). In general, reproducibility was reasonable as the variability values lying within half to twice the value expected are reported to show "acceptable performance" (Albert and Horwitz, 1997).

A high variability ratio was present in vitamin B_1 (total thiamin) analysis of pig liver and in total pyridoxine measurements of wholemeal flour. Component(s) in the pig liver matrix that have an effect on the quantitative measurement of thiamin cannot be ruled out. Further evaluation of HPLC chromatograms revealed that the interpretation of chromatograms in vitamin B_6 analysis may also cause difficulties. The interpretation of the chromatographic tracings will be simplified if pyridoxal and pyridoxamine are converted

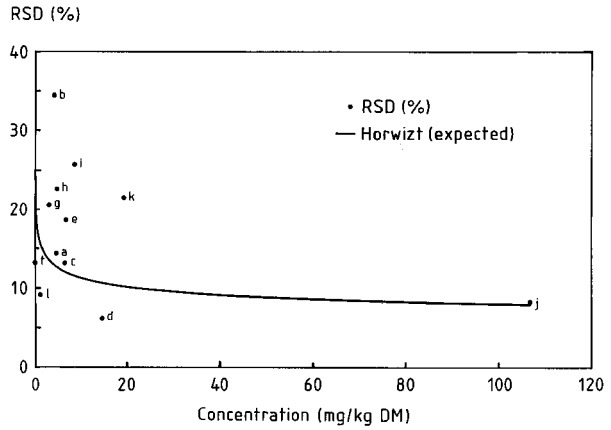

Figure 1. Relationship between the reproducibility RSD_R and concentration: (a) CRM 121, vitamin B_1; (b) CRM 121, vitamin B_6; (c) CRM 421, vitamin B_1; (d) CRM 421, vitamin B_2; (e) CRM 421, vitamin B_6; (f) CRM 421, vitamin B_{12}; (g) CRM 485, vitamin B_1; (h) CRM 485, vitamin B_6; (i) CRM 487, vitamin B_1; (j) CRM 487, vitamin B_2; (k) CRM 487, vitamin B_6; (l) CRM 487, vitamin B_{12}.

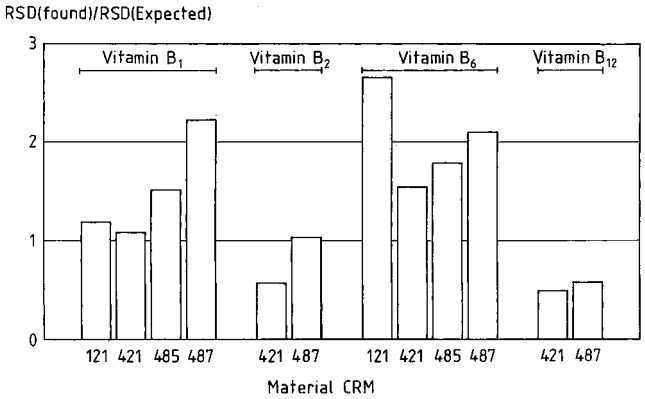

Figure 2. Ratio of RSD_R (found/expected) for each certified reference material and analyte.

to pyridoxine (Reitzer-Bergaentzle et al., 1993; Bergaentzle et al., 1995) as then only one analyte would need to be quantitated. This approach, adopted as an official method in France at present, was used in one laboratory.

The importance for the harmonization of the analytical methods for vitamin measurements cannot be overstated. Standardization of the methods, including the chromatographic separation and calibration procedures, requires more up-to-date collaborative data comprising various food matrices. Variations in the matrices of seemingly similar materials but actually different matrices can require revalidation of the analytical method; the effect of the dissimilarity of sample matrix (milk and soy-based infant formula compared to medical foods) on the analysis of fat-soluble vitamins has been shown by Chase et al. (1999). The ability to obtain pure commercial standard compounds may also be limited. At present, certain producers have indicated that some vitamin B_6 compounds are no longer under commercial production (personal communications), which can complicate the method validation. Therefore, any efforts producing reference materials with various food matrices for the vitamin analysis should be received with enthusiasm.

AOAC International's Task Force on Methods for Nutrition Labeling has proposed the use of a nine-sector fat−protein−carbohydrate triangle to categorize the different foods according to their fat, protein, and carbohydrate contents (Sharpless et al., 1997). In this approach, foods are divided into nine sectors so that each food material can be located in a certain sector on the basis of its proximate (fat, protein, and carbohydrate) content. This will guide the method validation as one or two reference materials are considered to represent other foods in that particular sector and be useful in the methodology and quality assessment for those foods. NIST's standard reference materials SRM 1563 (cholesterol and fat-soluble vitamins in coconut oil), SRM 1846 (infant formula), and SRM 2383 (baby food composite) (Sharpless et al., 1997, 1999a,b; Sharpless and Gill, 2000), all materials having reference or information values for their vitamin content, stand in sectors 1, 6, and 5, respectively.

CRM 121, CRM 421, CRM 485, and CRM 487 can be located in sectors 5, 7, 5, and 9, respectively. Thus, these new certified reference materials for B-group vitamins (B_1, B_2, B_6, and B_{12}) will cover two open sectors, namely, 7 and 9, in that "AOAC nine-sector triangle". This increases the coverage of reference materials for various foods, providing new tools to accomplish the quality assurance both in method validation and in efficiency testing of laboratories. However, more reference materials with variable types of matrices and analytes are inevitably still needed to cover the extensive array of food and feed samples in laboratories performing vitamin analysis.

In summary, reference materials as well as intercomparison studies on vitamin analyses are needed in method validation and in evaluation of a laboratory's analytical proficiency. The B-group vitamins dealt with in this study consist of compounds with miscellaneous chemical natures. Despite the variability in the final methodology, an acceptable level of agreement in the results was achieved, allowing the certification of four reference materials. Certified reference materials presented here provide a basis for further improvements in quality control programs in vitamin analysis. This work should be continued by harmonizing the analytical procedures internationally.

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