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Report 91.11 February 1991

INTERCOMPARISON OF METHODS FOR THE DETERMINATION OF VITAMINS IN FOODS. First interlaboratory trial

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ABSTRACT

INTERCOMPARISON OF METHODS FOR THE DETERMINATION OF VITAMINS IN FOODS. First interlaboratory trial.

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39 tables, 9 figures, 4 appendices

In order to assess the state of the art of vitamin analysis in foods, an intercomparison of methods was set up. Eighteen laboratories in Europe specialised in vitamin analysis participated in this trial. Each laboratory received samples of dry foods in the form of homogeneous powders, milk powder, freeze-dried pork muscle and haricots verts beans. The food samples presently to be certified for major nutrients, had proved to be homogeneous with respect to these compounds. In addition homogeneity of retinol, α-tocopherol, vitamin B_1 and B_2 was checked, and was found to be adequate. Each laboratory was requested to perform the analyses by its own methods. Laboratories had to carry out at least three separate determinations on sub-samples taken from at least two of the sachets provided. Results had to be expressed on dry weight, as determined by a prescribed drying method.

- For retinol in milk powder, the coefficient of variation of reproducibility (CV_{Reprod}) was only 10% after excluding one laboratory using in inadequate method.

- For β-carotene results in milk powder agreed very well, CV_{Reprod} was 14%. The values reported for haricots verts beans ranged from 0.063 to 0.398 mg/100 g dry weight, CV_{Reprod} was 52%. A major part of this variability was due to differences in methodological principles and inadequate chromatographic resolution.
- After excluding results of two laboratories (calibration and limit of detection), a tight set of results for α-tocopherol in milk powder and haricots verts was obtained. CV_{Reprod} was 16% and 15% respectively.
- Reproducibility of the determination of vitamin B_1 in milk powder, pork muscle and haricots verts was poor, CV_{Reprod} ranged from 24% to 37%. Extraction/hydrolysis procedures proved to be an important source of variation.
- Differences between laboratories in the vitamin B_2 content in milk powder, pork muscle and haricots verts were very high, with CV_{Reprod} ranging from 28% to 74%. Again extraction/hydrolysis procedures were the most important source of variation.
- Variation in the results for vitamin B_6 in the three food samples was high, CV_{Reprod} ranged from 37% to 61%. A major part of this variability was due to differences in extraction/hydrolysis procedures and problems with identification of B_6 vitamers.
- The results reported for vitamin C in haricots verts agreed well, after rejecting two laboratories using inadequate methods. CV_{Reprod} was only 9.8%.
- Variation in the results for niacin obtained with microbiological methods in milk powder, pork muscle and haricots verts, was rather small: CV_{Reprod} = 9 - 15%.
- Summarising, results for retinol, β-carotene, α-tocopherol, vitamin C and niacin agreed rather well for most of the foods. Indicative values for these vitamins in these foods presently to be certified for major nutrients are proposed. For vitamin B_1, B_2, and B_6 extraction/hydrolysis procedures have to be studied.
- On the other hand only a few of these laboratories specialised in vitamin analysis were able to perform analyses of vitamin D_3, folic acid, and vitamin B_{12} .

  For each of these vitamins focused attention is needed, by comparing different procedures. Laboratories specialised in the analysis of these vitamins have to be invited.

Key words: intercomparison, vitamin analysis, HPLC, microbiological assay, reference materials, foods, indicative values, retinol, β-carotene, α-tocopherol, vitamin A, vitamin D_3, vitamin E, vitamin B_1, vitamin B_2, vitamin B_6, folic acid, vitamin B_{12}, vitamin C, niacin

CONTENTS

7. APPENDICES

1. INTRODUCTION

In order to improve the quality of vitamin analysis in foods, BCR started a programme including the following aspects: improvements in methodology, intercomparison of methods and preparation of reference materials.

As vitamins are a large group of compounds, differing greatly in chemical composition, physiological action and nutritional importance, priorities for activities had to be set. BCR invited a group of experts to review the status of vitamin analysis. As a result a summary of different aspects of vitamin analysis was prepared (Table 1). It was concluded that all vitamins except for biotin, pantothenic acid and Vitamin K1, ought to be studied.

The present intercomparison of methods for 11 vitamins in three foods, marks the first step of the programme. The purpose of this intercomparison is to asses the "state-of-the-art" of vitamin analysis and to identify problem areas. Participants were invited to apply own methods of analysis that they considered to give the most reliable results in their hands. In addition, a description of essential details of the methods used was asked. Participants (Appendix 1) carried out the analyses during the months of November, December 1989, and January 1990. Results of this intercomparison were discussed by the laboratories at a meeting in June 1990, guided by a preliminary statistical evaluation.

This report will give detailed data of this intercomparison, an evaluation of the results, and conclusions regarding the performance of the methods. Future work needed to improve the quality of vitamin analysis will be discussed.

Table 1. Summary of different aspects of vitamin analysis in foods

VITAMIN	NUTRITIONAL IMPORTANCE		LEGISLATION LABELLING	ANALYTICAL STATUS	IMPORTANT FOODS
Retinol	5	I, All	5*	Acceptable	Dairy products Fish
Carotenoids	4-5	All	0	Acceptable, but isomers are difficult	Vegetables, Fruits
Tocopherols	4-5	All	5	Acceptable, vitamers are difficult	All
D_3	4-5	I, Ethnic groups	5*	Difficult	Dairy, Fat products, Fish Meat
K_1	?	?	0#	Difficult	Vegetables, Liver
B_1	5	I, P, E, A	4	Doubts/ Acceptable	Cereals, Meat
B_2	4	E	4	Acceptable	Dairy, Cereals Meat
B_6	4	I, P, E	3-4	Doubts	All
Pantothenic acid	2-3	I, P, E, A	4	Doubts	All
Folates	4-5	I, P, E	4	Difficult	Vegetables, Meat, Cereals
B_{12}	5	Vegetarians, E	1	Difficult	Meat products, Dairy, Eggs
C	4-5	I, P, E, A	4	Acceptable	Vegetables, Fruits,
Biotin	1	-	-	Difficult	Liver, Eggs
Niacin	2	-	4	Acceptable	Cereals, Meat

Key
I = Infants
E = Elderly
P = Pregnant women
A = Adolescents

Figures 1 - 5 indicate importance:
0 = low
5 = high
* = dangerous in excess
# = commercial importance

2. MATERIALS AND METHODS

2.1 Protocol

Participating laboratories were invited to use their own (routine) methods, and asked to provide details of the methods used, together with representative chromatograms (if relevant). For each food sample and vitamin, laboratories had to carry out at least three separate determinations on three separately weighed sub-samples taken from at least two of the sachets provided. Results had to be expressed on a dry-weight basis as determined by drying at conditions prescribed. Instructions (Appendix 2) and reporting forms were sent, together with the samples to the participants.

Vitamin standards used as calibrants can be an important source of variation. To be able to estimate these errors originating from differences in vitamin standards used, a multivitamin reference mixture of known composition (Appendix 3) was sent together with the samples. Two separate determinations on two separately weighed sub-samples taken from two units had to be made. Guidelines for the preparation of stock solutions of this multivitamin mixture were given (Appendix 4). The enzymes needed also were provided. Participants were asked to report their results with and without a correction factor for the level found in the multivitamin reference mixture. This correction factor was calculated by each laboratory as the ratio of the given theoretical level in the multivitamin reference mixture and the mean value of the separate determinations.

2.2 Materials

Three dry foods in the form of homogeneous powders, milk powder,freeze-dried pork muscle and dried haricots verts beans were selected for this intercomparison. The samples were packed into double heat sealed laminated foil sachets, flushed with nitrogen. These foods currently are developed as reference materials for major nutrients, and proved to be homogeneous and stable with respect to the major nutrients, total protein nitrogen, total fat, carbohydrates, and ash (Hollman and Wagstaffe, 1990). Following the same procedure, homogeneity and stability of selected vitamins in the milk powder and pork muscle also were tested. In this, 10 samples of each product were taken at 100 package intervals and analysed for the vitamins of interest. To determine the analytical precision, one sample of each product was analysed for each vitamin ten times. All results were calculated on dry matter. For each vitamin and material, the standard deviation between the samples was compared with the standard deviation within the samples. The results (Table 2) show that there is no significant difference (F-test, 5% level) between these standard deviations. One exception has to be made for vitamin B_2 in

milk powder. The variation between the samples differs significantly (5% level) from the analytical variation. However, at 1% level these differences are not statistically significant. So, the variation in the vitamin content between samples was small, and could largely be ascribed to analytical error rather than to true differences between different samples of one material. Samples can thus also be regarded as homogeneous with respect to vitamins.

In order to study the stability of vitamins in the powdered dry materials, a 24 months stability study was carried out. Retinol, α-tocopherol, vitamin B_1 and vitamin B_2 in milk powder and pork muscle was monitored at regular intervals. Results did not show evidence for degradation, except for retinol. It showed that after packaging retinol content of milk powder stored at 4°C started to decrease. At six months a total decrease of about 10% was measured. However, after this period the level of retinol in milk powder proved to be stable. The samples used in this intercomparison were taken at about 1 year after packaging.

Table 2. Homogeneity of the food samples as judged by the variation in vitamin contents within and between samples (sachets)

	Within sample			Between samples		
	mean (mg/100 g)	n	swithin	n	sbetween	CVbetween
RETINOL						
milk powder	0.400	10	0.007	9	0.008	2.1%
α-TOCOPHEROL						
milk powder	0.678	9	0.019	10	0.031	4.6%
VITAMIN B_1						
milk powder	0.322	10	0.006	9	0.009	3.2%
pork muscle	2.661	9	0.042	9	0.035	1.4%
VITAMIN B_2						
milk powder	1.594	10	0.031	10	0.057	3.9%
pork muscle	0.775	9	0.011	10	0.019	2.5%

The multivitamin reference mixture was composed of lactose with vitamins A, E, and D_3 added in the form of beadlets made of gelatine, starch and saccharose, the other vitamins except for vitamin B_{12} were added as the pure substances. The composition of this multivitamin mixture is given in Appendix 3. The multivitamin mixture was packed into bottles of brown glass with screw caps. To get an impression of the variation between the samples, five bottles were randomly chosen and analysed for a number of fat-soluble and water-soluble vitamins in one laboratory. The variation between the samples (Table 3) was rather small compared to the analytical variation (CV_{repeat}) of the laboratories found in this intercomparison (Table 5), and can thus be regarded as homogeneous.

Table 3. Variation between different samples (five) of the multivitamin reference mixture

	mean (mg/100 g)	CVbetween
Retinol	322.2	2.7%
Carotene	219.3	2.7%
α-Tocopherol	4727	2.3%
Vitamin D_3		2.0%
Vitamin B_1	280	1.5%
Vitamin B_2	560	1.1%
Vitamin B_6	301	0.8%
Niacin	1270	0.6%

2.3 Statistical Analysis

Statistical evaluation followed the principles of the International Standardization norm ISO 5725 (ISO,1981) to calculate the coefficients of variation of reproducibility, CV_{Reprod}, and repeatability, CV_{repeat}. Individual extreme values were detected by Dixon test (extreme mean values) and poor replicates by Cochran test (extreme differences between replicates). Strictly speaking, ISO 5725 was designed to evaluate collaborative studies with one well-defined method. The aim of this intercomparison was to investigate the influence of different procedures routinely used by different laboratories.

Horwitz (1982) examined the results of more than 150 collaborative studies, organised by the Association of Official Analytical Chemists and found a general curve relating the reproducibility with the concentration of the analyte. It represents the reproducibility that can be obtained when all laboratories use the same rigidly defined standardised methods. Horwitz also derived an empirical equation that relates CV_{Reprod} to the concentration (C) of the analyte, expressed in negative powers of 10:

$$CV_{Reprod} = 2^{(1-0.5 \log C)}$$

In the tables this value is given as "Achievable CV_{Reprod}".

3. RESULTS

Laboratories carried out the analyses in the period November, December 1989, January 1990. However, lab 16 en 17 only were able to perform the analyses in March 1990.

Participants expressed their results, except for the multivitamin mixture, on dry-weight basis. The results for dry weight, as determined by the prescribed procedure (Appendix 2), are given in Table 28. Results agree rather well, as is shown by a CV_{Reprod} smaller than 1% (Table 4). Extreme values of laboratories 2 and 9 were still used to express the results of those laboratories on dry weight, because the samples might actually have gained or lost some moisture.

Table 4. Summary of the results for DRY WEIGHT (g /100 g)

	Milk Powder	Pork Muscle	Haricots Verts
Number of laboratories	15	14	16
Mean of means	97.880	97.269	96.110
Range	97.020-99.900	95.783-98.500	94.423-98.450
CV_{Reprod} %	0.6	0.8	0.9
CV_{repeat} %	0.2	0.3	0.2
Cochran	7*	9**	13*
Dixon	2**	-	9*, 2**

Dixon : laboratories with extreme mean values detected by Dixon test
Cochran : laboratories with poor replicates detected by Cochran test.
Laboratories giving outlying results with marginal significance (1% < P ≤ 5%) are marked with *, and outliers (P ≤ 1%) are marked with ** in the tables.

3.1 Multivitamin reference mixture

In planning the intercomparison, differences in vitamin standards used by different laboratories were judged to be potential important sources of variation. Therefore, the multivitamin mixture was sent together with the samples. Table 5 summarises the precision achieved by the different laboratories in analysing the multivitamin mixture. Especially results for the fat-soluble vitamins are very poor, compared to the CV_{Reprod} predicted by the Horwitz-equation (achievable CV_{Reprod}). As this equation was derived from collaborative studies using uniform methods, the real CV_{Reprod} in this intercomparison is expected to be somewhat higher. However, the differences found are quite high. Inhomogeneity of the multivitamin mixture can be ruled out, because results of Table 3 show that the variation is well within the analytical variation (CV_{repeat}, Table 5) found in this intercomparison. Segregation of the beadlets during shipping could have caused inhomogeneity. However, this could not have resulted in the large variation observed, because the sample size prescribed in the guidelines (Appendix 4) would have ensured a representative sample. This large CV_{Reprod} possibly could indicate differences in calibration procedures of the laboratories. Comparing CV_{Reprod} of the food samples with the CV_{Reprod} of the results corrected for the level found in the multivitamin mixture (Table 5), no decrease in the variation is noticeable. If differences in calibration of vitamin standards are an important source of variation between laboratories, CV_{Reprod} corrected is expected to be smaller than the uncorrected CV_{Reprod}. Most likely the poor results in the standard mixture are caused by the extraction procedures prescribed in the protocol. Participants were not familiar with these procedures. In addition, using hydrochloric acid in making the stock solutions for fat-soluble vitamins introduced a high risk because of instability of these vitamins towards acid. So, because of the extraction procedures prescribed, the multivitamin mixture failed to reveal possible calibration problems.

Conclusions

- Variability in the results for vitamins in the multivitamin mixture was high, especially for fat-soluble vitamins. This variability probably was mainly caused by inadequate extraction procedures.
- This intercomparison was not able to reveal possible effects of differences in calibration procedures of vitamin standards on the precision.
- For future intercomparisons, liquid solutions of vitamin standards should be used to avoid the kind of extraction problems with the powdered standards of the present intercomparison.

Table 5. Summary of the variation in the results for vitamins in the multivitamin mixture, and the effect of the correction factor on the reproducibility

	Multivitamin Mixture			Milk Powder		Pork Muscle		Haricots Verts	
	CV-r (%)	CV-R (%)	CV-a (%)	CV-R (%)	CV-R* (%)	CV-R (%)	CV-R* (%)	CV-R (%)	CV-R* (%)
Retinol	9.1	24	4.7	22	26	-	-	-	-
Carotene	7.3	35	5.1	14	16	-	-	52	46
α-Tocopherol	4.1	19	3.2	23	22	-	-	126	139
Vitamin D_3	5.5	39	9.8	-	-	-	-	-	-
Vitamin B_1	3.8	8.8	4.8	24	21	18	14	37	37
Vitamin B_2	5.0	7.9	4.3	28	27	74	73	35	33
Vitamin B_6	4.0	8.8	4.9	-	-	-	-	-	-
Vitamin C	3.9	8.9	2.6	-	-	-	-	231	255
Niacin	5.5	10	3.8	9.2	10	-	-	-	-

Key

CV-r = CV_{repeat}
CV-R = CV_{Reprod}
CV-R* = CV_{Reprod} of corrected results
CV-a = achievable CV_{Reprod}

3.2 Retinol

Thirteen laboratories reported values for retinol (Table 29). Results for milk powder show a CV_{Reprod} of 22% (Table 6), and are moderate compared to an interlaboratory study with enriched skimmed milk powder containing > 0.3 mg retinol/100 g (IDF, 1988). In this IDF study, participants applying a uniform HPLC method, the following precision data were found: CV_{Reprod} = 15%, CV repeat = 5%. Variation between laboratories, but also within laboratories, for the determination in the multivitamin mixture is very high, with coefficients of variation of 24% and 9% resp.(Table 6). It is not very likely that this variation is entirely due to differences in calibration of vitamin standards used by the participants, as the results for milk powder corrected for the level found in the multivitamin mixture do not show improvement (see paragraph 3.1).

All participants isolated vitamin A by alkaline saponification, followed by extraction of the retinol (Table 7). Conditions for extraction varied. Laboratories 4 and 17 used solid phase extraction instead of liquid-liquid extractions used by the other participants. Only HPLC methods, reversed phase as well as normal phase (laboratories 1,6,7,10, and 17) were applied. Both UV-detection and fluorescence detection (laboratories 1,4,9,15, and 17) were used.

The material used in this intercomparison was purchased from a commercial supplier, so the cis-isomers 13-cis, 9,13-di-cis, and 9-cis can be expected to be present in the samples. Woollard and Indyk (1986) had determined cis-isomers in different commercial samples of milk powder and found 13-cis as most predominant isomer in all samples. Levels ranged from 9% up to 20% of all-trans retinol. Laboratories 1,7 10, and 16 reported all-trans values, which are used for the statistical calculations and are shown in Figure 1. The majority of the laboratories did not separate 13-cis and all-trans retinol, so 13-cis is included in their results. However, the contribution of 13-cis to the total value for retinol depends on the detection chosen : UV-detection at 325 nm, E(1%,1cm) 13-cis = 0.92xE(1%,1cm) all-trans; fluorescence detection 314/485 nm, intensity 13-cis = 0.33x intensity all-trans. Laboratories 7 and 16 determined 13-cis, and found values of 10% and 13% of the all-trans retinol content respectively. This is in agreement with the data of Woollard (1986). However, it is documented that during the analytical procedure isomerisation can occur depending on the type of sample and conditions of saponification and extraction (Landers et al., 1986; Steuerle, 1985). Supposing the sample contains 13-cis retinol, about 11% of the all-trans, laboratories 3,6,8,11,and 14 should have reported a retinol content about 10% higher than the mean of the values reported by laboratories 1,7,10, and 16 only reporting all-trans. As Figure 1 demonstrates, this can only explain a small part of the variation observed.

Internal as well as external standards of retinyl esters or retinol were used as calibrants.

Using an internal standard gives the possibility to account for losses during saponification, extraction and concentration.

Results of laboratory 9 are very low (Figure 1) for both the multivitamin mixture and the milk powder. These low results possibly are caused by insufficient extraction after saponification and inadequate chromatographic resolution. Because of this, the statistical evaluation was repeated excluding results of laboratory 9 (Table 6). This time, results for milk powder agree very well. The CV_{Reprod} of 10% agrees very well with the achievable CV_{Reprod} obtained when methods were rigidly standardised.

Conclusions

- The results for retinol in milk powder agreed very well between laboratories after excluding poor results of one laboratory using an inadequate method.
- For milk powder, presently to be certified for major nutrients (see 2.2) an indicative value for retinol is proposed :

  0.267 mg/100 g dry weight (standard deviation : 0.023 mg/100 g; 12 sets)
- Precision possibly can be improved by taking into account the two isomers of retinol viz. 13-cis and all-trans. However, for this material only a marginal improvement can be expected.

Table 6. Summary of the results for RETINOL (mg /100 g dry weight)

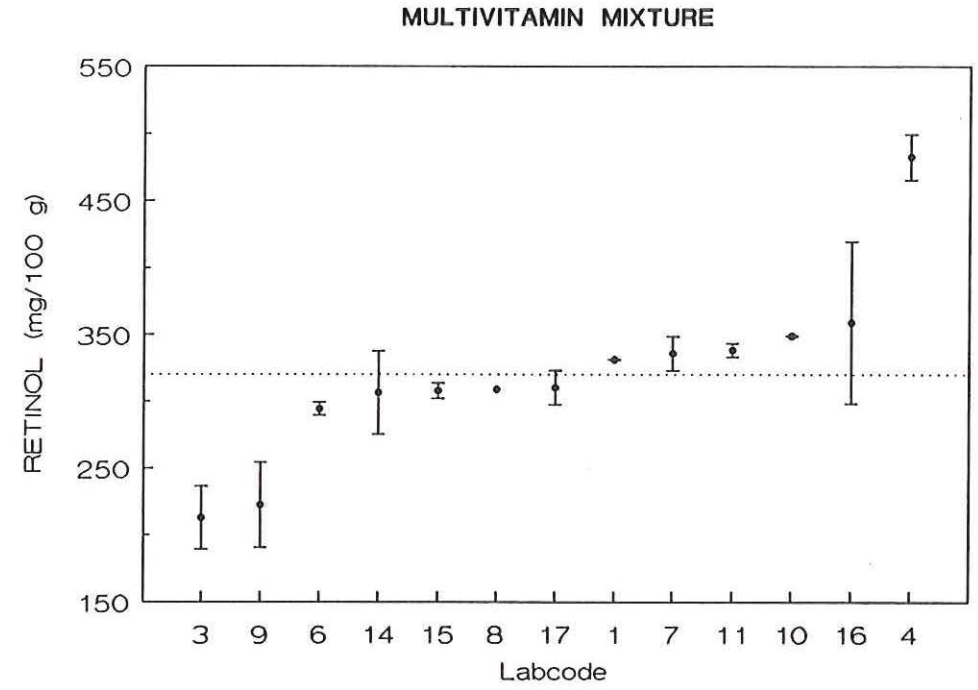
	Multivitamin Mixture	Milk Powder
Number of laboratories	13	13
Mean of means	320.03	0.254 (0.267)
Range	212.9 - 482.8	0.106 - 0.293
CV_{Reprod} %	24	22 (26)
CV_{repeat} %	9.1	6.6
Cochran	16*	4*
Dixon	4**	9*
Achievable CV_{Reprod} %	4.7	14

Table 6. - continued

	Multivitamin Mixture	Milk Powder
<u>Excluding lab 9</u>:		
Number of laboratories		12
Mean of means		0.267
Range		0.222 - 0.293
CV_{Reprod} %		10
CV_{repeat} %		6.7

Between brackets : values corrected for level found in multivitamin mixture by each laboratory.
Dixon : laboratories with extreme mean values detected by Dixon test
Cochran : laboratories with poor replicates detected by Cochran test.
Laboratories giving outlying results with marginal significance (1% < P ≤ 5%) are marked with *, and outliers (P ≤ 1%) are marked with ** in the tables.

Figure 1. Results of individual laboratories for RETINOL (mg/ 100 g dry weight)
Data represent the mean ± standard deviation of the separate determinations of each laboratory.



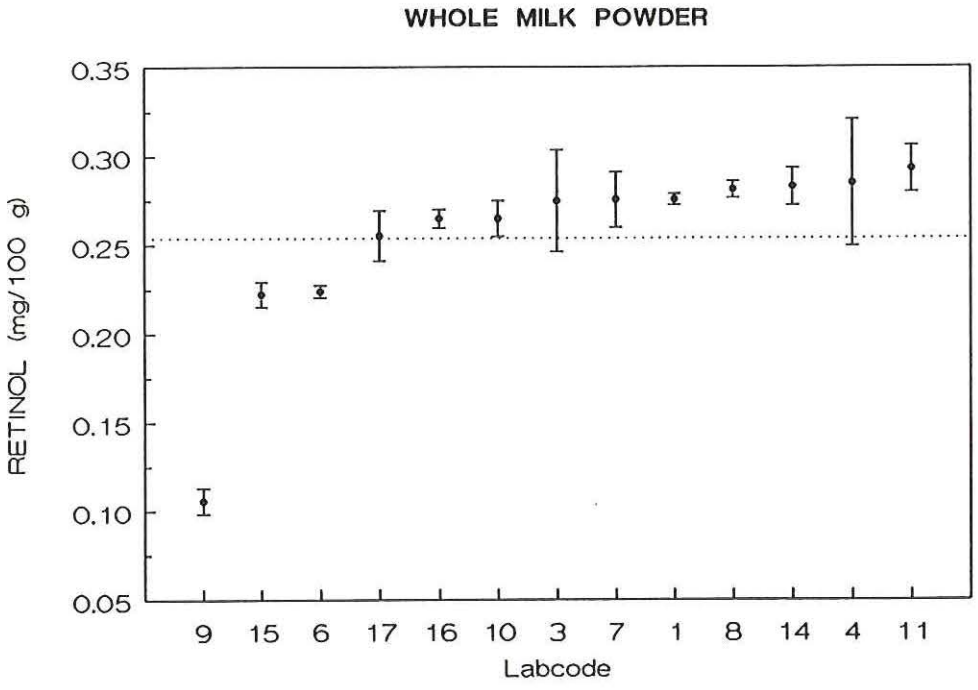


Table 7. Methods used for the determination of RETINOL

Lab-code	Extraction	Chromatography	Calibration/Calculation
Normal Phase HPLC			
1	sample weight: 10 grams alkaline saponification, extraction with diisopropyl-ether 100 ml, wash until neutral, inject	Polygosil Si-60 5 micron 250x4.6 n-hexane:methylenechloride:iso-propanol 900:90:12 flow: 2.0 ml/min tr: 600 sec detection: fluor 333/470 nm	retinyl acetate Sigma E(1%,1cm)=1830, ethanol 325 nm after saponific. standard as sample peak height (Ref. 1,2)
6	7 grams milk powder alkaline saponification, extraction petroleum ether: diethylether 1:1 80+80+80 ml, evaporate and redissolve in ?? (see vitamin E)	uPorasil 10 micron 300x4.0 iso-octane:isopropanol 985:15 flow: 2.0 ml/min tr: 500 sec detection: UV 340 nm	retinylacetate WHO calibration ?? standard as sample peak area (Ref.3)
7	20 grams alkaline saponification, extraction with diethyl-ether (3x),evaporate, redissolve in n-heptane	Kieselgel 5 micron 250x4.6 isopropanol:n-heptane (gradient) flow: 1.0 ml/min tr: 700 sec detection: UV 325 nm	retinylacetate USP E(1%,1cm)=1830 325 nm standard as sample peak area
10	30 grams alkaline saponification, extraction diethylether, evaporate??	Lichrosorb Si-60 5 um 250x4.6 iso-octane:isopropanol 985:15 flow: 1.0 ml/min tr: 950 sec detection: UV 312 nm peak area	retinylacetate USP E(1%,1cm)=1525 325 nm in isopropanol standard as sample
17	alkaline saponification, clean-up with Extrelut, elute with n-hexane, evaporate --> isooctane	Shand. APS Hypersil 3 um 100x4.6 isooctane:isobutanol 96:4 flow: 1.2 ml/min tr: 140 sec detection: fluor 328/477 nm	all-trans retinol, Fluka absorbance 325 nm; E(1%,1cm)=1835 peak area
Reversed Phase HPLC			
3	20 grams-->200 ml water alkaline saponification (cold), extraction petroleum ether 50+25+25, evaporate redissolve in methanol/BHT	uBondapak C-18 10 um 250x4.6 methanol:water 90:10 flow: 1.0 ml/min tr: 300 sec detection: UV 325 nm	retinylpalmitate calibration: Farmocopea standard as sample peak area (Ref. 4)
4	200 mg milk powder alkaline saponification, neutralisation clean-up with Extrelut, elute with n-hexane, evaporate and redissolve in methanol (see α-tocopherol)	Hypersil-ODS 3 micron 100x4.6 methanol flow: 1.0 ml/min tr: 110 sec detection: fluor 325/450 nm	retinol 99% Fluka # ?? absorbance prior assay internal standard 7-methoxycumarin-4-aceticacid-hexadecylamid peak area/height (Ref.5,6)
8	10 grams alkaline saponification, extraction with ether, evaporate?? (see α-tocopherol)	Lichrosorb RP-18 10 um 250x4.6 methanol:water 93:7 flow: 2.0 ml/min tr: 300 sec detection: UV 328 nm	standard type calibration external standard peak area

Table 7. - continued

Lab-code	Extraction	Chromatography	Calibration/Calculation
9	0.2 grams alkaline saponification, extraction with n-hexane 6+3, evaporate and redissolve in ethanol (see α-tocopherol)	Lichrosorb RP-18 5 um 250x4.0 methanol:water 97:3 flow: 1.0 ml/min tr: 300 sec detection: fluor 333/470 nm	all trans-retinol 70% Sigma # R-7632 E(1%,1cm)=1832, ethanol 325 nm, external standard peak area (Ref.7)
11	10 grams alkaline saponification, extraction dichloroethane 100 ml, wash until neutral, dilute with methanol (1+2)	Hypersil-5-ODS 5 micron 250x4.6 methanol:water 97:3 flow: 1.0 ml/min tr: 330 sec detection: UV 325 nm	retinol Serva #38280 E(1%,1cm)=1830 in isopropanol 325 nm external standard peak area (Ref. 8)
14	2 grams milk powder alkaline saponification, extraction with n-hexane 50+50 ml, wash until neutral, n-hexane--->250 ml evaporate aliquot, redissolve in methanol (see α-tocopherol, β-carotene)	Spherisorb S3ODS2 3 micron 150x4 methanol:water 95:5 flow: 1.0 ml/min tr: 240 sec detection: UV 325 nm	retinol Sigma # R-7632 E(1%,1cm)=1830 in methanol 325 nm external standard peak area (Ref. 9)
15	20 grams alkaline saponification, extraction with n-hexane 100+100+100+50+50 wash until neutral, evaporate redissolve in n-hexane, dilute 1+2 with methanol (see α-tocopherol, vit. D carotene)	Lichrospher 100 RP-18 5 micron 1 methanol:water 98:2 flow: 1.5 ml/min tr: 150 sec detection: fluor 325/470 nm	all-trans retinol, type absorbance; E(1%,1cm) external standard peak height (Ref. 7,10)
16	20 grams alkaline saponification + pyrogallol, extraction twice with diethylether:petrolether 1:1, evaporate (BHT) and redissolve in methanol:water 90:10 (see carotene, α-tocopherol)	Partisil ODS-2 10 micron 250x4.6 methanol:water 90:10 flow: 1.0 ml/min tr: 800 sec detection: UV 325 nm	all-trans retinol Aldrich # 22,302-6 absorbance 325 nm; E(1%,1cm)=1832 external standard peak area

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5 Fresenius Z. Anal. Chem. (1988) 330 p 143-145
6 Z. Lebensm. Unters. Forsch. (1988) 186 p 514-518
7 Brubacher et al. (eds.): Methods for the determ. of vitamins in food, COST 91, Elsevier (1985)
8 Grimm, Tiews, Z. Landwirtsch. Forsch. 27 (1972) p 42
9 JAOAC 63 (4) p. 894-898
10 Bognar A., Z. Lebensmittelunters. Forsch. (1986) 182 p 492-497.

3.3 β-Carotene

Reproducibility of the determination of carotene in haricots verts is very poor, results ranging from 0.063 to 0.398 mg/100 g dry weight (Table 30). For collaborative studies using uniform methods an analyte at this level should give a CV_{Reprod} of about 15% (empirical equation of Horwitz, 1982), as opposed to the CV of 52% obtained for haricots verts (Table 8). On the other hand, results for milk powder agree very well. CV_{Reprod} of 14% calculated for milk powder is identical to the achievable CV_{Reprod}. Differences between laboratories in the carotene content found in the multivitamin mixture are very high. Again correction of the results for the level found in the multivitamin mixture does not decrease the variability.

All laboratories, except laboratory 2, extracted carotenes after alkaline saponification (Table 9). Most participants subsequently used HPLC, both in normal phase (laboratories 7 and 14), and in reversed phase mode (laboratories 1,11,13,15, and 16). Laboratories 2, 3, and 8 used methods based on open column chromatography at atmospheric pressure, and consequently determined the total of all-trans carotenes (α, β, γ, and δ) and their stereoisomers, calculated as β-carotene (Brubacher et al.,1985). Of these laboratories, only laboratory 3 carried out analyses in the milk powder. Indyk (1987) determined carotenoids in milk powder using HPLC and found that β-carotene is the main carotenoid, no α-carotene was present. Several participants using HPLC confirmed that no α-carotene or other carotenes were present. Consequently, results of carotene in milk powder obtained with open column chromatography and HPLC should theoretically agree. In this intercomparison, results of laboratory 3 really agreed with HPLC results (Figure 2). Laboratory 16 determined α-carotene content of haricots verts and found 0.083 mg/100 g (21% of β-carotene content); probably no γ- and ε-carotene is present. So results of laboratories 2, 3 and 8 are expected to be higher than results of HPLC methods (Figure 2). However, in this intercomparison the results of these laboratories using open column chromatography at atmospheric pressure, found values lower than the trial mean. Laboratory 8 argued that its low values were due to technical problems with the aluminium oxide used in the column. Laboratory 14 reported problems with the extraction of haricots verts due to formation of emulsions, and judged its method to be less suitable for haricots verts. Laboratory 14 did not succeed in separating α- and β-carotene and probably reported α- + β-carotene. Chromatograms for haricots verts showing adequate resolution of α- and β-carotene were given by laboratories 11 and 16, reporting the highest β-carotene values, but also by laboratory 13 giving one of the lowest values. So, resolution of the carotene isomers seems to be not the only problem in the analysis of β-carotene.

Conclusions

- The results for β-carotene in milk powder agreed very well between laboratories.
- For milk powder, presently to be certified for major nutrients (see 2.2) an indicative value for β-carotene is proposed :

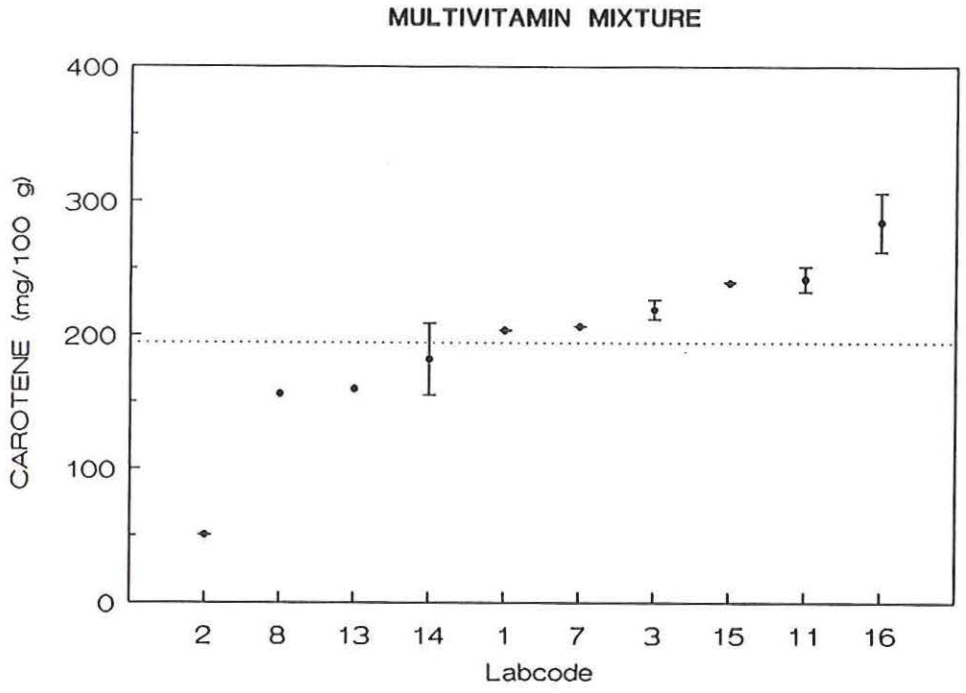
  0.119 mg/100 g dry weight (standard deviation : 0.016 mg/100 g; 7 sets).
- The reproducibility of the carotene determination between laboratories in haricots verts beans was very poor.
- Poor resolution between α- and β-carotene in haricots verts beans with HPLC methods does not explain the variability found.
- Methods based on open column chromatography at atmospheric pressure tended to give the lowest results, even in the haricots verts beans where both α- and β-carotene are present.

Table 8. Summary of the results for β-CAROTENE (mg /100 g dry weight)

	Multivitamin Mixture	Milk Powder	Haricots Verts
Number of laboratories	10	7	9
Mean of means	194.231	0.119 (0.107)	0.222 (0.252)
Range	50.48 - 284.33	0.098 - 0.146	0.063 - 0.398
CV_{Reprod} %	35	14 (16)	52 (46)
CV_{repeat} %	7.3	7.3	7.6
Cochran	-	-	-
Dixon	2*	3**	3**
Achievable CV_{Reprod} %	5.1	16	14

Between brackets : values corrected for level found in multivitamin mixture by each laboratory.
Dixon : laboratories with extreme mean values detected by Dixon test
Cochran : laboratories with poor replicates detected by Cochran test.
Laboratories giving outlying results with marginal significance (1% < P ≤ 5%) are marked with *, and outliers (P ≤ 1%) are marked with ** in the tables.

Figure 2. Results of individual laboratories for β-CAROTENE (mg/ 100 g dry weight)
Data represent the mean ± standard deviation of the separate determinations of each laboratory.



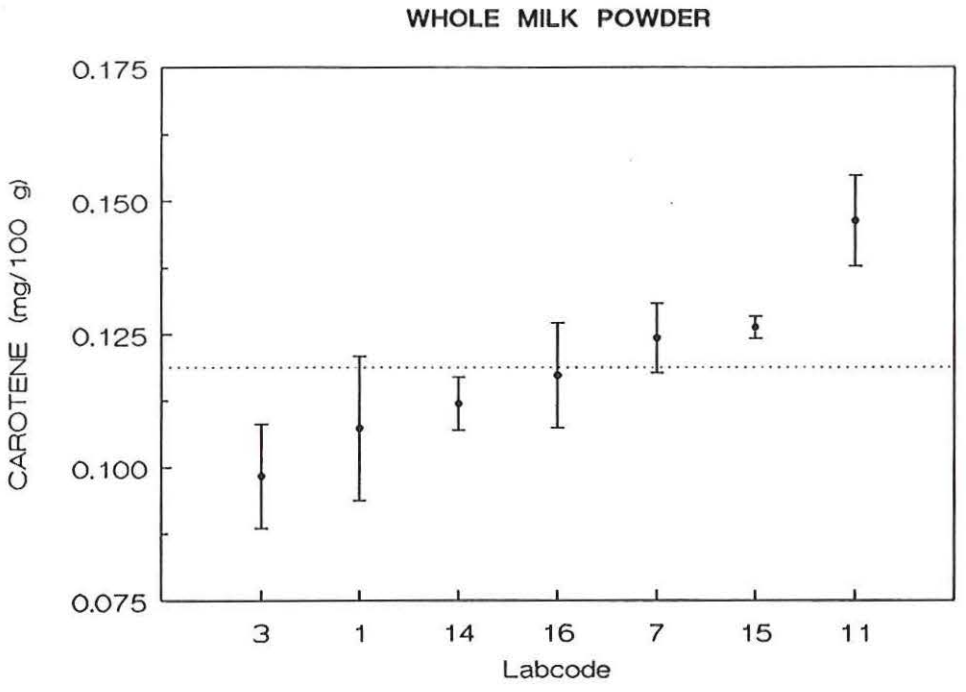


Figure 2. - continued

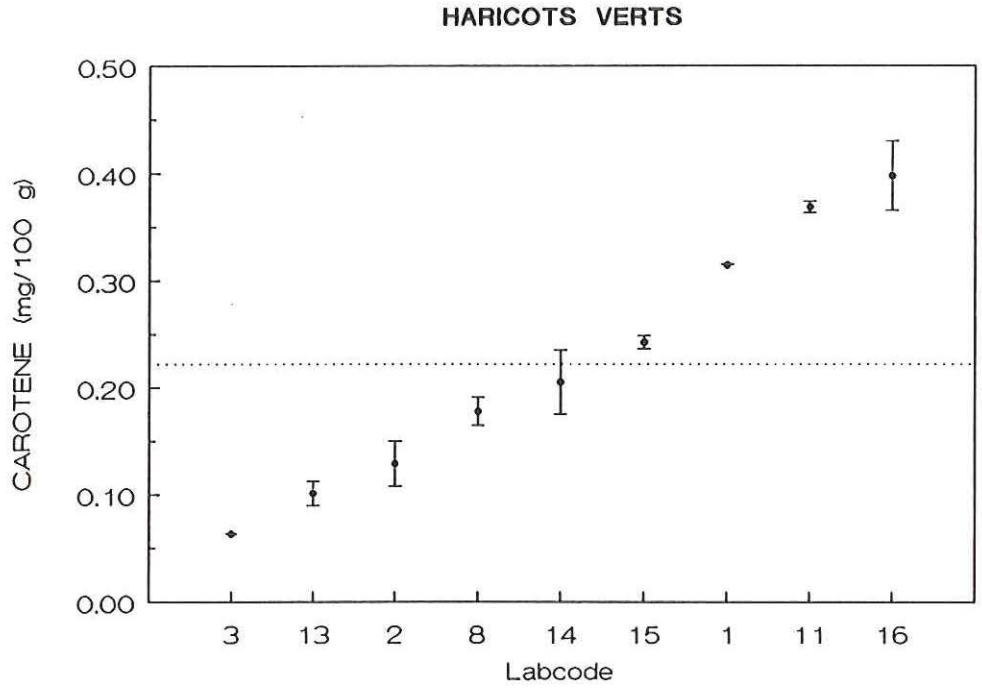


Table 9. Methods used for the determination of CAROTENE

Lab-code	Extraction	Chromatography	Calibration/Calculation
Normal Phase HPLC			
7	sample weight: 20 grams alkaline saponification, extraction with diethylether, evaporate?? (see retinol)	Kieselgel 5 micron 250x4.6 isopropanol:n-heptane (gradient) flow: 1.0 ml/min tr: 140 sec detection: VIS 450 nm	standard type, purity ?? calibration ?? external standard ?? peak area/height ??
14	2 grams milk powder, 1.5 grams haricots verts alkaline saponification, extraction with n-hexane 50+50 ml, wash until neutral, n-hexane--->250 ml evaporate aliquot, redissolve in n-hexane (see retinol, α-tocopherol)	Partisil 5 micron 250x4.6 n-hexane:abs. ethanol 9999:1 flow: 1.0 ml/min tr: 300 sec detection: VIS 450 nm peak area	β-carotene type I Sigma E(1%,1cm)=2592 in n-hexane, 453 nm external standard (Ref.1)
Reversed Phase HPLC			
1	10 grams alkaline saponification, extraction with 100 ml diiso- propylether, wash until neutral, evaporate and re- dissolve in mobile phase	Hypersil-ODS 5 micron 250x4.6 acetonitril:chloroform:aceton: water 750:150:100:20 flow:1.5 ml/min tr:950 sec detection: VIS 445 nm	β-carotene Merck E(1%,1cm)=2500 in hexane 445 nm external standard peak height (Ref.2,3)
11	10 grams alkaline saponification, extraction with diisopropyl- ether, wash until neutral, evaporate and redissolve in n-hexane/BHT	Hypersil-ODS 5 micron 250x4.6 methanol:acetonitril:chloroform: water 5:85:8:2 flow: 1.8 ml/min tr: 1800 sec detection: VIS 445 nm	β-carotene Sigma E(1%,1cm)=2600 in hexane 445 nm external standard peak area (Ref.3)
13	5 grams alkaline saponification, extraction with diethylether 4x100 ml, wash with water until neutral evaporate and redissolve in tetrahydrofuran (see α-tocopherol)	C-18 5 micron 220x4.6 acetonitrile:dichloromethane: methanol 70:20:10 flow: 1.8-2.0 ml/min tr: 500 sec detection: VIS 450 nm (see α-tocopherol)	β-carotene Sigma E(1%,1cm):2592 in petroleum ether 453 nm external standard peak height (Ref.4)
15	20 grams alkaline saponification, extraction with n-hexane 100+100+100+50+50 wash until neutral, evaporate redissolve in n-hexane/BHT (see retinol, vit. D, vit. E)	Lichrospher 100 RP18 5 um 125x4. acetonitril: methanol:dichloro- methane 36:40:24 flow:1.5 ml/min tr:400 sec detection: UV 450 nm	β-carotene(cryst.) Serva absorbance; E(1%,1cm)=?? external standard peak height(Ref.4,5)

Table 9. - continued

Lab-code	Extraction	Chromatography	Calibration/Calculation
16	20 grams alkaline saponification + pyrogallol, extraction twice with diethylether:petroleum ether 1:1, evaporate (BHT) and redissolve in mobile phase (see vit. A, vit. E)	Zorbax ODS 5 micron 250x4.6 acetonitrile:dichloromethane: methanol 70:20:10 flow: 1.3 ml/min tr: 1300 sec detection: UV 450 nm	β-carotene type IV Sigma absorbance 450 nm (E1%,1cm)=2592 in hexane external standard ?? peak area
Other methods			
2	5 grams + 40 ml acetone, 60 ml n-hexane filter and wash with 2x60 ml acetone and 25 ml n-hexane, wash pooled extracts with 5x50 ml water, n-hexane -->100 ml, containing 9 ml acetone	activated magnesia+diatomaceous earth 1+1, elute with n-hexane: acetone 9:1 collect in 10 ml measure absorbance at 436 nm	β-carotene(biochemistry) Merck, mg b-carotene/l= 4533xA436+0.025 in n-hexane:aceton 9:1 (Ref.6)
3	milk powder: alkaline saponification, extraction with diethylether 100+100+100-->volume wash aliquot with ether-saturated water until neutral, evaporate and redissolve in n-hexane haricots verts: 2-5 grams extract exhaustively with n-hexane/acetone, alkaline saponification without heating	deactivated aluminiumoxid-90, elute with n-hexane measure absorbance at 450 nm E(1%,1cm)=2590 in n-hexane	no standard used, calculated with E(1%,1cm)=2590 in n-hexane (Ref.4)
8	5 grams alkaline saponification, extraction with 100 ml petroleum ether, wash with water, evaporate aliquot and redissolve in petroleum ether	aluminiumoxide neutral activity elute with diethylether:petrol. ether 1:3, evaporate, redissolve in petroleum ether and measure at 452 nm	no standard used, calculated with E(1%,1cm) in petroleum ether (Ref.7)

References for the determination of carotene:

1 JAOAC 63 (4) p. 894-898
2 Speek, Vitamin analysis in body fluids and foodstuffs with HPLC, Thesis University of Amsterdam (1989)
3 Speek et al. Food Chem. 19 (1986) p. 65-74
4 Brubacher, Muller-Mulot, Southgate (Eds.): Methods for the determination of vitamins in food, recommended by COST 91, Elsevier (1985)
5 Bognar A., Z. Lebensmittelunters. Forsch. (1986) 182 p 492-497. Amtliche Sammlung von Untersuchungsmethoden nach par. 35 LMBG.
6 AOAC 13th edition 1980 method 43.014-43.017
7 Strohecker R., Henning H.M., Vitaminbestimmungen, Verlag Chemie,

3.4 Vitamin D_3

Only 2 laboratories ,1 and 15, were able to determine vitamin D_3 in milk powder (Table 31). Laboratories 9 and 17 reported that the level was below their limit of detection. Results for the multivitamin mixture are very poor ranging from 949 to 3420 μg cholecalciferol/100 g (Table 10). Laboratory 9 is indicated to give extreme low values, and very poor duplicates.

In Table 11 a summary of the methods used is given. It has to be pointed out that in literature there is no consensus on E(1%,1 cm) values for cholecalciferol: 459 and 474 are found. The limited number of laboratories of this group of laboratories specialised in vitamin analysis participating in this intercomparison, able to perform vitamin D_3 analyses, and the even smaller number of laboratories that can determine vitamin D_3 in unfortified milk powder, indicate that vitamin D_3 analysis is very difficult. In spite of problems associated with the limit of detection, unfortified milk powder is very relevant for vitamin D_3 analysis. Therefore, vitamin D_3 requires special attention.

Conclusions

- The variation in vitamin D_3 values reported in the multivitamin mixture is very high. However, as only a limited number of participants were able to perform vitamin D_3 analysis, conclusions are difficult.
- A project focused on vitamin D_3 analysis is badly needed. Laboratories specialised in vitamin D_3 analysis have to be invited.

Table 10. Summary of the results for VITAMIN D_3 (µg cholecalciferol/100 g dry weight)

	Multivitamin Mixture	Milk Powder
Number of Labs	5	3
Mean of means	2671.4	
Range	949 - 3420	<0.1 - 0.270
CV_{Reprod} %	39	
CV_{repeat} %	5.5	
Cochran	9**	
Dixon	9*	
Achievable CV_{Reprod} %	9.8	

Dixon : laboratories with extreme mean values detected by Dixon test
Cochran : laboratories with poor replicates detected by Cochran test.
Laboratories giving outlying results with marginal significance (1% < P ≤ 5%) are marked with *, and outliers (P ≤ 1%) are marked with ** in the tables.

Table 11. Methods used for the determination of VITAMIN D_3

Lab-code	Extraction	Chromatography	Calibration/Calculation
1	sample weight: 10 grams alkaline saponification digitinon/methanol precipitation, clean-up with RP-18 cartridge	Polygosil Si-60 5 micron 250x4.6 n-hexane:isopropanol 995:5 flow: 1.5 ml/min tr: 720 sec detection: UV 264 nm radio-assay	cholecalciferol Sigma E(1%,1cm)=474.4 in ethanol,264 nm, radio-assay,Logit-logtransformation of standard curve (Ref.1,2)
9	4-5 grams, dissolved in 10 volumes water, extraction with diethylether evaporate and redissolve in methanol	Lichrosorb RP-18 5 micron 250x4. methanol:water 97:3 flow: 2.0 ml/min tr: 360 sec detection: UV 265 nm	cholecalciferol Sigma absorbance,E(1%,1cm)= ?? external standard ?? peak area (Ref.3)

Table 11. - continued

Lab-code	Extraction	Chromatography	Calibration/Calculation
15	20 grams alkaline saponification, extraction with n-hexane 100+100+100+50+50 wash until neutral, evaporate redissolve in n-hexane (see retinol, vit. E, carotene)	clean-up: Lichrospher Si-60 5 um 250x4.0 n-hexane:isopropanol 99:1 flow: 1.5 ml/min, inject 1000 ul collect fraction tr: 700-950 sec evaporate, redissolve in methano analytical: Lichrospher RP18 5 micron 250x4. methanol:water 95:5 flow: 2.0 ml/min tr: D2=660, D3=720 sec detection: UV 264 nm	vitamin D2, and vit.D3 by weight external standard peak height (Ref.4)
16	20 grams alkaline saponification + pyro- gallol, extraction twice with diethylether:petroleum ether 1:1, evaporate (BHT), clean-up with Seppak, evaporate, redissolve in n-hexane	clean-up: cholecalciferol Partisil-PAC 5 micron 250x4.6 n-hexane:amylalcohol 99:1 flow: 1.0 ml/min, inject 200 ul collect fraction tr: 840-1020 se evaporate, redissolve in analyti mobile phase analytical: Zorbax ODS 7-8 micron 250x4.6 acetonitril:methanol 90:10 flow: 1.0 ml/min tr: 1400 sec detection: UV 265 nm	cholecalciferol Sigma E(1%,1cm) ergocalciferol = 475 in ethanol at 265 nm,internal standard ergocalciferol Sigma , blank correction peak height
17	25 grams alkaline saponification, extraction twice with hexane, wash with 1 M NaOH, 40% etha- nol (2x), water until neutral, evaporate, redissolve in tetra- hydrofuran:chloroform:isooctane 2:10:88, inject into semi- preparative column	claen-up: semi-preparative normal phase column analytical: Vydac TP 201 5 micron 250x4.6 chloroform:methanol:acetonitril flow: 1,0 ml/min, tr: 480 sec detection: UV 265 nm	cholecalciferol, ergo- calciferol, Merck internal standard ergo- calciferol peak area

References for the determination of vitamin D_3

1 van den Berg et al., J. Agric. Food Chem., 34, 1986, p. 254-268

2 Hollis and Frank, Meth. of Enzym. 123, 1986, p. 167-176

3 Brubacher, Muller-Mulot, Southgate (Eds.): Methods for the determination of vitamins in food, recommended by COST 91, Elsevier (1985)

4 Bognar A., Z. Lebensmittelunters. Forsch. (1986) 182 p 492-497. Amtliche Sammlung von Untersuchungsmethoden nach par. 35 LMBG.

3.5 α-Tocopherol

Results for milk powder, with a CV_{repeat} of 14% and a CV_{Reprod} of 23% (Table 12), compare favourable to the results of a collaborative study of tocopherols in vegetable oils and fats organised by IUPAC (Pocklington et al., 1988). In this IUPAC study a CV_{repeat} of 5% and a CV_{Reprod} of 31% was found for a sample with a tocopherol content of 1.7 mg/100 g. Values reported for haricots verts are very poor ranging from 0.251 to 2.484 mg/100 g dry weight (Table 32). Results for the multivitamin mixture again are very poor, even when outlying results (laboratory 6) are excluded from the statistical evaluation given in Table 12. Similar to retinol and carotene, correction for the level found in the multivitamin mixture by each laboratory (values between brackets in Table 12) does not improve these results.

All participants used alkaline saponification, mostly identical with the procedure used for retinol and carotene (Table 13). The tocopherols were extracted with hexane or petroleum ether, mixtures of petroleum ether and diethylether, diisopropylether, and dichloroethane. Laboratory 4 and 17 used solid phase extraction, and again laboratory 4 (see retinol) showed a high variation within the laboratory. All but one (laboratory 10) used reversed phase HPLC (laboratories 4,8,9,11, and 13) or normal phase HPLC. Most of the laboratories used fluorescence detection, only laboratories 8,13, and 14 used UV-detection. No effects of this choice of chromatography and detection on the results was apparent (Figure 3). Laboratory 10 applied a continuous flow method with fluorescence detection. This laboratory commented that for the determination of vitamin E in milk powder and haricots verts, its method reached the detection limit.

Laboratory 14 reported difficulties during the extraction of the haricots verts caused by formation of emulsions. The results given by this laboratory are very high and have not been included in Figure 3. High results of laboratory 4 can be explained because of the calibration procedure. Using tocopherol as a standard, without checking the content by determination of E(1%,1 cm), leads to erroneous results, because the tocopherol supplied never has 100% purity. High variation within laboratories 16 (Figure 3) is caused by one outlying result.

Excluding results of laboratory 4 (calibration) and 10 (limit of detection), for milk powder a tight set of results is obtained. CV_{Reprod} is comparable to the CV_{Reprod} achievable (Horwitz, 1982) when uniform methods are used (Table 12). Rejecting results of laboratories 4 and 10 and one high result of laboratory 16, also for haricots verts beans an indicative value is feasible. However, stability of α-tocopherol in haricots verts has to be proven.

Conclusions

- The results for α-tocopherol in milk powder and haricots verts agreed rather well between laboratories after rejecting two laboratories using inadequate methods.
- For milk powder and haricots verts, presently to be certified for major nutrients (see 2.2) indicative values for α-tocopherol are proposed :

  milk powder 0.603 mg/100 g dry weight
  (standard deviation : 0.092 mg/ 100 g; 11 sets)

  haricots verts 0.335 mg/100 g dry weight
  (standard deviation : 0.046 mg/100 g; 10 sets).
- Using α-tocopherol as a standard, without checking the content by determination of E(1%,1 cm), leads to erroneous results.

Table 12. Summary of the results for α-TOCOPHEROL (mg /100 g dry weight)

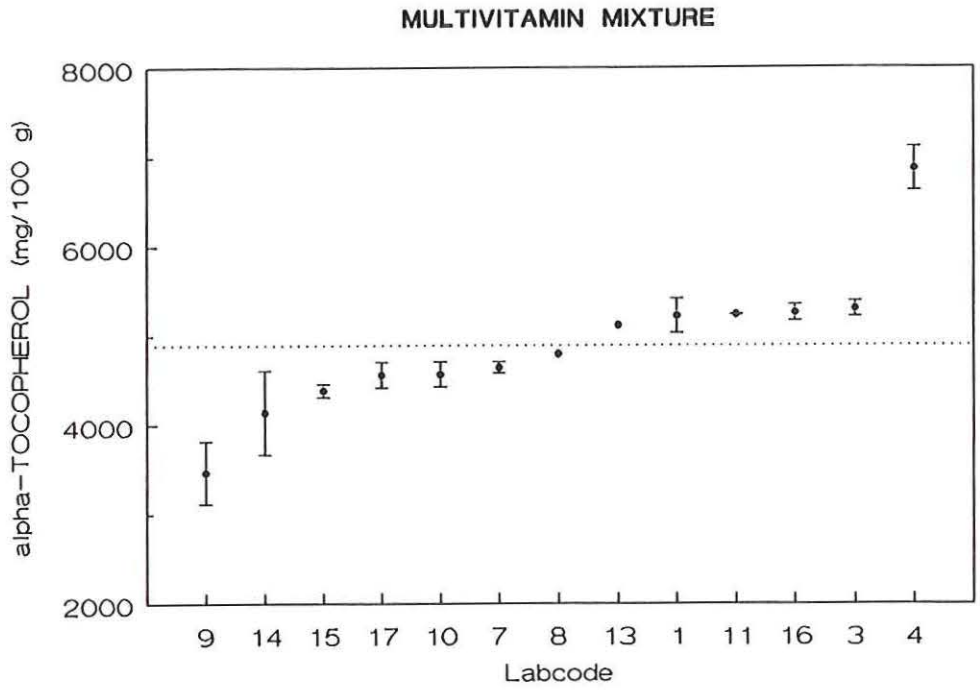
	Multivitamin Mixture	Milk Powder	Haricots Verts
Number of laboratories	13	13	12
Mean of means	4891.28	0.637 (0.620)	0.539 (0.568)
Range	3467.5 - 6870.0	0.503 - 0.867	0.251 - 2.484
CV_{Reprod} %	19	23 (22)	126 (139)
CV_{repeat} %	4.1	14	67
Cochran	-	10**	14**
Dixon	4*	-	14**
Achievable CV_{Reprod} %	3.2	12	12

Table 12. - continued

	Multivitamin Mixture	Milk Powder	Haricots Verts
Excluding laboratories:		4 and 10	10 and 14
Number of laboratories		12	10
Mean of means		0.603	0.335
Range		0.503 - 0.771	0.251 - 0.406
CV_{Reprod} %		16	15
CV_{repeat} %		7.0	8.1

Between brackets : values corrected for level found in multivitamin mixture by each laboratory.
Dixon : laboratories with extreme mean values detected by Dixon test
Cochran : laboratories with poor replicates detected by Cochran test.
Laboratories giving outlying results with marginal significance (1% < P ≤ 5%) are marked with *, and outliers (P ≤ 1%) are marked with ** in the tables.

Figure 3. Results of individual laboratories for α-TOCOPHEROL (mg /100 g dry weight) Data represent the mean ± standard deviation of the separate determinations of each laboratory.



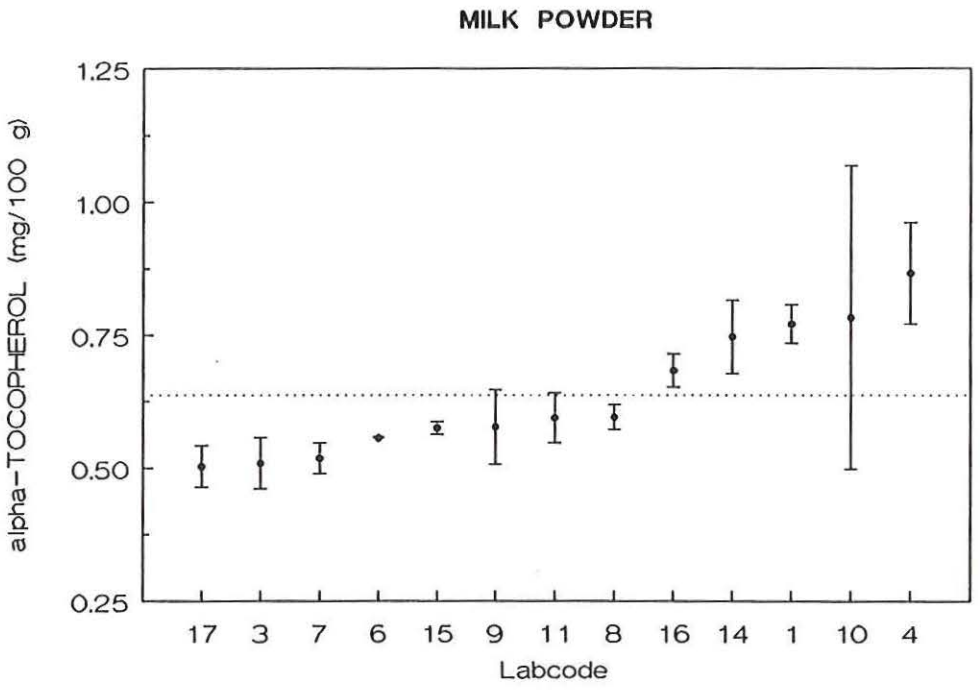


Figure 3. - continued

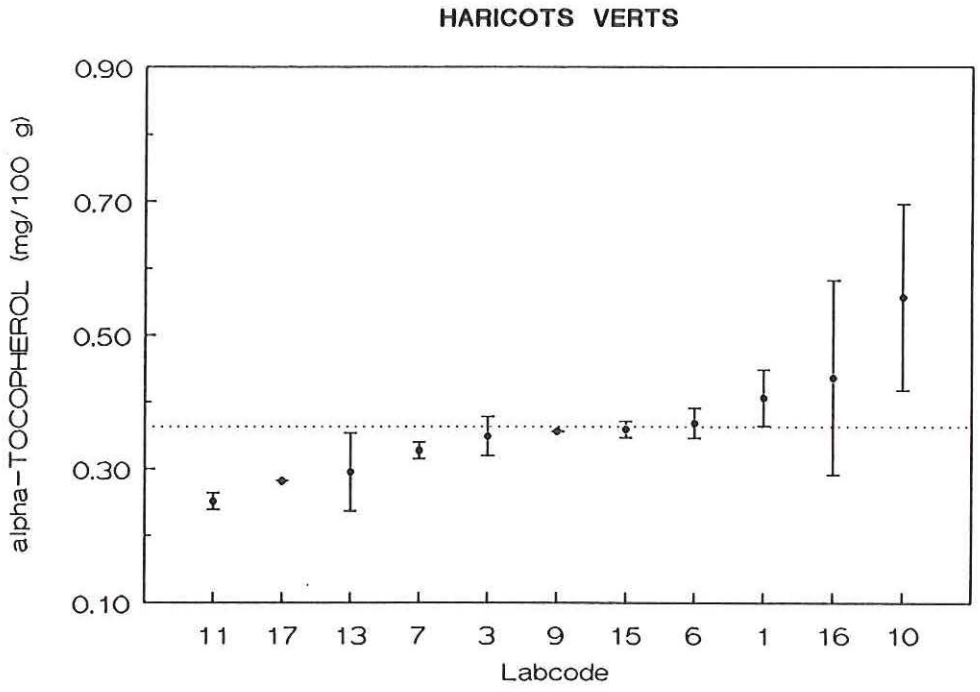


Table 13. Methods used for the determination of α-TOCOPHEROL

Lab-code	Extraction	Chromatography/Principle	Calibration/Calculation
Normal Phase HPLC			
1	sample weight: 10 grams alkaline saponification, extraction with 100 ml diiso-propylether, wash until neutral, inject	Polygosil Si-60 5 micron 250x4.6 n-hexane:diisopropylether 900:10 flow: 1.5 ml/min tr: 600 sec detection: fluor 296/320 nm	dl-α-tocopherylacetate, Sigma, E(1%,1cm)=70.75, ethanol,292 nm after saponification,standard treated as sample peak height (Ref.1,2)
3	20 grams-->200 ml water alkaline saponification (cold) extraction with petroleum ether 50+25+25 ml wash till neutral, evaporate redissolve in n-hexane/BHT	Lichrosorb Si-60 5 um 250x4.6 2-propanol:n-hexane 10:990 flow: 1.0 ml/min tr: 250 sec detection: fluor 290/325 nm	dl-α-tocopherylacetate Merck, by weight (x0.911 = tocopherol),standard treated as sample peak area
6	7 grams haricots verts alkaline saponification, extraction petroleum ether: diethylether 1:1 80+80+80 ml, evaporate, redissolve in ?? (see retinol)	uPorasil 10 micron 300x4.0 iso-octane:isopropanol 996:4 flow: 2.0 ml/min tr: 330 sec detection: fluor 290/325 nm	dl-α-tocopherol Merck calibration ?? external standard peak height (Ref.3)
7	5-20 grams alkaline saponification, extraction with diethylether, evaporate, redissolve in n-heptane	Kieselgel 5 micron 250x4.6 isopropanol:n-heptane flow: 1.0 ml/min tr: 360 sec detection: fluor 290/327 nm	dl-α-tocopherylacetetate 99% laRoche, E(1%,1cm)= 400 at 520 nm after reaction with $FeCl_3$+ α,α'-dipyridil, standard treated as sample peak area/height ??
14	2 grams milk powder, 1.5 grams haricots verts alkaline saponification, extraction with n-hexane 50+50 ml, wash until neutral, n-hexane-->250 ml evaporate aliquot, redissolve in n-hexane (see retinol, carotene)	Spherisorb S5W 5 micron 250x4.6 n-hexane:isopropanol 985:15 flow: 1.0 ml/min tr: 300 sec detection: UV 295 nm peak area	dl-α-tocopherol Fluka by weight external standard (Ref.4)
15	20 grams alkaline saponification, extraction with n-hexane 100+ 100+100+50+50, wash until neutral, evaporate redissolve in n-hexane/BHT (see retinol, vit. D, carotene)	Lichrospher Si-60 5 um 125x4.0 n-hexane:dioxan 97:3 flow: 1.0 ml/min tr: 500 sec detection: fluor 293/326 nm	dl-α-tocopherol (biochemistry) ?? calibration ?? external standard peak height (Ref.3,5)

Table 13. - continued

Lab-code	Extraction	Chromatography/Principle	Calibration/Calculation
16	20 grams alkaline saponification, extraction with petroleum ether:diethylether 1:1 evaporate (BHT), redissolve in n-hexane:2-propanol 1:1	Lichrosorb Si-60 5 micron 250x4. n-hexane:2-propanol 995:5 flow: 1.2 ml/min tr: α=560, β=1070, γ=1200, δ=220 detection: fluor 290/330 nm	Tocopherol isomers (α, β,γ,δ) Merck absorbance α-tocopherol E(1%,1cm)= 75.8 external standard peak area
17	alkaline saponification, Extrelut, elute hexane, evaporate --> isooctane	Shandon APS Hypersil 3 um 100x4. isooctane:isobutanol 96:4 flow: 1.2 ml/min tr: 180 sec detection: fluor 295/327 nm	dl-α-tocopherol, Merck calibration ?? area/height ??
Reversed Phase HPLC			
4	200 mg milk powder alkaline saponification, neutralisation clean-up with Extrelut, elute with n-hexane, evaporate and redissolve in methanol (see retinol)	Hypersil-ODS 3 micron 100x4.6 methanol flow: 1.0 ml/min tr: 240 sec detection: fluor 290/330 nm	α-tocopherol Merck calibration ?? internal standard dimethyltocol peak height (Ref.6,7)
8	10 grams alkaline saponification, extraction with ether, evaporate ??	Lichrosorb RP-18 10 um 250x4.6 methanol:water 93:7 flow: 2.0 ml/min tr: 700 sec detection: UV 292 nm (see retinol)	standard type ?? calibration ?? external standard ?? peak area
9	0.2 grams alkaline saponification, extraction with n-hexane 6+3, evaporate and redissolve in ethanol (see retinol)	Lichrosorb RP-18 5 um 250x4.0 methanol:water 97:3 flow: 2.5 ml/min tr: 330 sec detection: fluor 293/326 nm	dl-α-tocopherol 95% Sigma, UV-294 E(1%,1cm) = 71 in ethanol external standard ?? peak area (Ref.3)
11	10 grams alkaline saponification, extraction dichloroethane 100 ml, wash until neutral, dilute with methanol (1+1)	Hypersil-5-ODS 5 micron 250x4.6 methanol flow: 1.0 ml/min tr: 620 sec detection: fluor 290/330 nm	α-tocopherylacetate Merck, by weight (x0.911= tocopherol), standard treated as sample peak area (Ref.8)
13	5 grams alkaline saponification, extraction with diethylether 4x100 ml, wash with water until neutral evaporate and redissolve in tetrahydrofuran (see carotene)	C-18 5 micron 220x4.6 acetonitrile:dichloromethane:met 70:20:10 flow: 1.8-2.0 ml/min tr: 220 sec detection: UV 294 nm	d-α-tocopherol(+67%) Sigma, E(1%,1cm):72-76 ethanol,294 nm external standard ?? peak height (Ref.3)

Table 13. - continued

Lab-code	Extraction	Chromatography/Principle	Calibration/Calculation
Other methods			
10	30 grams alkaline saponification, extraction with diethylether	continuous flow method detection: fluor	dl-α-tocopherol 98-100% laRoche, E(1%, 1cm):75, ethanol 292 nm external standard "peak height" (Ref.9)

References for the determination of vitamin E:

1 Speek, Vitamin analysis in body fluids and foodstuffs with HPLC, Thesis University of Amsterdam (1989)
2 Fragner, Chemie und Biochemie, Band I, VEB Gustav Fisher Verlag, Jena (1965), p 1565
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3.6 Vitamin B_1

Reproducibility of the determination of vitamin B_1 in milk powder, pork muscle and haricots verts, CV_{Reprod} = 24% - 37% (Table 14), is very poor compared to the CV_{Reprod} of < 15% predicted by the Horwitz-equation (Horwitz, 1982). CV_{Reprod} for the determination of vitamin B_1 in the multivitamin mixture is somewhat higher than can be expected. So, no serious calibration problems are evident. Correction of the results reported in milk powder, pork muscle and haricots verts for the level found in the multivitamin mixture (values between brackets in Table 14) by each laboratory does not reduce the variability.

Most of the participants extracted vitamin B_1 by autoclaving, or boiling in acid (laboratories 5 and 9), followed by enzymatic hydrolysis of the phosphorylated thiamin (Table 15). Takadiastase or mixtures of Takadiastase and papaine (laboratory 12), or phosphatase (laboratory 16) were used. Laboratory 9 did not apply enzymatic dephosphorylation. Laboratory 7 used a mild extraction, boiling with a buffer pH 4.5, followed by enzymatic hydrolysis (overnight) with amyloglucosidase. Laboratory 8 did not give details on the extraction procedures used. Two different types of HPLC methods were used. Laboratories 1, 2, 3, and 11 separated thiamin on the HPLC column, followed by a post column reaction of thiamin to thiochrome, which was measured by fluorescence detection. Only laboratory 2 used UV-detection and consequently did not convert thiamin to thiochrome. Laboratories 5, 12, 15, and 17 converted thiamin to thiochrome prior to injection into the system and thus thiochrome was chromatographed. Laboratories 7, 8, and 9 did not use an HPLC method but applied a fluorometric procedure called thiochrome method. In this method thiamin is converted to thiochrome and is measured fluorometrically after extraction in isobutanol. Only laboratory 16 used a microbiological assay.

Laboratory 9 reported the lowest results in all samples (Figure 4), except for the pork muscle. These low results are caused by the omission of a dephosphorylation step before the oxidation of thiamin to thiochrome. Thus laboratory 9 was not able to measure the fraction of vitamin B_1 present as phosphorylated thiamin after the extraction. Laboratory 2 also finds very low results in all samples except for the multivitamin mixture. This laboratory did not succeed in getting baseline separated peaks, probably leading to erroneous results. UV-detection used by this laboratory caused a broad background of peaks interfering with thiamin. Because results of laboratories 2 and 9 are not reliable, they are not shown in Figure 4. Comparing the results of the microbiological assay (laboratory 16) with the results of the other methods, this assay gives high results in all samples (Figure 4). However, no clear-cut conclusions can be made because only one laboratory used a microbiological assay. Thiochrome methods also show a tendency to

produce high results, but only with milk powder and pork muscle. These methods are expected to give higher results than HPLC methods, because they are less specific. Again conclusions are difficult because only few laboratories used these thiochrome methods. No effects of the different types of HPLC methods, separation as thiamin followed by post-column derivatisation or pre-column derivatisation and separation as thiochrome, were apparent. Probably incomplete extraction/hydrolysis of the vitamers was the most important source of variation between laboratories. Comparing extraction procedures and results of other laboratories, laboratory 5 concluded that its own extraction/hydrolysis procedure had not been sufficient.

Rejecting results of laboratories 2, 5, and 9 a tight set of results remains for milk powder, pork muscle and haricots beans. So, indicative values for these materials can be issued.

Conclusions

- The results for vitamin B_1 in milk powder, pork muscle and haricots verts agreed well between laboratories after rejecting poor results of three laboratories using methods with poor extraction/hydrolysis procedures and/or an inadequate chromatographic method.
- For milk powder, pork muscle and haricots verts, presently to be certified for major nutrients (see 2.2) indicative values for vitamin B_1 are proposed :

  milk powder 0.320 mg thiaminchloride.HCl/100 g dry weight
  (standard deviation : 0.041 mg/100 g; 8 sets)

  pork muscle 3.126 mg thiaminchloride.HCl/100 g dry weight
  (standard deviation : 0.285 mg/100 g; 9 sets)

  haricots verts 0.220 mg thiaminchloride.HCl/100 g dry weight
  (standard deviation : 0.038 mg/100 g; 9 sets)
- Extraction/hydrolysis procedures are an important source of variation between laboratories. Optimum conditions need to be studied.
- UV-detection after reversed phase separation of thiamin is not suitable for the determination of vitamin B_1 in these foods.
- No serious calibration problems were apparent.

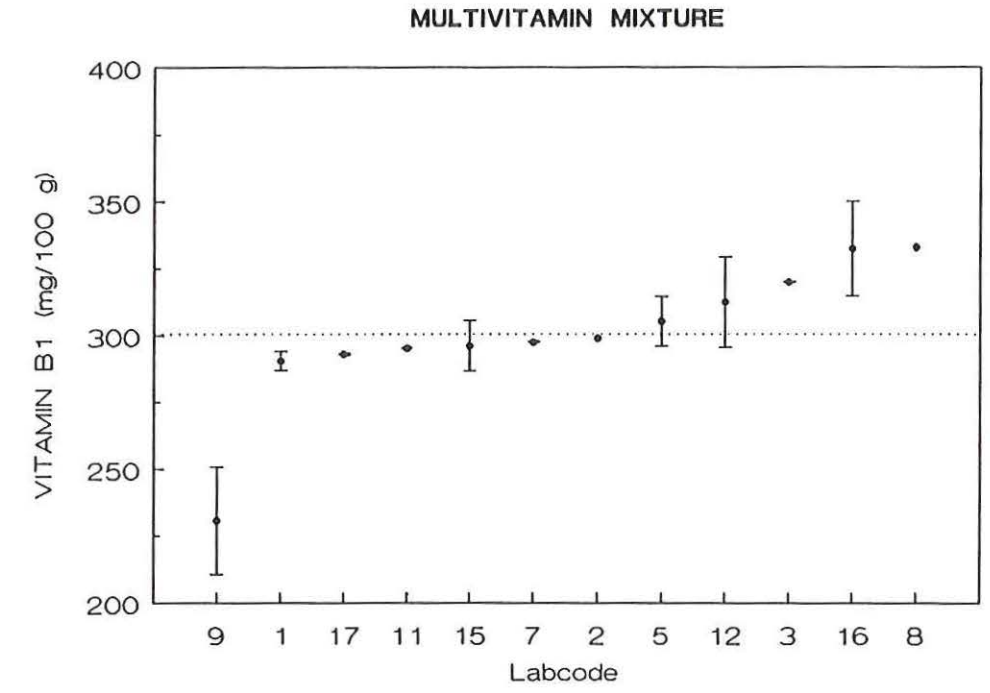
Table 14. Summary of the results for VITAMIN B_1 (mg thiamin chloride.HCl/100 g dry weight)

	Multivitamin Mixture	Milk Powder	Pork Muscle	Haricots Verts
Number of laboratories	12	11	12	12
Mean of means	300.5	0.284 (0.285)	2.884 (2.873)	0.187 (0.183)
Range	230.7 - 333.0	0.157 - 0.385	1.59 - 3.63	0.016 - 0.294
CV_{Reprod} %	8.8	24 (21)	18 (14)	37 (37)
$CV_{repeatr}$ %	3.8	5.1	4.0	4.8
Cochran	-	-	-	-
Dixon	9**	-	-	-
Achievable CV_{Reprod} %	4.8	14	9.6	15
Excluding laboratories 2, 5 and 9:				
Number of laboratories		8	9	9
Mean of means		0.320	3.126	0.220
Range		0.259 - 0.385	2.78 - 3.63	0.282 - 0.359
CV_{Reprod} %		14	9.9	17
$CV_{repeatr}$ %		4.7	3.8	4.6

Between brackets : values corrected for level found in multivitamin mixture by each laboratory.
Dixon : laboratories with extreme mean values detected by Dixon test
Cochran : laboratories with poor replicates detected by Cochran test.
Laboratories giving outlying results with marginal significance (1% < P ≤ 5%) are marked with *, and outliers (P ≤ 1%) are marked with ** in the tables.

Figure 4. Results of individual laboratories for VITAMIN B_1 (mg thiaminchloride.HCl/100g dry weight)

Data represent the mean ± standard deviation of the separate determinations of each laboratory.



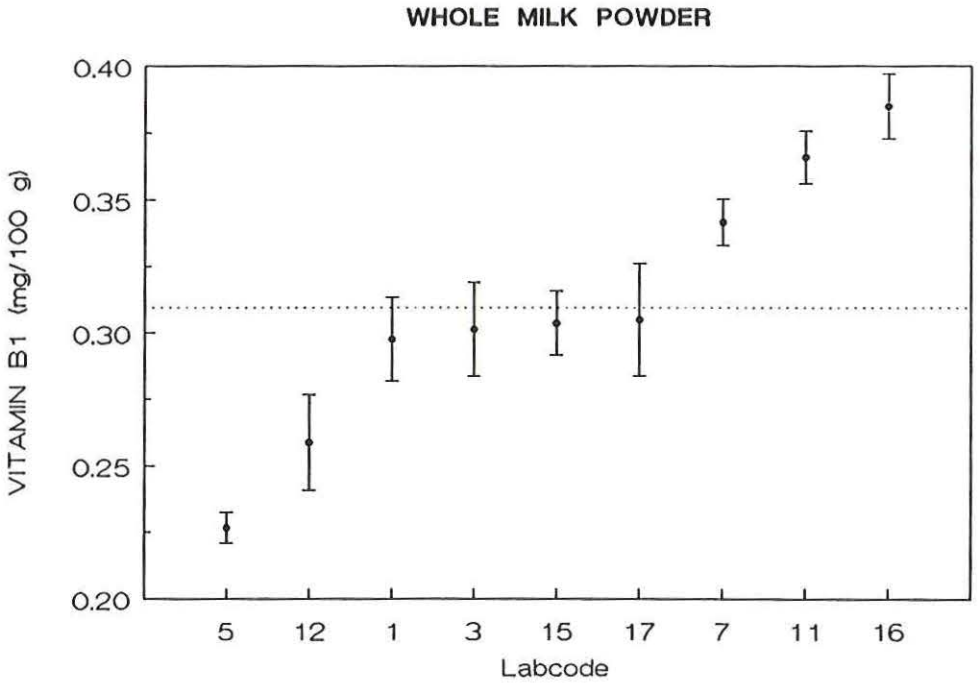
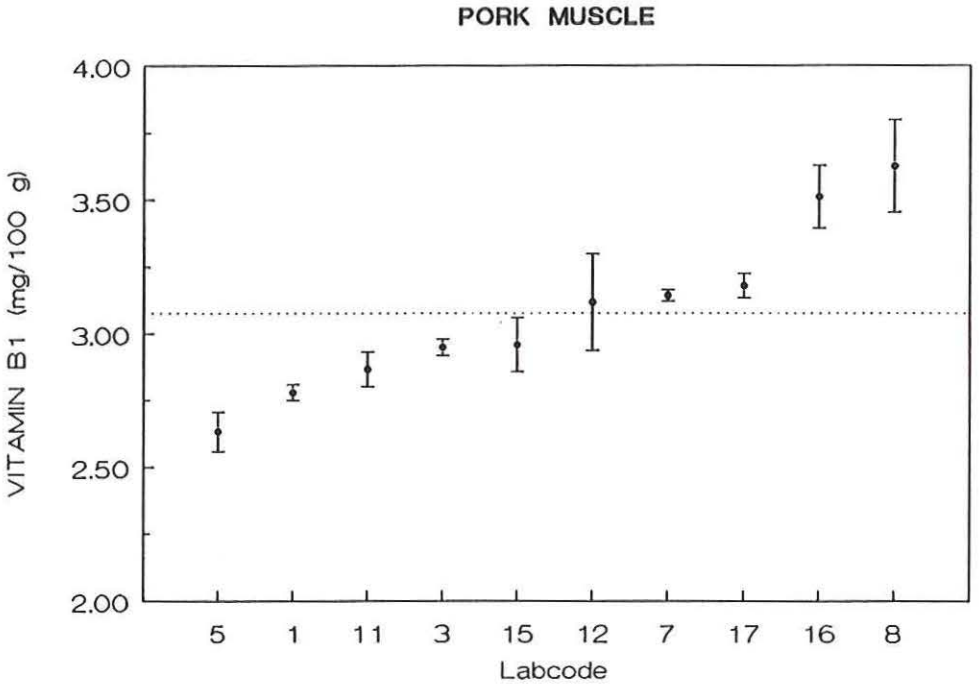


Figure 4. - continued



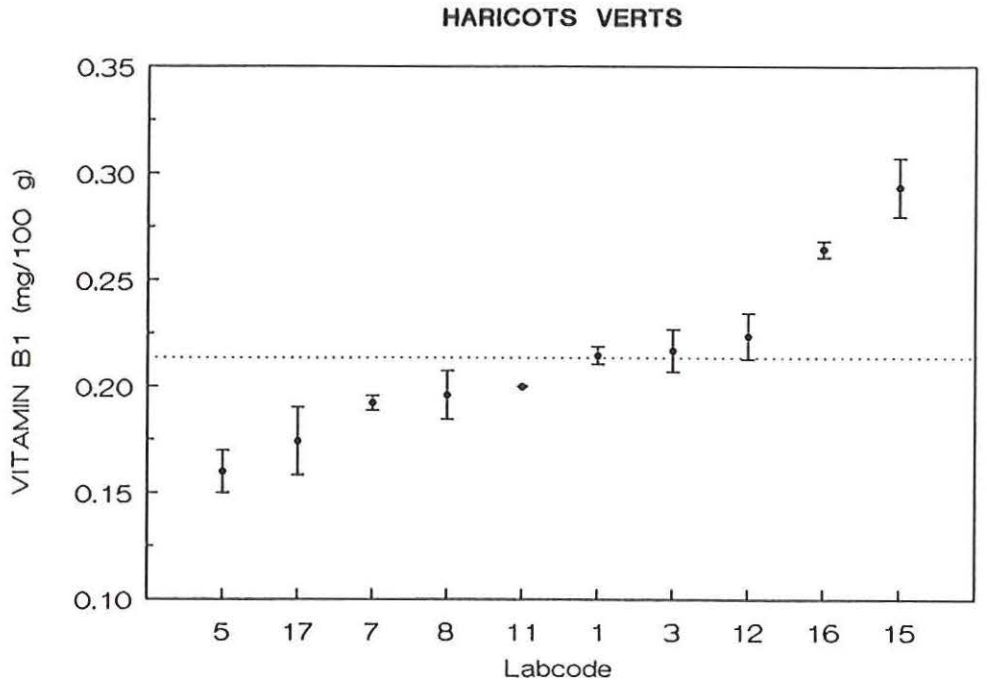


Table 15. Methods used for the determination of VITAMIN B_1

Lab-code	Extraction	Chromatography/Principle	Calibration/Calculation
HPLC-methods, separation as thiamin			
1	sample weight: 5 grams autoclave 15 min, 120°C, 0.15 M HCl Takadiastase 1h, 45°C	8-Si-10 10 um Radial-Pak module 0.05 M phosphate buffer pH 7.4: ethanol3000:1100 flow: 3.0 ml/min tr: 720 sec detection: fluor 367/418 nm post column reaction	thiamine.HCl Sigma E(1%,1cm)=397.3, ethanol 248 nm; standard treated as sample peak height (Ref.1,2)
2	5-10 grams autoclave 15 min, 121°C, 0.30 M HCl -->pH 4.0-4.5 + 5 ml 6% Takadiastase 3h, 48°C, filter and dilute clean-up: CG-50 resin and Seppak	uBondapak C18 10 micron 300x3.9 methanol:water:acetic acid 31: 68.5:.5, 1-hexaneSO_3Na, 1-heptaneSO_3Na 5mM flow: .5/.7 ml/min tr: 800 sec detection: UV- 254 nm	thiamine.HCl Sigma calibration ?? standard treated as sample peak area
3	10 grams solid, 2 grams liquid autoclave 15 min, 121°C, 0.05M H_2SO_4 Takadiastase 3h, 45°C	uBondapak C18 10 micron 250x4.6 methanol:water:acetic acid 250:735:15 Pic-B6 5mM flow: 1.0 ml/min tr: 420 sec detection: fluor: 360/450 nm post column reaction, flow = 1.5 ml/min, 0.33 g $K_3Fe(CN)_6$ + 15 g NaOH/1000 ml	thiamine.HCl USP by weight standard treated as sample peak height
11	0.5 grams autoclave 30 min, 121°C, 0.1M H_2SO_4 --> pH 4.0 Takadiastase 18h 45°C---> 100 ml (see vitamin B2)	Hypersil-ODS 5 micron 250x4.6 0.0075M TEACl+0.007M heptaneSO_3Na + 0.063M KH_2PO_4 in 600 ml water, pH-->3.5 +300 ml methanol--> 1000 ml water flow: 1.0 ml/min tr: 500 sec detection: fluor 368/420 nm post column reaction, flow = 0.1 ml/min 0.02N $K_3Fe(CN)_6$ +2.5 M KOH in water	thiamine.HCl BDH by weight, after drying standard treated as sample peak height
HPLC-methods, separation as thiochrome			
5	3 grams 30 min, 100°C, 0.1 M HCl -->pH 4.5, Takadiastase 2h, 50°C, filter 20 ml, conversion to thiochrome 5 ml+3 ml alkaline $K_3Fe(CN)_6$ 10 min	Pherisorb ODS2 10 micron 250x5.0 methanol:water 40:60 flow: 1.5 ml/min tr: 240 sec detection: fluor 375/435 nm	thiamine.HCl Koch Light calibration ?? external standard peak height (Ref.3)

Table 15. - continued

Lab-code	Extraction	Chromatography/Principle	Calibration/Calculation
12	4-5 grams autoclave 20 min, 121°C, 0.1M HCl -->pH 4.5, Taka-diastase/papaine 2-3h 45°C, centrifuge and filter, 1 ml+ 1 ml oxidising agent (15% NaOH+ 4% $K_3Fe(CN)_6$ +1.5 ml iso-butanol	uBondapack C-18 10 um 300x3.9 methanol:water 35:65 flow: 1.5 ml/min tr: 180 sec detection: fluor 365/435 nm peak	thiamine.HCl Merck 99.5% by weight external standard ?? area/height ?? (Ref.4,5)
15	1-3 grams autoclave 30 min, 121°C, 0.1M H_2SO_4 --> pH 5.0 Taka-diastase 45°C, 15h --> pH 2, filter. 10 ml+15 ml iso-butanol+ 1.5 ml NaOH+ 0.4 ml 5% K3Fe(CN)6 (see vitamin B2)	Nucleosil 120 C18 5 um 250x4.0 methanol:isobutanol:acetonitril 800:100:100 flow: 1.0 ml/min tr: 140 sec detection: fluor 370/420 nm	thiamine.HCl Merck by weight after drying standard treated as sample peak height (Ref.6)
17	5 grams autoclave 30 min, 121°C, 0.1M HCl -->pH 4.5 enzym 45°C 18 h, add 2 ml TCA 50%, heat 5 min, -->pH 4.5, volume to 200 ml 0.1 ml 0.03M $K_3Fe(CN)_6$+1.0 ml 4M NaOH+ 2 ml sample, mix 5 sec, filter after 3 min and inject after 5 min	Cromasil C18 5 micron 250x4.6 methanol:water 40:60 pH 4.5 HAc flow: 1.0 ml/min tr: 260 sec detection: fluor 375/440 nm only for pork muscle: Hamilton PRP-1 5 micron 150x4.1 acetonitril:water 10:90 flow: 1.0 ml/min tr: 470 sec	thiamine.HCl Fluka calibration ?? peak area
Other methods			
7	5 grams heat 5 min, 100°C acetate-buffer pH 4.5 AMG 200 L (NOVO) 16h at 37°C	thiochrome method clean-up: BIOREX 70 ionexchange detection: fluor 365/435 nm	Thiamine.HCl USP E(1%,1cm)=418, 246 nm internal standard
8	2 or 3 grams-->250 ml pork muscle; 10 grams-->250 ml haricots verts	thiochrome method	thiamindichlorid type?? calibration ?? standard addition ? (Ref.7)
9	5 grams heat 10 min, 100°C, 0.1M HCl add 3M HCl until precipitate forms, heat 30 min, 100°C and filter, 25 ml extract +1.5 ml oxidising agent (0.04% $K_3Fe(CN)_6$ in 15% NaOH) +6.5 ml isobutanol	thiochrome method detection: fluor 380/435 nm	thiamine.HCl Sigma by weight, correction moisture external standard (Ref.8)

Table 15. - continued

Lab-code	Extraction	Chromatography/Principle	Calibration/Calculation
16	1-2 grams autoclave 30 min, 121°C, 0.1M HCl-->pH 4.5, Takadiastase/ phosphatase 37°C, 18h, autoclave 20 min, 100°C	microbiological method Lactobacillus Vidridescens ATCC 12706	thiamin.HCl 99% Fluka by weight after drying (Ref.9)

References for the determination of vitamin B_1

1 Speek, Vitamin analysis in body fluids and foodstuffs with HPLC, thesis University of Amsterdam 1989
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3.7 Vitamin B_2

Eleven to thirteen laboratories submitted values for vitamin B_2 (Table 34). Differences between laboratories in the vitamin B_2 content found in milk powder, pork muscle and haricots verts are very high, with CV_{Reprod} ranging from 28% to 74% (Table 16). For collaborative studies using standardised methods a CV_{Reprod} of only 11% to 15% is to be expected (Horwitz, 1982). Discrepancies in vitamin B_2 values for the multivitamin mixture between laboratories are rather small. So, no serious calibration problems are evident. Correction of the results reported in milk powder, pork muscle and haricots verts for the level found in the multivitamin mixture (values between brackets in Table 16) by each laboratory does not reduce the variability.

Extraction by autoclaving or boiling with acid was the extraction method of choice for all participants, except laboratory 4 (Table 17). In addition laboratories using HPLC methods mostly applied an enzymatic dephosphorylation step with Takadiastase (laboratories 1,2,5,11,12,15,17). Laboratory 4 used a different extraction procedure to be able to determine the B_2 vitamers riboflavin, flavinmononucleotide (FMN), and flavin adenine dinucleotide (FAD) separately. HPLC methods all involved reversed phase columns and fluorescence detection, except for laboratory 2 using UV-detection. Three laboratories applied microbiological assays. These laboratories differed in the choice of the microorganisms, laboratories 7 and 8 used Lactobacillus casei, laboratory 16 used Enterococcus falcalis, also known as Streptococcus faecalis subspp. zymogenes.

Laboratory 16 reported extreme high values in all samples except for the multivitamin mixture (Figure 5). However, laboratories 7 and 8 also used microbiological methods and found values close to the results of laboratories 11 and 15. The response of both microorganisms is equal for riboflavin and FMN, so no explanation is apparent for the high values of laboratory 16. Laboratory 2 did not obtain adequate chromatographic resolution for the haricots verts. Because of the background of interfering peaks, a reliable determination of the content was not possible. Results of laboratory 9 are very low because the enzymes used, amylase and trypsin, did not have phosphatase activity.

In Figure 5 two groups of results are noticeable : high results reported by laboratories 11 and 15, and low results reported by the other laboratories. Comparing the extraction procedures (Table 17), it is evident that laboratories 11 and 15 used the most rigorous procedures concerning acid as well as enzymatic hydrolysis. The possibility that these differences are caused by inadequate autoclave and/or enzymatic hydrolysis procedures was examined by laboratory 11. Two autoclave extraction (H_2SO_4) periods, 15 and 30 min, in combination with two enzymatic hydrolysis (45°C, Takadiastase, supplied by Serva) periods, 3 h and 18 h, were compared. It showed that 15 min autoclave/3 h enzyme treatment was insufficient for both pork muscle (34% of 'total' value reached

with 30 min autoclave/18 h enzyme) and haricots verts(78%); 30 min autoclave/3 h enzyme only gave 48% of the total vitamin B_2 in pork muscle and 91% of the total value for beans. Extending the autoclave extraction from 15 to 30 min had no effect on the vitamin B_2 content in both of the materials, provided that the enzymatic hydrolysis lasted for 18 hours.

Problems with enzymes used for the extraction/dephosphorylation of vitamin B_1/B_2 are known. Takadiastase was used by most of the participants, however, experience of laboratory 1 shows that the performance of Takadiastase varies between different suppliers. Other laboratories reported positive experience with Claradiastase. These enzymes really are mixtures of enzymes with different substrate specificities and activities. Specifications are difficult to obtain. Problems arise when a supplier stops producing a good performing enzyme mixture, which has occurred in the past (MacBride et al.,1983; Schrijver, 1987).

In conclusion, extraction procedures have to be studied. Objectives would be to develop specifications for phosphatase activity and to optimise autoclave extraction and enzymatic hydrolysis.

Conclusions

- Variability in the results for vitamin B_2 in milk powder, pork muscle and haricots verts was high.
- Extraction/hydrolysis procedures are the most important source of variation between laboratories. Optimimum conditions need to be studied.
- No serious calibration problems were apparent.

Table 16. Summary of the results for VITAMIN B_2 (mg riboflavin/100 g dry weight)

	Multivitamin Mixture	Milk Powder	Pork Muscle	Haricots Verts
Number of laboratories	13	13	11	12
Mean of means	579.70	1.441(1.493)	0.502(0.516)	0.318(0.329)
Range	505.3-639.8	0.795-2.535	0.083-1.153	0.175-0.564
CV_{Reprod} %	7.9	28 (27)	74 (73)	35 (33)
CV_{repeat} %	5.0	7.0	4.2	6.0

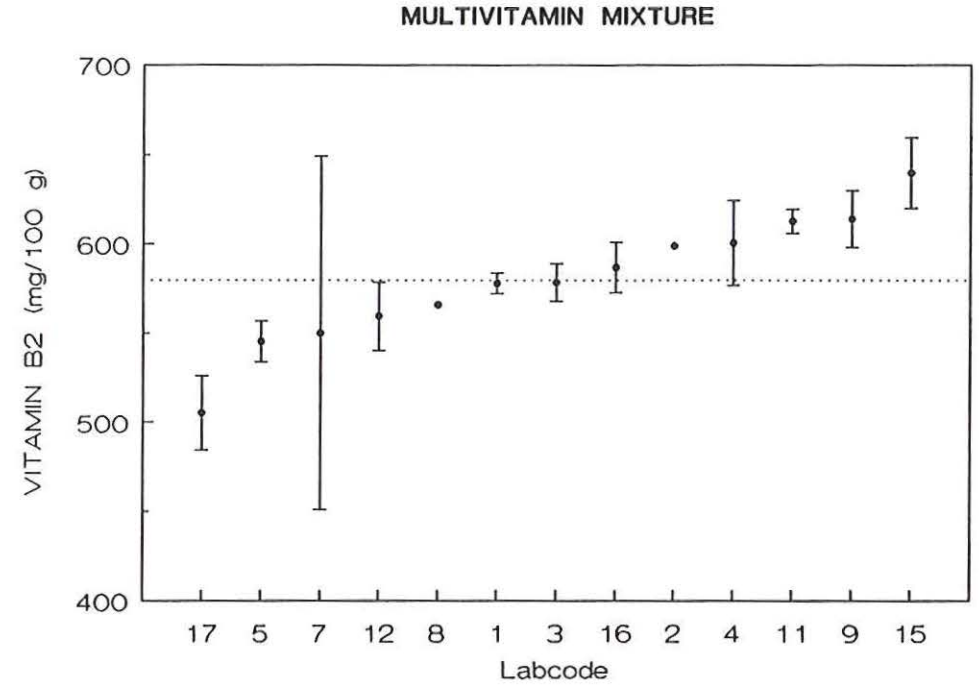
Table 16. - continued

	Multivitamin Mixture	Milk Powder	Pork Muscle	Haricots Verts
Cochran	7**	4*	-	-
Dixon	-	-	-	-
Achievable CV_{Reprod} %	4.3	11	13	13

Between brackets : values corrected for level found in multivitamin mixture by each laboratory.
Dixon : laboratories with extreme mean values detected by Dixon test
Cochran : laboratories with poor replicates detected by Cochran test.
Laboratories giving outlying results with marginal significance ($1\% < P \leq 5\%$) are marked with *, and outliers ($P \leq 1\%$) are marked with ** in the tables.

Figure 5. Results of individual laboratories for VITAMIN B_2 (mg riboflavin/100 g dry weight)

Data represent the mean ± standard deviation of the separate determinations of each laboratory.



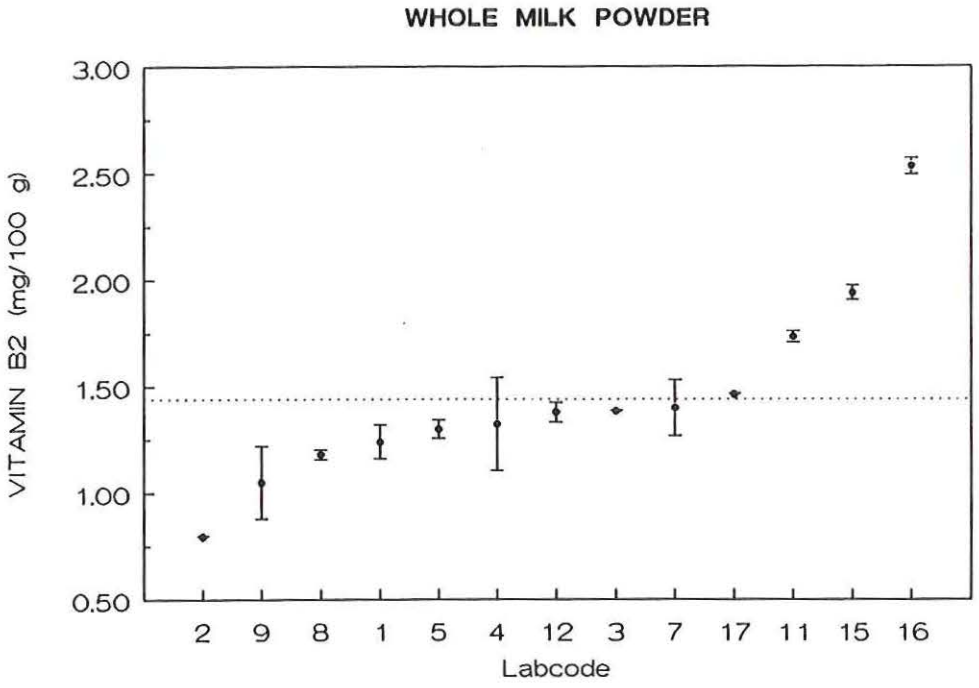
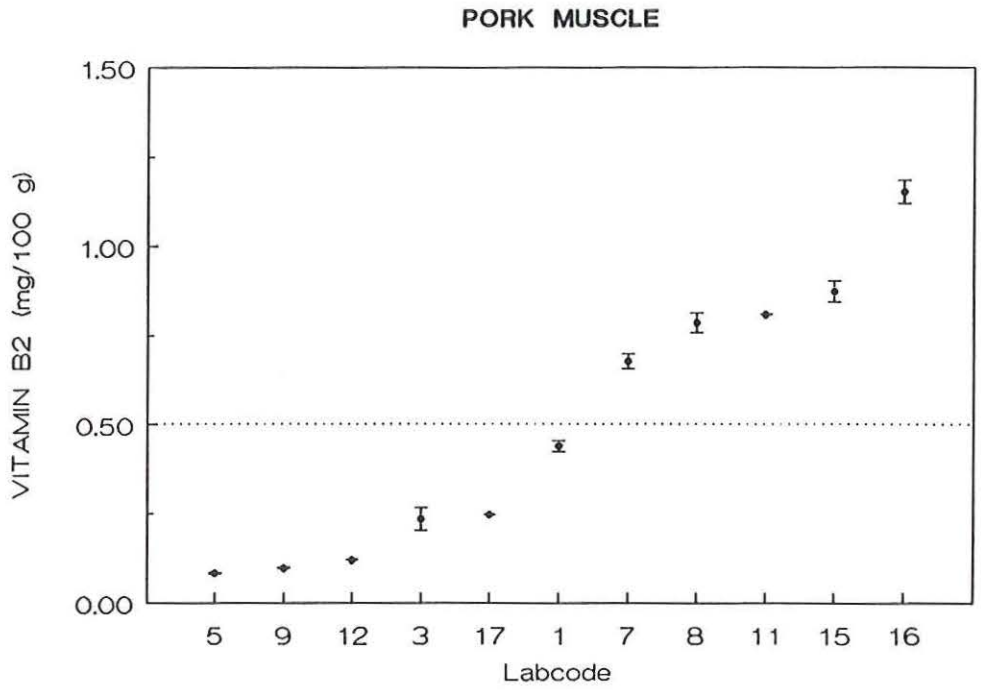


Figure 5. - continued



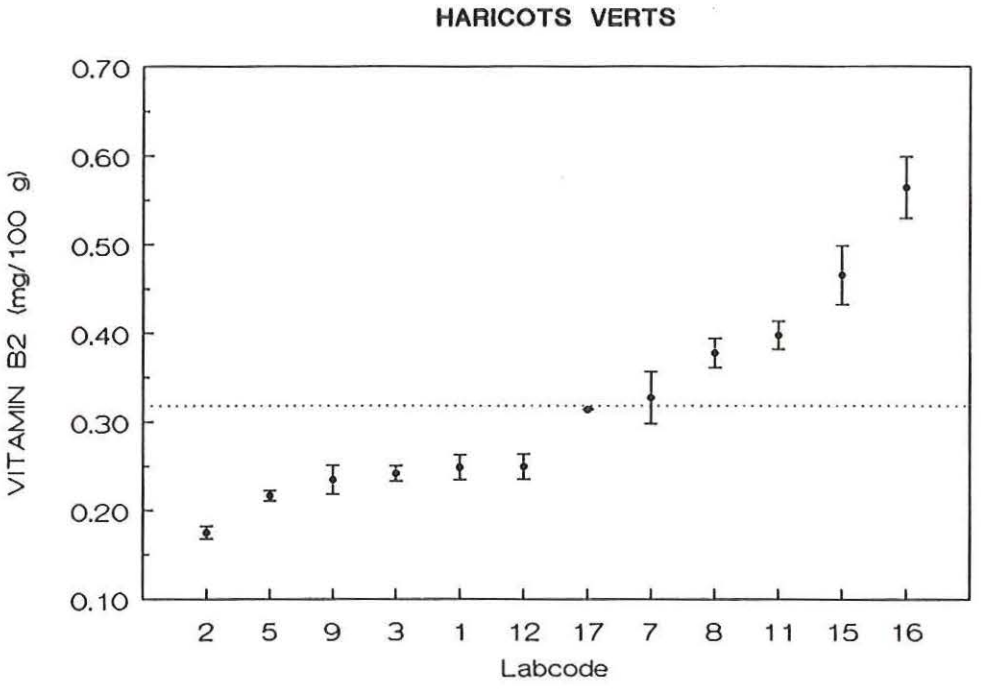


Table 17. Methods used for the determination of VITAMIN B_2

Lab-code	Extraction	Chromatography/Principle	Calibration/Calculation
HPLC-methods			
1	sample weight: 5 grams autoclave 15 min, 120°C, 0.15 M HCl Takadiastase 1h, 45°C	Hypersil-ODS 5 micron 125x4.6 methanol:water 20:80 flow: 1.2 ml/min tr: 480 sec detection: fluor FSA 404/FSA 115	riboflavin Sigma by weight external standard peak height (Ref.1,2)
2	5-10 grams autoclave 15 min, 121°C, 0.30M HCl -->pH 4.0-4.5 + 5 ml 6% Takadiastase 3h, clean-up: Florisil and Seppak	uBondapak C18 10 micron 300x3.9 methanol:water:acetic acid 31: 68.5:.5 1-hexaneSO_3Na, 1-heptaneSO_3Na 5mM flow: .5/.7 ml/min tr: 1100 sec detection: UV 254 nm (see vitamin B1)	standard type ?? by weight standard treated as sample peak height
3	10 grams solid, 2 grams liquid autoclave 15 min, 121°C, 0.05M H_2SO_4 (No dephosphorylation)	uBondapak C18 10 micron 250x4.6 methanol:water:acetic acid 250: 735:15 Pic-B6 5mM flow: 1.0 ml/min tr: 400 sec detection: fluor: 440/530 nm	riboflavin USP by weight external standard peak area
4	1 ml milk, 200 mg milk powder +10 ml 4M urea: formic acid 12% 1:1, 1 ml on 100 mg silica gel RP-18, elute with methanol: formic acid 10% 1:4 --> FAD, FMN and riboflavin	Supelcosil LC-18 3 micron 75x4.6 100mM KH_2PO_4 pH 2.9 15% acetonitril, flow: 1.0 ml/min tr: FAD= 60, FMN= 90, riboflavin= 140 sec detection: fluor 450/530 nm	FAD, FMN, riboflavin biochem. Merck , absorbance 450 nm E(1%,1cm)= ??, internal standard sorboflavin area/height ??
5	3 grams heat 30 min, 100°C, 0.1 M HCl-->pH 4.5, Takadiastase 2h, 50°C	Pherisorb ODS2 10 micron 250x5.0 methanol:water 40:60 flow: 1.5 ml/min tr: 220 sec detection: fluor 450/540 nm (see vitamin B1)	riboflavine Koch Light calibration ?? external standard peak height (Ref.3)
9	5 grams heat 30 min, 100°C, 0.1M H_2SO_4-->pH 4.5, amylase/ trypsin 90 min, 45°C, dilute 1+1 with methanol: water 7:3	Lichrosorb RP-18 5 um 250x4.0 methanol:$(NH_4)_2HPO_4$:dioxane 350:650:10 flow: 1.0 ml/min, tr: 400 sec detection: fluor 453/521 nm	riboflavin Sigma by weight, correction moisture external standard peak area (Ref.4)
11	0.5 grams autoclave 30 min, 121°C, 0.1M H_2SO_4-->pH 4.0, Takadiastase 18h 45°C--->100 ml, (see vitamin B1)	Hypersil-ODS 5 micron 250x4.6 0.0075M TEACl+0.007M heptane-SO_3Na+0.063M KH_2PO_4 in 600 ml water, pH-->3.5 +300 ml methanol -->1000 ml water flow: 1.0 ml/min tr: 500 sec detection: fluor 468/520 nm (see vitamin B1)	riboflavin BDH by weight, after drying standard treated as sample peak height

Table 17. - continued

Lab-code	Extraction	Chromatography/Principle	Calibration/Calculation
12	4-5 grams autoclave 20 min, 121°C, 0.1M HCl-->pH 4.5, Taka- diastase/papaine 2-3h 45°C, (see vitamin B1)	Apex ODS 3 micron 150x4.6 methanol:water 50:50 flow: 0.5 ml/min tr: 280 sec detection: fluor 450/510 nm	riboflavin Merck 99% by weight external standard ?? peak area/height ?? (Ref.5)
15	1-3 grams autoclave 30 min, 121°C, 0.1M H_2SO_4--> pH 5.0, Taka- diastase 45°C, 15h--> pH 2, (see vitamin B1)	Lichrosph.100 RP-18 5 um 125x4.0 methanol:sodiumacet. buffer pH 5 35:65 flow: 0.5 ml/min tr: 500 sec detection: fluor 467/525 nm	riboflavin 99% Merck by weight after drying standard treated as sample peak height/area ?? (Ref.6)
17	5 grams autoclave 30 min, 121°C, 0.1M HCl-->pH 4.5 enzym 45°C 18 h, add2 ml TCA 50%, heat 5 min, -->pH 4.5	Shandon ODS Hypersil 250x4.6 methanol:water 2:3 pH 4.5 with HAc flow: 1.0 ml/min tr: 300 sec detection: fluor 440/520 nm	riboflavin,WHO reference substance, by weight ?? standard treated as sample peak area
Microbiological methods			
7	5 grams autoclave 30 min,121°C 0.1M HCl, -->pH 4.5	Lactobacillus Casei ATCC-7469	WHO reference substance E(1%,1cm)=320 at 445 nm titration of acid (Ref.7)
8	10 grams -->250 ml	Lactobacillus Casei ATCC-7469 Bacto riboflavin assay medium	riboflavin type ?? calibration ?? (Ref.8)
16	1-2 grams autoclave 30 min, 121°C, 0.1M HCl, -->pH 4.5, filter	Enterococcus Falcalis ATCC-10100 riboflavin assay medium Difco 0942-15-8	FMN-Na.$2H_2O$ by weight

References for the determination of vitamin B_2

1 Speek, Vitamin analysis in body fluids and foodstuffs with HPLC, thesis University of Amsterdam 1989
2 van de Weerdhof et al., J. Chromatogr., 83 (1973) p. 455-460
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3.8 Vitamin B_6

There is a rather large variability in total vitamin B_6 values reported in milk powder, pork muscle and haricots verts, CV_{Reprod} ranging from 37% to 61% (Table 18), whereas the empirical equation of Horwitz (1982) predicts $CV_{Reprod} < 15\%$. Results for the multivitamin mixture agree rather well. So, no serious calibration problems are evident.

Two laboratories, 8 and 16, applied microbiological assays with different microorganisms (Table 19). Laboratories 1, 11, and 12 used reversed phase HPLC with fluorescence detection, and separated pyridoxine (PN), pyridoxamine (PM) and pyridoxal (PL) after dephosphorylation (Table 19). Laboratory 15 also separated PL, PN, and PM, but did not apply enzymatic dephosphorylation. Extraction and chromatographic conditions, together with the choice of excitation/emission wavelengths and pH in the cell of the detector, ensured that phosphorylated vitamers were included in the results. Laboratory 18 used gradient elution and was able to separate the phosphorylated vitamers as well. Therefore, a mild extraction procedure, preventing dephosphorylation was applied. Laboratory 9 did not apply dephosphorylation of the B_6 vitamers, and consequently was not able to determine the phosphorylated vitamers. Furthermore, only pyridoxine was quantified. So, laboratory 9 reported very low results in all samples except for the multivitamin mixture. Results of laboratory 9 are not included in Figure 7.

Results of laboratory 16 were high in each food sample (Figure 7). Laboratory 8 only analysed one food sample, pork muscle. Results do not agree very well with laboratory 16 also using a microbiological assay.

Figure 6 gives a summary of the results of the laboratories using HPLC methods to separate the different B_6 vitamers. Contrary to the other laboratories, laboratory 12 did not find PN in the haricots verts and pork muscle, and did not find PL in the haricots verts. This accounts for the relatively low results for total vitamin B_6 reported by laboratory 12. Contrary to the other laboratories, laboratory 1 did not detect any PM in pork muscle, but instead of this a relatively high level of PN. Laboratory 1 confirmed this finding after reexamining the results. Only laboratory 15 and 18 found PN in milk powder. Laboratory 11 used β-glucosidase to dephosphorylate the B_6 vitamers. These results are reported in Tables 18 and 35 and Figures 6 and 7. In addition, laboratory 11 also performed analyses in the haricots verts, using acid phosphatase instead of β-glucosi- PN in haricots verts is bound as pyridoxine-β-glucoside. In this case PN content only was 0.02 mg/100 g. It follows that the major part of PN is bound as pyridoxine-β-glucoside.

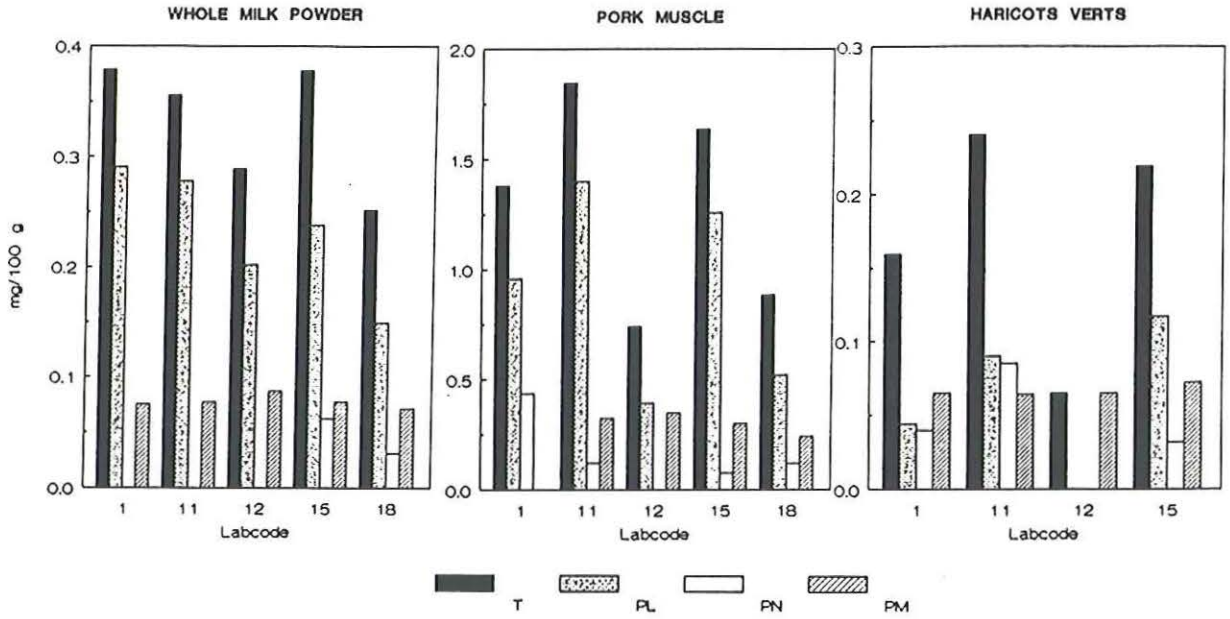


Figure 6. Results for individual B_6 vitamers determined with HPLC methods.
T = Total vitamin B_6; PL = pyridoxal; PN = pyridoxine; PM = pyridoxamine.

Results recalculated after rejecting laboratory 9 still show a large variation between laboratories (Table 18).

Two problem areas are recognised. Firstly, the identification of the different vitamin B_6 vitamers proves to be difficult, f.i. PN in milk powder. Consequently, total vitamin B_6 values as determined with HPLC by summation of the individual vitamers, will be erroneously. Secondly, the enzymatic hydrolysis in connection to pyridoxine-β-glucoside in haricots verts beans. As was shown by results of laboratory 11, alkaline phosphatase did not hydrolyse these glucosides, whereas β-glucosidase obviously liberated PN. Takadiastase probably hydrolyses only a fraction of these glucosides. As the bioavailability of these glucosides is less than the bioavailability of PN or PNP, it is important to know whether or not the vitamin B_6 value includes these glucosides. In addition, extraction and enzymatic hydrolysis of the other vitamers also have to be studied.

Conclusions

- Variation in the results for vitamin B_6 in milk powder, pork muscle and haricots verts beans was high.
- Extraction and hydrolysis procedures for the B_6 vitamers need to be studied. The pyridoxine-β-glucoside in vegetable products needs special attention.
- Identification of the B_6 vitamers with HPLC methods has to be studied.

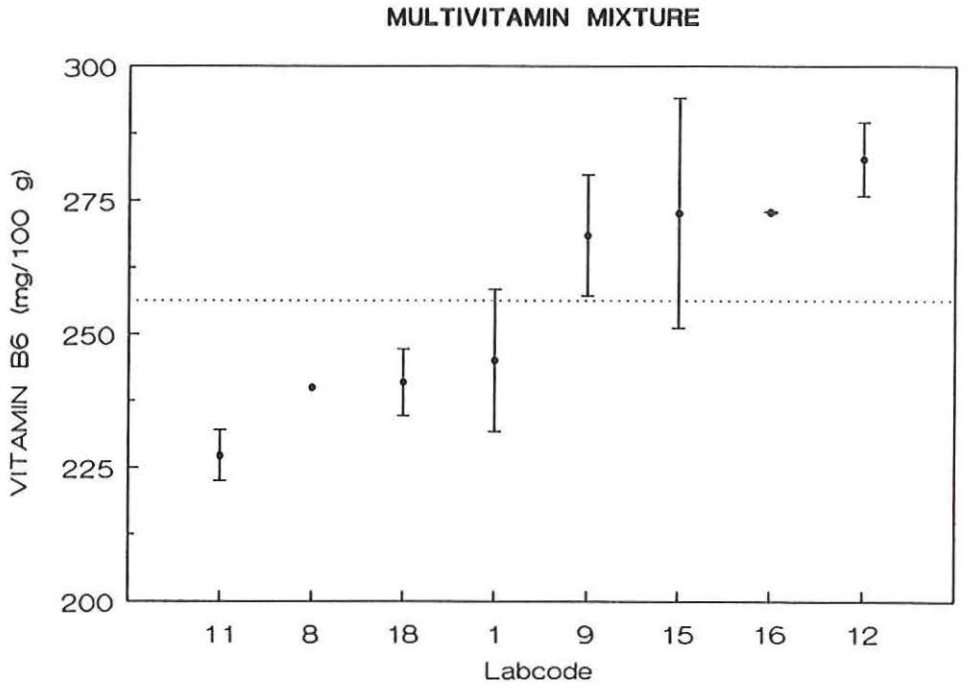
Table 18. Summary of the results for VITAMIN B_6 (mg pyridoxine/100 g dry weight)

	Multivitamin Mixture	Milk Powder	Pork Muscle	Haricots Verts
Number of laboratories	8	7	8	6
Mean of means	256.263	0.300	1.166	0.173
Range	227.3-282.8	0.066-0.381	0.158-1.845	0.043-0.313
CV_{Reprod} %	8.8	37	47	61
CV_{repeat} %	4.0	8.0	5.9	4.6
Cochran	-	-	16**	-
Dixon	-	9*	-	-
Achievable CV_{Reprod} %	4.9	14	11	15
<u>Excluding laboratory 9</u>:				
Number of laboratories		6	7	5
Mean of means		0.339	1.310	0.199
Range		0.252 - 0.381	0.74 - 1.85	0.065 - 0.313
CV_{Reprod} %		18	35	51
CV_{repeat} %		7.4	5.6	4.5

Between brackets : values corrected for level found in multivitamin mixture by each laboratory.
Dixon : laboratories with extreme mean values detected by Dixon test
Cochran : laboratories with poor replicates detected by Cochran test.
Laboratories giving outlying results with marginal significance ($1\% < P \leq 5\%$) are marked with *, and outliers ($P \leq 1\%$) are marked with ** in the tables.

Figure 7. Results of individual laboratories for VITAMIN B_6 (mg pyridoxine/100 g dry weight)

Data represent the mean ± standard deviation of the separate determinations of each laboratory.



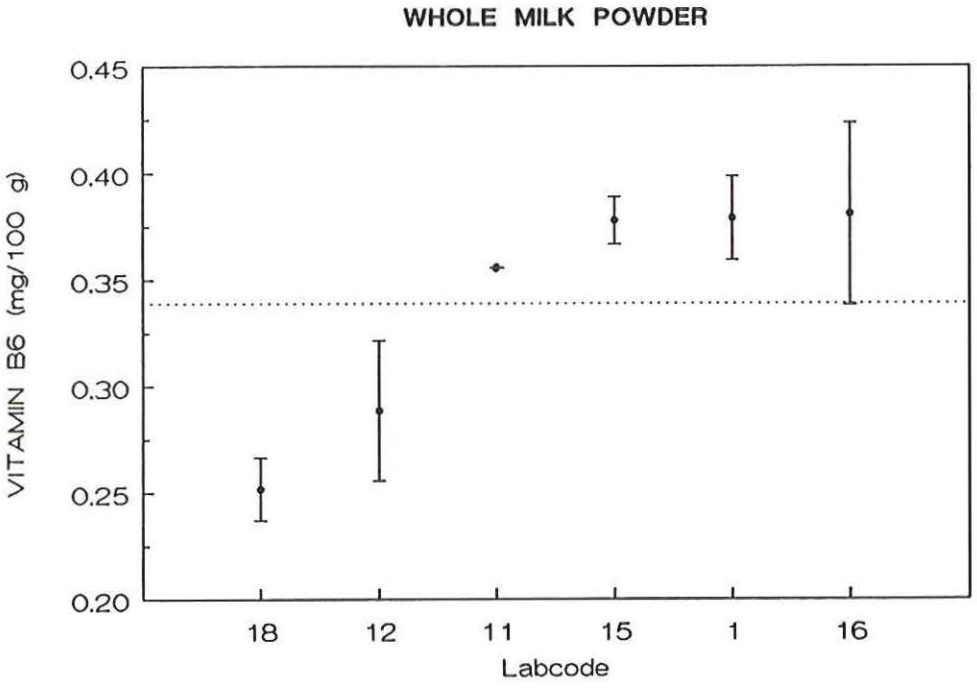
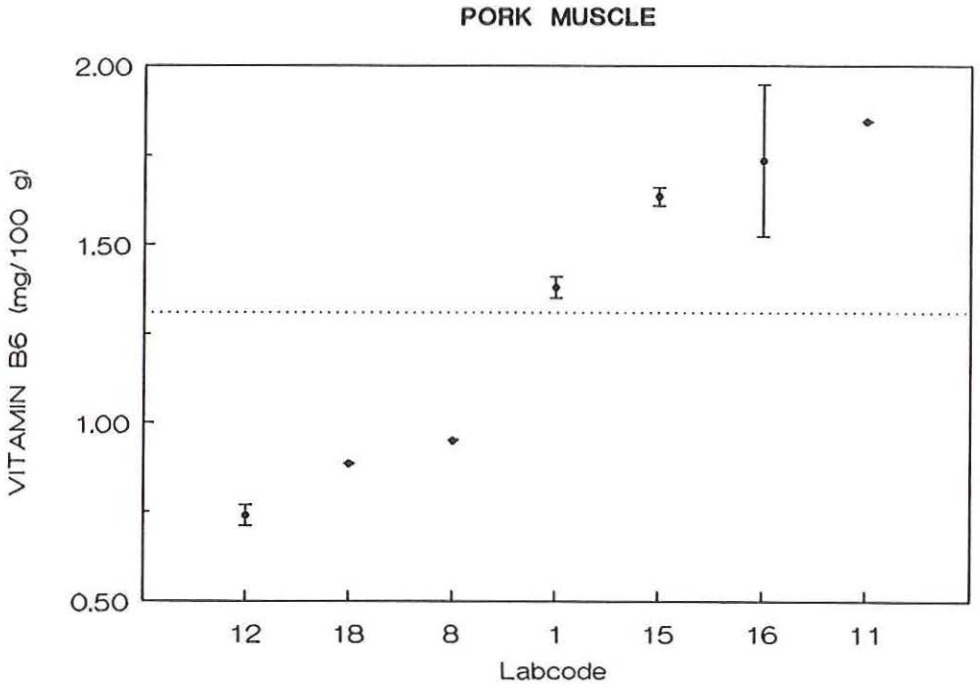


Figure 7. -continued



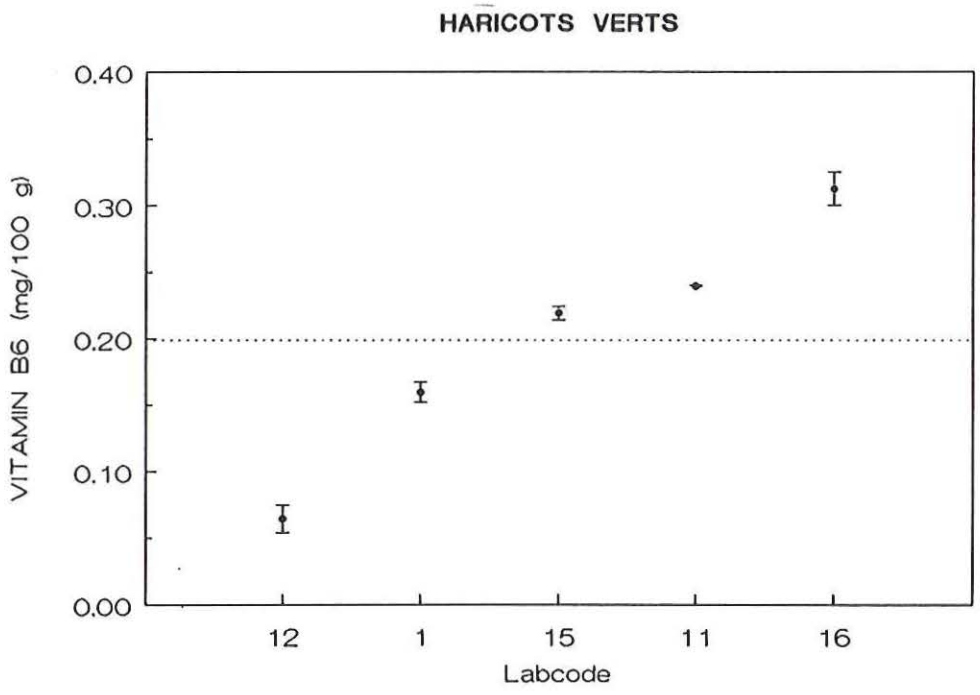


Table 19. Methods used for the determination of VITAMINE B_6

Lab-code	Extraction	Chromatography/Principle	Calibration/Calculation
HPLC-methods			
1	sample weight: 5 grams trichloroacetic acid 5% Takadiastase 1h, 45°C	Hypersil-ODS 5 micron 125x4.6 0.1M KH_2PO_4 buffer pH 2.1 +30 ml ethanol + PIC-B8 flow: 1.1 ml/min tr: PL=660, PN=1200, PM=1500 sec detection: fluor 333/375 nm	PL.HCl, PN.HCl, PM.2HCl Sigma; by weight internal standard deoxypyridoxine peak height (Ref.1,2,3,4)
9	5 grams heat 30 min, 100°C, 0.1M H_2SO_4, dilute -->100 ml,	Lichrosorb RP-18 5 um 250x4.0 0.04M H_2SO_4 flow: 1.5 ml/min tr: PN=360 sec detection: fluor 290/395 nm	PN.HCl Sigma by weight after drying external standard ?? peak height/area ?? (Ref.5)
11	0.5 grams lean pork, 1.0 grams milk powder, 3.0 grams haricots verts trichloroacetic acid 5%, +sodiumacetate (pH-->4.8) +β-glucosidase or phosphatase 18h 37°C, +TCA 60%,	Lichrospher RP-18 5 um 375x4.0 0.0075M TEACl+0.0075M heptane-SO_3Na+0.075M KH_2PO_4 in 600 ml water, pH-->2.6 +85 ml acetonitrile-->1000 ml water flow: 0.8 ml/min tr: Pl=680, PN=890, PM=1190 sec post column reaction, flow: 0.1 ml/min 0.5M K_2PO_4 detection: fluor 325/385 nm	PL.HCl, PN.HCl. PM.2HCl Sigma; by weight, after drying standard treated as sample peak height
12	4-5 grams autoclave 10 min, 121°C, 0.1M H_2SO_4,-->pH 4.6, amyloglucosidase 2h, 45-50°C,	Spherisorb ODS2 5 um 250x4.6 0.04M H_2SO_4:methanol 99:1 flow: 1.5 ml/min tr: PM=130, PL=330, PN=480 sec detection: fluor 290/395 nm	PL.HCl 99% Merck PN.HCl 99.5% Merck PM.2HCl 99% Merck calibration ?? external standard ?? peak height ?? (Ref.6,7)
15	1-3 grams autoclave 30 min, 120°C, 0.1M H_2SO_4	Nucleosil 120-5 5 micron 250x4.0 0.04M H_2SO_4 (gradient, wash with methanol:water 95:5 +12 min 2.0 ml/min) flow: 1.5 ml/min tr: PM=110, PL=260, PN=360 sec detection: fluor 290/395 nm	PL.HCl, PN.HCl, PM.2HCl type= ??; by weight standard treated as sample peak height/area ??

Table 19. - continued

Lab-code	Extraction	Chromatography/Principle	Calibration/Calculation
18	0.5 grams Ultraturrax 0°C 0.5M perchloric acid, centrifuge, -->pH 7.5, 10 min 0°C, dephosphorylation (for indirect measurement PNP) pH 4.0, 30 min, 25°C	Lichrosorb RP-18 5 um 125x4.0 gradient: A= methanol, B= 0.03M phosphate buffer pH 2.7 +0.004M octanesulfonic acid flow: 1.5 ml/min tr: PLP=115, PMP=200,PL=580,PN=650,PM=830 sec post column reaction, flow 0.07 ml/min 0.5M phosphate buffer pH 7.5+$NaHSO_3$ detection: fluor 340/400 nm	PLP.HCl, PMP.HCl,PL.HCl PN.HCl, PMP.HCl, Merck calibration ?? internal standard deoxypyridoxine peak height (Ref.8)
Microbiological methods			
8	5 grams--->250 ml 60 min, 100°C, 1.0M HCl	Neurospora sitophila ATCC 9276 assay medium ??	PN.HCl type ?? calibration ?? (Ref.9)
16	1-2 grams autoclave 5h, 121°C, 0.44M H_2SO_4, -->pH 4.5,	Saccharomyces Carlsbergensis ATCC 9080 pyridoxine assay medium Difco 0951-15-2	PN.HCl >98% Fluka calibration ?? (Ref.10)

References for the determination of vitamin B_6

1 Speek, Vitamin analysis in body fluids and foodstuffs with HPLC, thesis University of Amsterdam 1989
2 Coburn et al., Biochem. 129, 1983, p. 310-317
3 Tryfiates et al., J. Chromatogr. 227, 1982, p. 181-186
4 Morita et al., J. Chromatogr. 202, 1980, p. 134-138
5 Brubacher, Muller-Mulot, Southgate (Eds.); Methods for the determination of vitamins in foods, recommended by COST 91, Elsevier 1985
6 J. Human Diet., 1, 1988, p. 309-320
7 J. Human Diet., 2, 1989, p. 159-172
8 J. Chromatogr. 463, 1989, p. 207-211
9 Schweizerisches Lebensmittelbuch, Zweiter Band 1989, Kap. 62/8.2.1
10 Bell, J. Lab. Practice, 23, 5, 1974, p. 235-242 and 253

3.9 Folic acid

Only laboratory 1 reported results for folic acid (Tables 20, 36). However, this does not reflect the actual interest in folic acid. Generally there is agreement on the importance of folic acid from a nutritional point of view. In particular, analyses of folic acid in foods could be helpful in resolving discussions about intake of folic acid. Generally folic acid is not routinely determined in most of the laboratories. A contamination problem prevented laboratory 16 from participating. Laboratories 15 and 18 are working on HPLC methods for folic acid.

Conclusion

- From a nutritional point of view, folic acid is important. A project focused on folic acid is needed. Laboratories specialised in the analysis of folic acid have to be invited.

Table 20. Method used for the determination of FOLIC ACID

Lab-code	Extraction	Principle	Calibration/Calculation
Microbiological method			
1	sample weight: 2 grams autoclave 10 min, 120°C in phosphate buffer pH 6.1 containing 0.2% sodium-ascorbate, chicken-pancreas, 18h 37°C	Lactobacillus casei NCIB 10463 Folic acid assay medium Difco # 0822-15-9	pteroylglutamic acid Sigma; calibration ?? (Ref.1,2,3)

References for the determination of folic acid

1 Difco manual dehydrated culture media and reagents for microbiology, 10th edition 1984, p. 1090-1093
2 Williams, Microbiological assays in official methods of analysis of the AOAC, 14th edition, 1984, p. 862-873
3 Kavanagh, Analytical microbiology, Academic Press, New York, 1963, p. 423-488

3.10 Vitamin B_{12}

Only three laboratories reported results (Table 21 and 37).
Laboratories 7 and 16 used a microbiological assay with Lactobacillus leichmanii (Table 22). The level of agreement is expected to be within 10%. No explanation is apparent for the high results of laboratory 16. The radio-assay method with Intrinsic Factor (laboratory 1) normally gives a good correlation with the microbiological methods using L. leichmanii, although the specificity differs. Care has to be taken during storage because of the risk for increase in vitamin B_{12}.

Conclusions

- Because only three laboratories participated, conclusions are premature.
- A project focused on the determination of vitamin B_{12} is needed. Laboratories specialised in the analysis of vitamin B_{12} have to be invited.

Table 21. Summary of the results for VITAMIN B_{12} (µg cyanocobalamin/100 g weight)

	Multivitamin Mixture	Milk Powder	Pork Muscle
Number of laboratories	3	3	3
Mean of means	929.2	3.260	2.436
Range	837.5 - 985.0	2.567 - 4.053	1.600 - 3.883

Table 22. Methods used for the determination of VITAMIN B_{12}

Lab-code	Extraction	Principle	Calibration/Calculation
Microbiological methods			
7	sample weight: 5 grams autoclave 10 min, 121°C, 0.5M H_2SO_4	Lactobacillus leichmannii ATCC # 7830 B12 assay medium USP,Difco #0457 turbidimetric 650 nm	standard type USP E(1%,1cm) = 203, 360 nm (Ref.1)
16	1-2 grams 0.1M sodiumacetate buffer + sodiumcyanide 1%, autoclave 10 min, 121°C	Lactobacillus leichmannii ATCC # 7830 B12 assay medium USP, Difco # 0457-15-1	vitamin B12 >98% Fluka by weight after drying (Ref.2)
Other method			
1	5 grams autoclave 15 min 121°C in 0.1M sodium acetate buffer pH 4.6 containing 0.005% sodiumcyanide	Radio-assay with pure Intrinsic Factor (IF) as binding protein	cyanocobalamin Merck absorbance 368 nm 0.5M NaOH/0.5% KCN , Mol. ext.coeff. = 30.808 Logit-log transformation of standard curve (Ref.3,4)

References for the determination of vitamin B_{12}

1 Ph Nord., 1960 (with modifications)
2 Bell J.G., J. Lab. Practice, 23, 5, 1974, p. 235-242 and 252
3 van den Berg et al., Lancet I, 1988, p. 243-244
4 Lau et al., Blood 26, 1965, p. 202-214

3.11 Vitamin C

Differences between laboratories in the vitamin C content found in haricots verts are unacceptably high, ranging from 0.4 to 366 mg /100g (Table 38). Variation between laboratories in vitamin C values for the multivitamin mixture are somewhat higher than expected (Table 23). However, no serious calibration problems are evident from these data, as correction of the results reported in haricots verts for the level found in the multivitamin mixture by each laboratory (values between brackets in Table 23) does not reduce the variability.

HPLC methods with fluorescence detection were used by most of the participants (Table 24). Fluorescence detection requires oxidation of ascorbic acid to dehydroascorbic acid and a reaction with o-phenylenediamine to form a fluorescent quinoxaline. Laboratories 1, 3, and 11 applied enzymatic oxidation of the ascorbic acid, whereas laboratory 15 oxidised ascorbic acid with activated carbon. These laboratories thus determined total vitamin C, i.e. the sum of ascorbic and dehydroascorbic acid. On the other hand, laboratories 7, 9, and 14 also using HPLC applied UV-detection and consequently only were able to determine ascorbic acid, because dehydroascorbic acid is a non-absorbing compound in UV. Laboratories 10, 12, and 16 used fluorometric methods, also measuring the quinoxaline compound. Laboratory 2 used the classical indophenol titration to determine ascorbic acid. This method is not as specific as HPLC methods, because of possible interferences from other reducing substances present in the sample extract. Laboratory 9 had problems with chromatographic resolution. Possibly the wrong peak was identified as vitamin C, accounting for the extreme high values (Table 38).

In Figure 8 results of laboratory 9 are not shown. Results of laboratory 2 are rather high, probably because of the lack of specificity of the indophenol titration. Comparing results of laboratories 7 and 14, who only determined ascorbic acid, no differences with the results of methods determining total vitamin C are evident. So, probably dehydroascorbic acid content of the haricots verts was low.

After rejecting results of laboratories 2 and 9, recalculation shows that the remaining laboratories agree very well (Table 23).

Conclusions

- There was a good agreement between the results reported for vitamin C in haricots verts, after excluding two laboratories using inadequate methods.
- For haricots verts, presently to be certified for major nutrients (see 2.2) an indicative value for vitamin C is proposed, provided stability can be proven :

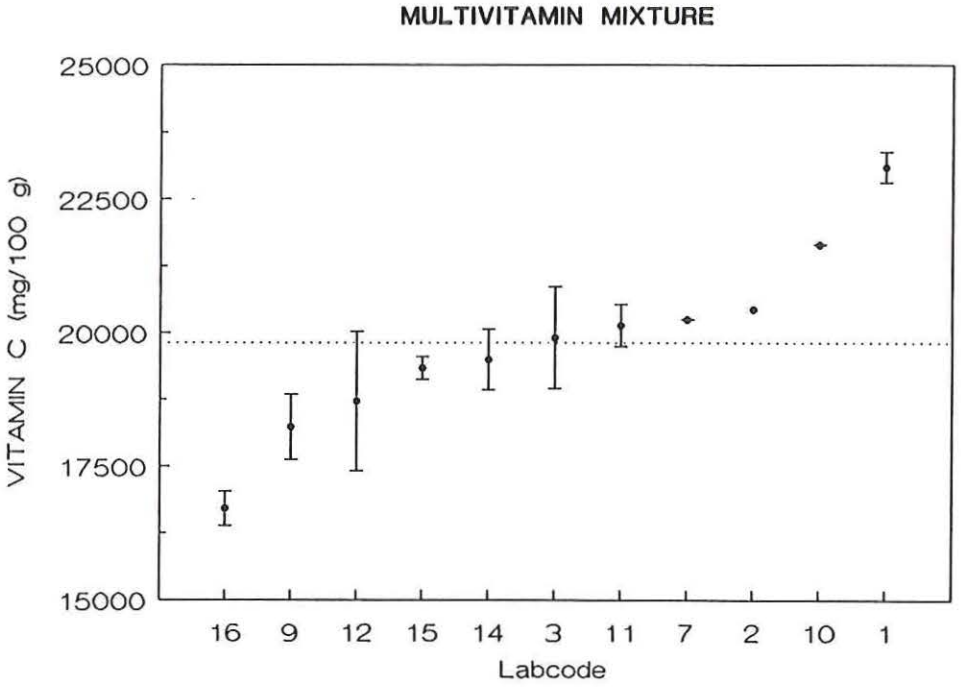
  15.42 mg/100 g dry weight (standard deviation : 1.33 mg/100 g; 9 sets).

Table 23. Summary of the results for VITAMIN C (mg /100 g dry weight)

	Multivitamin Mixture	Haricots Verts
Number of laboratories	11	11
Mean of means	19817.8	50.887 (50.675)
Range	16710 - 23100	12.940 - 365.6
CV_{Reprod} %	8.9	231 (255)
CV_{repeat} %	3.9	20
Cochran	-	9**
Dixon	-	9**
Achievable CV_{Reprod} %	2.6	6.3
Excluding laboratories 2 en 9:		
Number of laboratories		9
Mean of means		15.424
Range		12.940 - 17.367
CV_{Reprod} %		9.8
CV_{repeat} %		4.0

Between brackets : values corrected for level found in multivitamin mixture by each laboratory.
Dixon : laboratories with extreme mean values detected by Dixon test
Cochran : laboratories with poor replicates detected by Cochran test.
Laboratories giving outlying results with marginal significance (1% < P ≤ 5%) are marked with *, and outliers (P ≤ 1%) are marked with ** in the tables.

Figure 8. Results of individual laboratories for VITAMIN C (mg /100 g dry weight)
Data represent the mean ± standard deviation of the separate determinations of each laboratory.



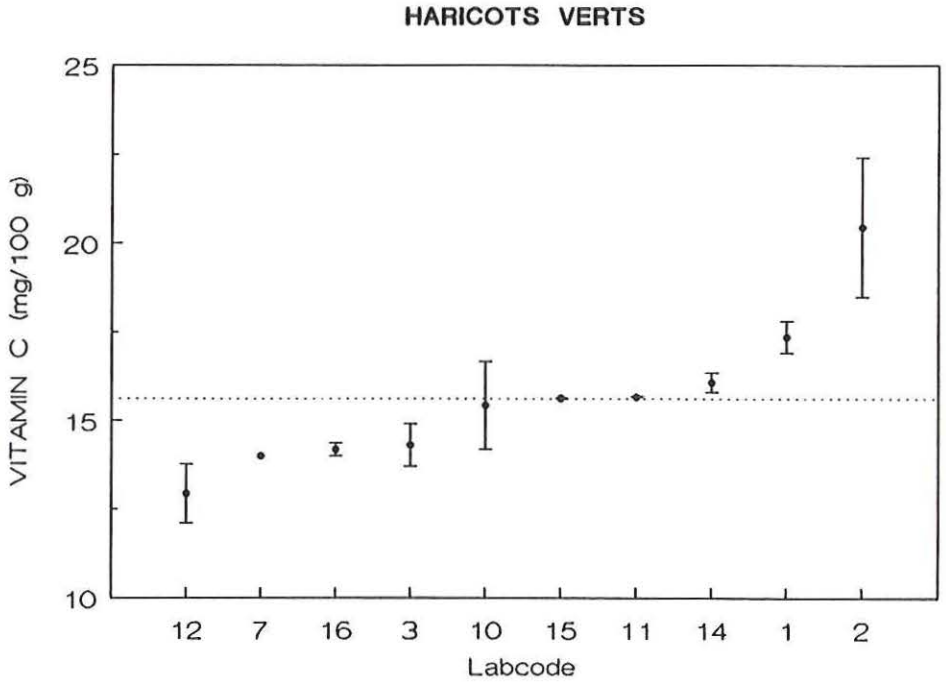


Table 24. Methods used for the determination of VITAMIN C

Lab-code	Extraction	Principle	Calibration/Calculation
HPLC-methods			
1	sample weight: 5 grams trichloroacetic acid 5% ascorbate oxidase, o-phenylenediamine (OPDA)	Hypersil-ODS 3 micron 125x4.6 0.8M phosphate buffer pH 7.8: methanol 800:150 flow: 1.2 ml/min tr: 420 sec detection: fluor 367/418 nm	ascorbic acid Merck by weight standard treated as sample peak height (Ref.1,2)
3	6-8 grams metaphosphoric acid 1%, 3 ml aliquot+2ml sodium- acetate buffer + ascorbate- oxidase 5 min, 37°C + 0.5 ml OPDA, 30 min, 37°C	8NCV-18 RCM-cartridge 4 um, 125x3.9; 0.08M phosphate buffer pH 7.8:methanol 800:200 flow: 1.0 ml/min tr: 480 sec detection: fluor: 355/425 nm	ascorbic acid Merck by weight external standard peak area (Ref.3)
7	15 grams metaphosphoric acid + EDTA	RP-18 5 micron 250x4.0 2M acetate buffer, tetrahexyl- ammonium bromide, water flow: 1.0 ml/min tr: 480 sec detection: UV 243 nm	standard ?? calibration ?? external standard ?? area/height ??
9	0.5 grams metaphosphoric acid/acetic acid, centrifuge, re-extract	Lichrosorb RP-18 5 um 250x4.0 5.5mM heptaneSO_3 + 50 mg EDTA + 24 ml acetic acid + 5 ml triethyl- amine + 150 ml methanol + 800 ml water flow: 1.0 ml/min tr: 150 sec detection: UV 254 nm	ascorbic acid Sigma by weight external standard ?? peak area
11	2 grams metaphosphoric acid 1%, 3 ml aliquot +2ml sodium- acetate buffer + ascorbate- oxidase 5 min, 37°C + 0.5 ml OPDA30 min, 37°C	Chromspher RP-18 5 um 100x3.0 0.08M phosphate buffer pH 7.8: methanol 915:85 flow: 1.0 ml/min tr: 160 sec detection: fluor 355/425 nm	ascorbic acid Merck by weight standard treated as sample peak area (Ref.2,3)
14	5 grams metaphosphoric acid/acetic acid	Partisil P5 PAC 5 um 250x4.6 acetic acid 0.75%:methanol 75:25 flow: 2.0 ml/min tr: 385 sec detection: UV 248 nm	ascorbic acid Fisons calibration ?? external standard peak area (Ref.4)
15	10-12.5 grams metaphosphoric acid/acetic acid centrifuge and filter + activated carbon + OPDA	Spherisorb ODS 10 um 250x4.0 acetate buffer pH 5.2: methanol 1:1 flow: 1.0 ml/min tr: 160 sec	ascorbic acid Merck by weight, afeter drying standard treated as sample peak area (Ref.5)

Table 24. - continued

Lab-code	Extraction	Principle	Calibration/Calculation
Other methods			
2	2 grams acetic acid/metaphosphoric acid, 5 min ultrasonic	titration with 2,6-dichloro-phenolindophenol AOAC-procedure	ascorbic acid Merck by weight (Ref.6)
10	10 grams metaphosphoric acid 5%	continuous flow method detection: fluor, wavelengths ??	ascorbic acid >98% HlR by weight external standard (Ref.7)
12	1-2 grams acetic acid/metaphosphoric acid + activated charcoal + OPDA	AOAC-microfluorimetric method	ascorbic acid 99.7% BDH calibration ?? standard treated as sample
16	5 grams acetic acid/metaphosphoric acid + activated carbon + OPDA	continuous flow method detection: fluor: 350/427 nm	ascorbic acid 99.7% Fisons; by weight standard treated as sample (Ref.8,9)

References for the determination of vitamin C

1 Speek, Vitamin analysis in body fluids and foodstuffs with HPLC, thesis University of Amsterdam 1989
2 Speek et al., J. Agr. Food Chem. 32 1984 p. 352-355
3 NEN-3468 draft method
4 Leatherhead Food RA Methods manual, 2nd Edition
5 Bognar A., Bestimmung von Vitamin C in Lebensmitteln mittels HPLC. Deutsch. Lebensm. Rundschau 84 1988 p. 73-76
6 AOAC 1984 14th Edition
7 Bourgeois C., et al., Analusis, 17, 9, 1989, p. 519-525
8 Deutsch M.J., Weeks C.E., JAOAC, 48, 1965, p. 1248-1256
9 Edberg D.C., JAOAC, 60, 1, 1977, p. 126-131

3.12 Niacin

Results for milk powder agree very well, CV_{Reprod} is only 9.1% (Tables 25 and 39). There is a large variability in niacin values reported for pork muscle and haricots verts. Thus, niacin content in pork muscle ranged from 11.05 to 27.26 mg/100 g. For haricots verts the range was 1.317 to 4.525. Results for multivitamin mixture agree rather well. So, no serious calibration problems are evident. Correction of the results reported in milk powder for the level found in the multivitamin mixture (values between brackets in Table 15) by each laboratory does not reduce the variability.

Laboratory 2 used HPLC with UV-detection after autoclave extraction, and only determined niacin in the pork muscle and haricots verts (Table 26). However, difficulties with the purification of the extract and chromatographic resolution were reported. All other laboratories used microbiological methods with an identical microorganism, Lactobacillus plantarum (Table 26). Extraction procedures were based on acid hydrolysis using autoclave heating.

Compared with the other laboratories, laboratory 2 gives extreme low values for pork muscle and extreme high values for haricots verts (Figure 9). Because of the difficulties reported, results of laboratory 2 were judged to be not reliable. The results obtained with microbiological methods are satisfactory with all samples, so indicative values can be given. No problems with stability are expected. There is no obvious explanation for the rather low value produced by laboratory 1 in pork muscle.

Conclusions

- The results for niacin obtained with microbiological methods in milk powder, pork muscle and haricots verts agreed well between laboratories.
- For milk powder, pork muscle and haricots verts, presently to be certified for major nutrients (see 2.2) indicative values for niacin are proposed :

  milk powder 0.831 mg niacinamide/100 g dry weight
  (standard deviation : 0.066 mg/100 g; 7 sets)
  pork muscle 24.2 mg niacinamide/100 g dry weight
  (standard deviation : 2.64 mg/100 g; 6 sets)
  haricots verts 1.706 mg niacinamide/100 g dry weight
  (standard deviation : 0.249 mg/100 g; 7 sets)

Table 25. Summary of the results for NIACIN mg niacinamide/100 g dry weight)

	Multivitamin Mixture	Milk Powder	Pork Muscle	Haricots Verts
Number of laboratories	7	7	7	8
Mean of means	1317.40	0.831 (0.823)	22.362	2.058
Range	1151.4-1546.0	0.742-0.898	11.05-27.26	1.317-4.525
CV_{Reprod} %	10	9.2 (10.2)	21	44
CV_{repeat} %	5.5	5.3	3.1	4.5
Cochran	16**	-	-	-
Dixon	-	-	-	2**
Achievable CV_{Reprod} %	3.8	12	7.1	10
Excluding laboratory 2:				
Number of laboratories			6	7
Mean of means			24.247	1.706
Range			19.53-27.26	1.317-2.173
CV_{Reprod} %			11	15
CV_{repeat} %			3.1	4.5

Between brackets : values corrected for level found in multivitamin mixture by each laboratory.
Dixon : laboratories with extreme mean values detected by Dixon test
Cochran : laboratories with poor replicates detected by Cochran test.
Laboratories giving outlying results with marginal significance (1% < P ≤ 5%) are marked with *, and outliers (P ≤ 1%) are marked with ** in the tables.

Figure 9. Results of individual laboratories for NIACIN (mg niacinamide/100 g dry weight)

Data represent the mean ± standard deviation of the separate determinations of each laboratory.

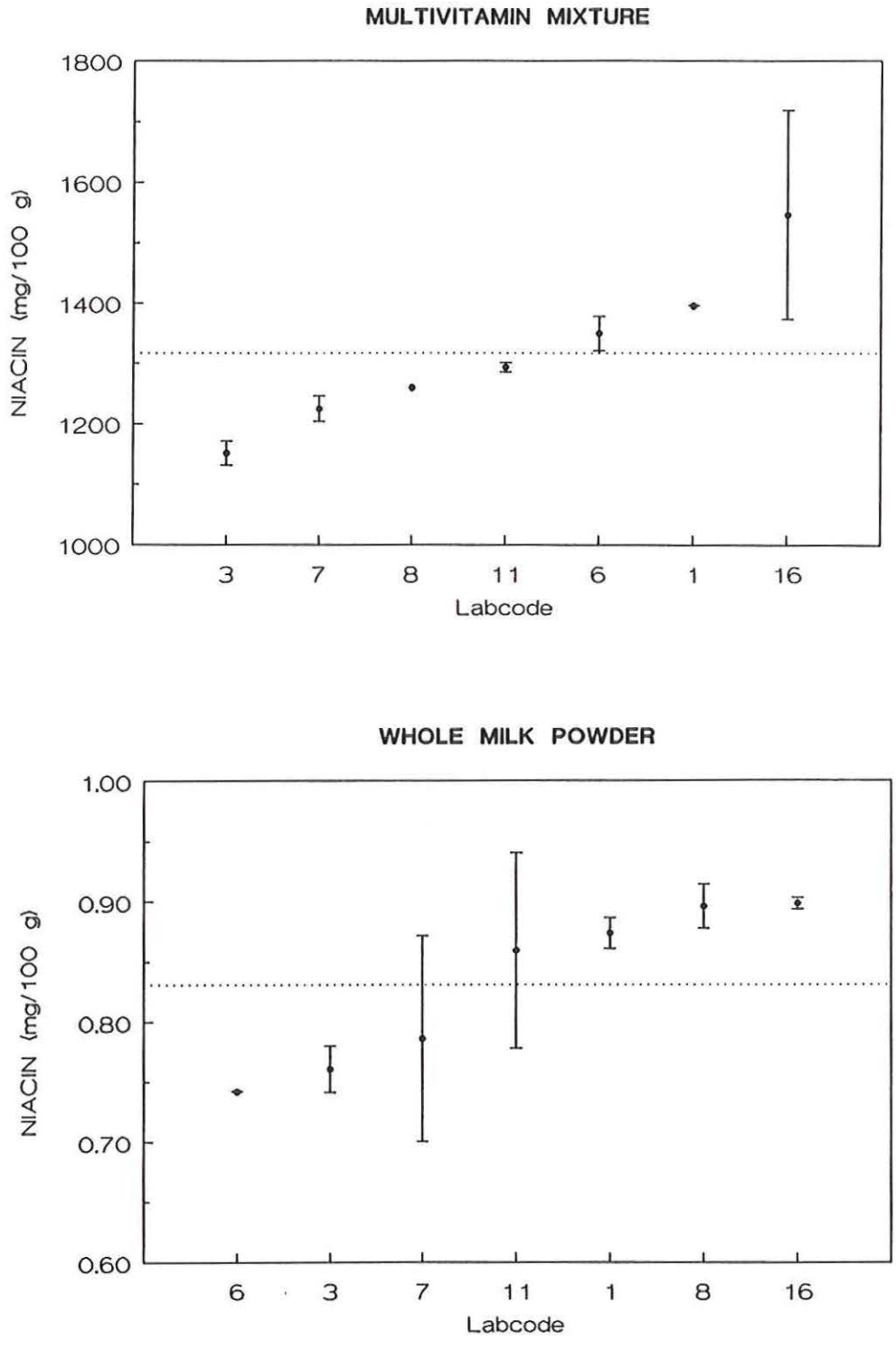
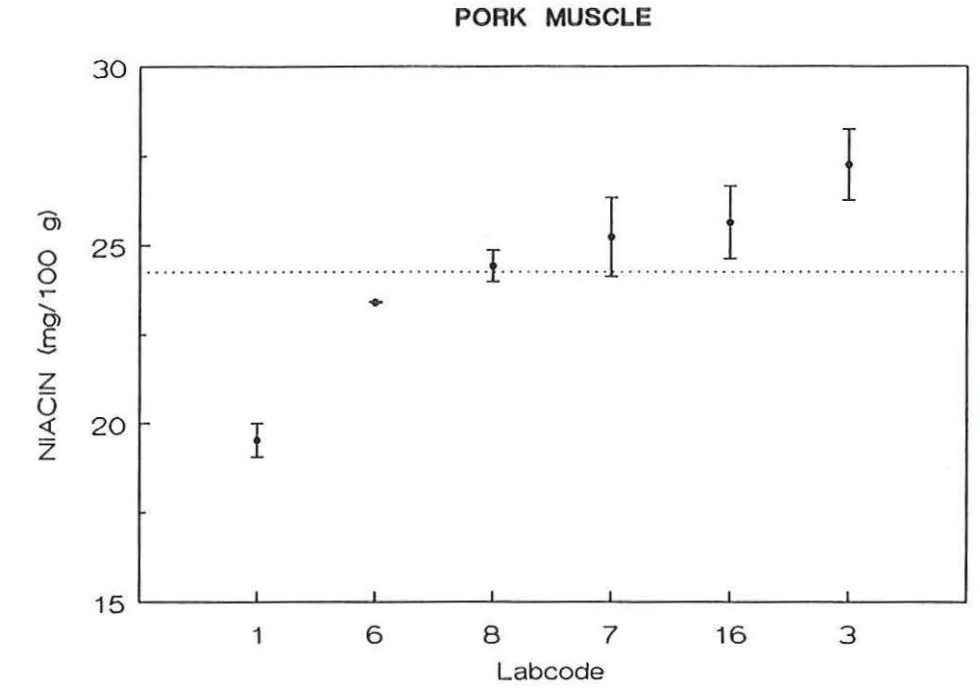


Figure 9. - continued



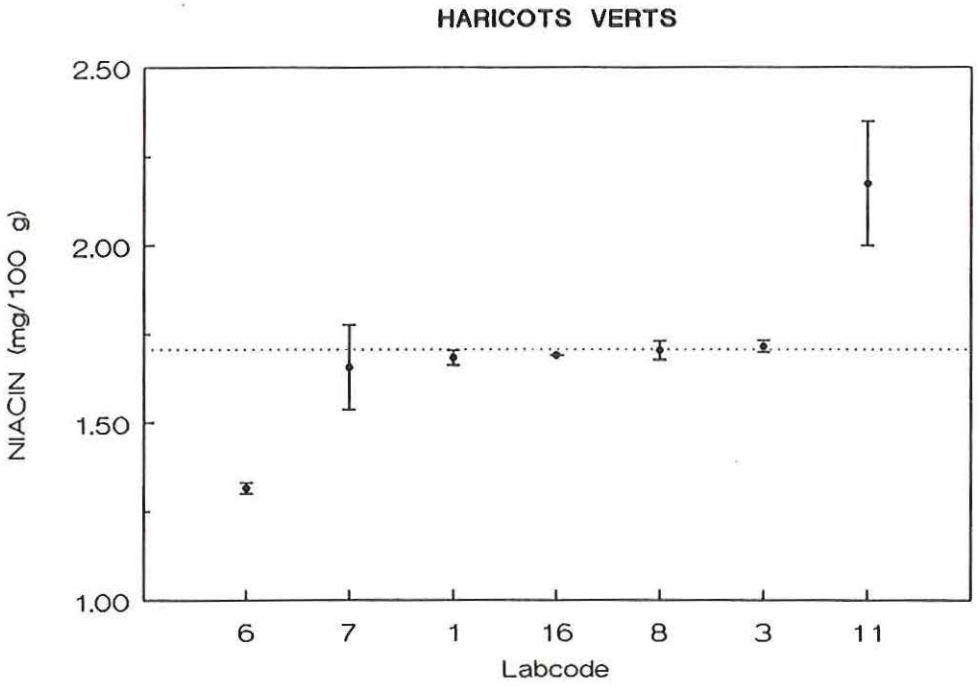


Table 26. Methods used for the determination of NIACIN

Lab-code	Extraction	Principle/Chromatography	Calibration/Calculation
Microbiological methods			
1	sample weight: 2 grams autoclave 15min, 120°C, 1M HCl	Lactobacillus plantarum ATCC 8014 niacin assay medium: Difco # 0322-15-4	niacinamide Sigma by weight (Ref.1,2)
3	sample weight: portion containing 20 ug autoclave 30 min, 121°C, 0.5M H_2SO_4; pH --> 4.5 +test medium, autoclave 10 min, 121°C, + 1 drop inoculum, incubate 72h, 37°C, titrate until pH 6.8 with 0.1M NaOH	Lactobacillus plantarum ATCC 8014 niacin assay medium: Difco # 0322	niacin USP calibration ??
6	2-3 grams autoclave 30 min, 121°C, 1M H_2SO_4	Lactobacillus plantarum ATCC 8014 niacin assay medium: Difco # 0322-15-4	niacin USP calibration ?? (Ref.3)
7	5 grams autoclave 15 min, 121°C acetic acid/acetate/KCN	Lactobacillus plantarum ATCC 8014 assay medium ?? turbidimetric at 650 nm	Niacin USP by weight (Ref.4)
8	10 grams	Lactobacillus plantarum ATCC 8014 assay medium Difco # ??	nicotinamide type ?? calibration ?? (Ref.5)
11	3 grams autoclave 15 min, 120°C, 2M HCl	Lactobacillus plantarum ATCC 8014 niacin assay medium: Difco # 0322-15-4 turbidimetric at 639 nm	nicotinic acid calibration ??
16	1-2 grams autoclave 15 min, 121°C, 1M HCl, -->pH 4.5	Lactobacillus plantarum ATCC 8014 niacin assay medium: Difco # 0322-15	nicotinic acid >99.5% Fluka, by weight (Ref.3)
Reversed Phase HPLC-method			
2	10 - 20 grams autoclave 15 min, 121°C, 0.1M HCl; pH--> 4-4.5 Takadiastase 3 h, 45°C	Spherisorb ODS2 10 um 300x34 0.01M acetate buffer:methanol 90:10, 0.005M tetrabutyl-ammoniumbromide flow: 1.4 ml/min tr: 750 sec detection: UV 254 nm	nicotinic acid Sigma by weight standard treated as sample peak height

References for the determination of niacin

1 Difco Manual dehydrated culture medium and reagents for microbiology 10th Edition, 1984, p.1090
2 Williams, Microbiological assays of the AOAC,14th Edition, 1984, p.862-873
3 Bell J.G., J. Lab. Practice, 23, 5, 1974, p. 235-242 and 252
4 Ph Nord., 1960 (with modifications)
5 Schweizerisches Lebensmittelbuch, Zweiter Band, 1989, Kapitel 62/12.2-12.2.1

4. DISCUSSION

A summary of the results of this intercomparison on methods for the determination of vitamins in foods is given in Table 27. In this intercomparison experienced food laboratories participated. The choice of method was left to the participants subject to the requirements of achieving the best level of accuracy. The reproducibility (CV_{Reprod}) shown gives an impression of the state of the art of vitamin analysis. As reproducibility depends on the concentration of the analyte in the sample, the approach of Horwitz (1990) was followed. In this, the ratio (Table 27) was calculated, with CV_{Reprod} found in this intercomparison as the numerator, and as denominator the achievable CV_{Reprod}. From collaborative studies with uniform methods Horwitz(1982) had derived that the achievable CV_{Reprod} is only a function of concentration, independent of analyte, matrix and method. It represents the analytical variation caused by different laboratories with different operators using different equipment, but using the same well-defined method. In this intercomparison laboratories used different methods. So, when different procedures used by different laboratories do not have a strong influence on the results, the ratio will be close to 1. This proved to be true for the analysis of retinol and β-carotene in milk powder, α-tocopherol in milk powder and haricots verts, vitamin B_1 in all of the food samples, vitamin C in haricots verts, and niacin in the three food samples. Results agreed well, mostly after excluding one or two laboratories because of inadequate methods used. One of these laboratories had little experience in the analysis of vitamins in foods, but was specialised in vitamin analysis in body fluids and tissues. The other laboratory had chosen only UV-detection, which proved to be inadequate for the water-soluble vitamins B_1, B_2, and niacin. Indicative values of retinol, β-carotene, α-tocopherol, vitamin B_1, vitamin C, and niacin issued in these foods will be helpful for food analysts.

For future intercomparisons, calibration of vitamin standards as a source of variation between laboratories has to be studied. The present intercomparison failed to identify the role of different calibration procedures, because of the extraction procedures prescribed for the multivitamin mixture.

High performance liquid chromatography was the method of choice of most of the participants for the determination of fat-soluble vitamins. Future intercomparisons for retinol need to take into account all-trans and 13-cis retinol, because of the different response depending on detection. As was evident from the results of β-carotene in haricots verts, the traditional methods based on open column chromatography proved to be biased. In the evaluation of results of α-tocopherol, calibration procedures as a source of variation was emphasised.

Only 8 out of 12 laboratories applied HPLC methods for vitamin B_1. However, extrac-

tion/hydrolysis procedures probably were the most important source of variation between laboratories. Three laboratories used a microbiological assay for the determination of vitamin B_2, as opposed to 10 participants using HPLC. Microbiological results did not agree very well. The large differences between all laboratories analysing vitamin B_2 was caused by inadequate extraction/hydrolysis procedures. Results for vitamin B_6 were discrepant. Identification of the different B_6 vitamers and extraction/ hydrolysis procedures were judged to be major sources of variation. Optimisation of extraction/hydrolysis procedures for vitamin B_1, B_2, and B_6 will be an important step in the improvement of methods for the determination of these vitamins.

Results for vitamin C agreed well, as long as HPLC and fluorometric methods were used. As expected, the indophenol titration proved to give extreme high results. The similarity in values reported for niacin, probably was caused by all laboratories using a microbiological assay with an identical microorganism.

Determination of vitamin D_3, folic acid, and vitamin B_{12} proves to be very difficult. Only a few of these laboratories specialised in vitamin analysis were able to perform the analyses. For each of these vitamins focused attention is needed, by comparing different procedures. Laboratories specialised in the analysis of these vitamins have to be invited.

Table 27. Summary of the differences between laboratories, expressed as CV_{Reprod}, found in this intercomparison.

	Multivitamin Mixture		Milk Powder		Pork Muscle		Haricots Verts	
	CVR (%)	ratio	CVR (%)	ratio	CVR (%)	ratio	CVR (%)	ratio
Retinol	24	5.1	10	0.7	-	-	-	-
ß-Carotene	35	6.9	14	0.9	-	-	52	3.7
Vitamin D_3	39	4.0	-	-	-	-	-	-
α-Tocopherol	19	5.9	16	1.3	-	-	15	1.2
Vitamin B_1	8.8	1.8	14	0.7	9.9	1.0	17	1.2
Vitamin B_2	7.9	1.8	28	2.6	74	5.9	35	2.6
Vitamin B_6	8.8	1.8	18	1.3	35	3.2	51	3.5
Folic acid	not sufficient data							
Vitamin B_{12}	not sufficient data							
Vitamin C	8.9	3.4	-	-	-	-	9.8	1.3
Niacin	10	2.6	9.2	0.8	11	1.5	15	1.5

Key
CVR = CV_{Reprod}
ratio = CV_{Reprod} / Achievable CV_{Reprod}

5. REFERENCES

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6. DATA FOR INDIVIDUAL LABORATORIES

Table 28. Results of individual laboratories for DRY WEIGHT (g/100 g)

WHOLE MILK POWDER

Lab	Results					Mean	St Dev
1	97.07	97.48	97.52			97.357	0.249
2	99.8	100.0				99.900	0.141
3	97.733	97.798	97.553			97.695	0.127
5	97.73	97.70				97.715	0.021
6	98.0	97.9	97.9			97.933	0.058
7	97.7	97.1	97.1			97.300	0.346
8	98.8					98.800	
9	96.95	97.12	96.99			97.020	0.089
10	97.7	98.0	97.6			97.767	0.208
11	97.86	97.66	97.68			97.733	0.110
12	97.9	97.9	97.6	97.6		97.750	0.173
14	97.9	97.8	97.7	97.7	97.8	97.780	0.084
15	97.68	97.70	97.66	97.70	97.68	97.684	0.017
16	98.09	97.99	97.88			97.987	0.105
17	97.7	97.9	97.7	97.8		97.775	0.096

PORK MUSCLE

Lab	Results				Mean	St Dev
1	96.91	96.79	97.11		96.937	0.162
2	98.7	98.3			98.500	0.283
3	97.158	97.171	97.376		97.235	0.122
5	97.12	97.20			97.160	0.057
6	97.6	97.5	97.3		97.467	0.153
7	96.88	96.94	96.90		96.907	0.031
8	98.0				98.000	
9	94.81	96.20	96.34		95.783	0.846
10	97.2	97.1	97.1		97.133	0.058
11	97.24	97.40	97.30		97.313	0.081
12	96.1	96.1	96.5	95.8	96.125	0.287
15	97.91	97.89	97.86		97.887	0.025
16	97.71	97.65	97.72		97.693	0.038
17	97.5	97.3	97.9	97.8	97.625	0.275

Table 28. - continued

HARICOTS VERTS

Lab	Results					Mean	St Dev
1	95.95	96.08	96.10			96.043	0.081
2	98.3	98.6				98.450	0.212
3	95.758	95.310	95.599			95.556	0.227
5	95.57	95.59				95.580	0.014
6	96.3	95.9	96.2			96.133	0.208
7	95.6	95.8	95.3			95.567	0.252
8	96.4					96.400	
9	94.41	94.73	94.13			94.423	0.300
10	96.0	96.2	96.2			96.133	0.115
11	96.18	96.10	95.92			96.067	0.133
12	97.4	97.8	97.3	97.4		97.475	0.222
13	95.22	95.78	96.23			95.743	0.506
14	95.9	95.8	95.8	95.8	95.8	95.820	0.045
15	96.25	96.28	96.26			96.263	0.015
16	96.42	96.43	96.01			96.287	0.240
17	95.8	96.0	95.9	95.6		95.825	0.171

Table 29. Results of individual laboratories for RETINOL (mg /100 g dry weight)

MULTIVITAMIN MIXTURE

Lab	Results					Mean	St Dev
1	329.0	333.7				331.350	3.323
3	196.205	229.506				212.855	23.547
4	469	504	469	489		482.750	17.017
6	291	298				294.500	4.950
7	345	327				336.000	12.728
8	309					309.000	
9	245	200				222.500	31.820
10	347	351				349.000	2.828
11	335	342				338.500	4.950
14	283.0	295.0	342.0			306.667	31.182
15	304	312				308.000	5.657
16	358	425	279	374		359.000	60.503
17	292	314	313	322		310.250	12.816

WHOLE MILK POWDER

Lab	Results					Mean	St Dev
1	0.277	0.278	0.272			0.276	0.003
3	0.291	0.242	0.292			0.275	0.029
4	0.314	0.289	0.323	0.239	0.260	0.285	0.036
6	0.220	0.226	0.226			0.224	0.003
7	0.282	0.287	0.258			0.276	0.016
8	0.279	0.287	0.278	0.286	0.277	0.281	0.005
9	0.108	0.100	0.115	0.100		0.106	0.007
10	0.255	0.275	0.265			0.265	0.010
11	0.281	0.307	0.291			0.293	0.013
14	0.271	0.289	0.289			0.283	0.010
15	0.223	0.229	0.215			0.222	0.007
16	0.269	0.267	0.259			0.265	0.005
17	0.258	0.240	0.268			0.255	0.014

Table 30. Results of individual laboratories for β-CAROTENE (mg /100g dry weight)

MULTIVITAMIN MIXTURE

Lab	Results			Mean	St Dev
1	203.4	203.7		203.550	0.212
2	52.78	48.18		50.480	3.253
3	224.2	213.9		219.050	7.283
7	208	205		206.500	2.121
8	156			156.000	
11	235.3	248.5		241.900	9.334
13	159.55			159.550	
14	201.0	163.0		182.000	26.870
15	237.7	240.2		238.950	1.768
16	302	260	291	284.333	21.779

WHOLE MILK POWDER

Lab	Results			Mean	St Dev
1	0.109	0.120	0.093	0.107	0.014
3	0.104	0.104	0.087	0.098	
7	0.124	0.131	0.118	0.124	0.007
11	0.143	0.156	0.140	0.146	0.009
14	0.107	0.112	0.117	0.112	0.005
15	0.124	0.128	0.127	0.126	0.002
16	0.122	0.124	0.106	0.117	0.010

Table 30. - continued

HARICOTS VERTS

Lab	Results					Mean	St Dev
1	0.316	0.316	0.313			0.315	0.002
2	0.1143	0.1438				0.129	0.021
3	0.063	0.067	0.060			0.063	
8	0.19	0.17	0.18	0.19	0.16	0.178	0.013
11	0.372	0.363	0.372			0.369	0.005
13	0.113	0.101	0.09			0.101	0.012
14	0.214	0.172	0.230			0.205	0.030
15	0.237	0.242	0.249			0.243	0.006
16	0.379	0.435	0.380			0.398	0.032

Table 31. Results of individual laboratories for VITAMIN D_3 (ug cholecalciferol/100 g dry weight)

MULTIVITAMIN MIXTURE

Lab	Results			Mean	St Dev
1	3010	3108		3059	69.296
9	748	1150		949	284.257
15	2804			2804	
16	3420			3420	
17	3135	3134	3106	3125	16.462

Table 31. - continued

MILK POWDER

Lab	Results				Mean	St Dev
1	0.154	0.128	0.154		0.145	0.015
15	0.36	0.25	0.24	0.23	0.270	0.061
17	<0.1	<0.1	<0.1			

Table 32. Results of individual laboratories for α-TOCOPHEROL (mg /100 g dry weight)

MULTIVITAMIN MIXTURE

Lab	Results				Mean	St Dev
1	5087	5359			5223.0	192.3
3	5241.845	5363.724			5302.8	86.2
4	7120	6680	7040	6640	6870.0	245.2
6	31300	42400			36850	7848.9
7	4690	4600			4645.0	63.6
8	4799				4799.0	
9	3218	3717			3467.5	352.8
10	4469	4668			4568.5	140.7
11	5248	5235			5241.5	9.2
13	5117.67				5117.7	
14	4475	3810			4142.5	470.2
15	4440	4336			4388.0	73.5
16	5340	5160	5280		5260.0	91.7
17	4413	4466	4650	4716	4561.3	144.8

Table 32. - continued

MILK POWDER

Lab	Results					Mean	St Dev
1	0.813	0.756	0.745			0.771	0.037
3	0.513	0.459	0.555			0.509	0.048
4	0.993	0.937	0.767	0.796	0.840	0.867	0.096
6	0.563	0.554	0.553			0.557	0.006
7	0.516	0.549	0.491			0.519	0.029
8	0.62	0.60	0.56	0.61	0.59	0.596	0.023
9	0.523	0.529	0.585	0.674		0.578	0.070
10	0.505	1.075	0.77			0.783	0.285
11	0.584	0.646	0.554			0.595	0.047
14	0.825	0.724	0.693			0.747	0.069
15	0.588	0.574	0.564			0.575	0.012
16	0.652	0.715	0.684			0.684	0.032
17	0.468	0.496	0.545			0.503	0.039

HARICOTS VERTS

Lab	Results				Mean	St Dev
1	0.455	0.384	0.380		0.406	0.042
3	0.350	0.377	0.319		0.349	0.029
6	0.388	0.373	0.344		0.368	0.022
7	0.317	0.341	0.324		0.327	0.012
9	0.363	0.359	0.351	0.352	0.356	0.006
10	0.405	0.585	0.680		0.557	0.140
11	0.259	0.237	0.258		0.251	0.012
13	0.355	0.238	0.293		0.295	0.059
14	1.096	3.569	2.786			
15	0.359	0.371	0.347		0.359	0.012
16	0.604	0.363	0.342		0.436	0.146
17	0.288	0.276	0.282		0.282	0.006

Table 33. Results of individual laboratories for VITAMIN B_1 (mg thiamin chloride.HCl/100 g dry weight)

MULTIVITAMIN MIXTURE

Lab	Results					Mean	St Dev
1	288	293				290.50	3.54
2	299					299.00	
3	321	319				320.00	1.41
5	300	316	300			305.33	9.24
7	298	297				297.50	0.71
8	333					333.00	
9	216.5	244.9				230.70	20.08
11	294.3	296.2				295.25	1.34
12	325	329	298	298		312.50	16.82
15	289.5	302.8				296.15	9.41
16	345	320				332.50	17.68
17	292.5	294.0	292.7			293.07	0.81

WHOLE MILK POWDER

Lab	Results					Mean	St Dev
1	0.315	0.294	0.284			0.298	0.016
2	0.17	0.20				0.185	0.021
3	0.281	0.313	0.310			0.301	0.018
5	0.23	0.22	0.23			0.227	0.006
7	0.349	0.344	0.332			0.342	0.009
9	0.152	0.163	0.155			0.157	0.006
11	0.355	0.369	0.374			0.366	0.010
12	0.264	0.264	0.271	0.268	0.227	0.259	0.018
15	0.305	0.315	0.291			0.304	0.012
16	0.383	0.374	0.398			0.385	0.012
17	0.321	0.313	0.281			0.305	0.021

Table 33. - continued

PORK MUSCLE

Lab	Results					Mean	St Dev
1	2.81	2.78	2.75			2.780	0.030
2	1.55	1.63				1.590	0.057
3	2.981	2.948	2.920			2.950	0.031
5	2.69	2.55	2.66			2.633	0.074
7	3.15	3.16	3.12			3.143	0.021
8	3.70	3.62	3.42	3.87	3.52	3.626	0.172
9	2.27	2.35	2.13			2.250	0.111
11	2.93	2.87	2.80			2.867	0.065
12	2.86	3.31	3.26	3.03	3.13	3.118	0.181
15	3.040	2.989	2.845			2.958	0.101
16	3.645	3.471	3.422			3.513	0.117
17	3.17	3.14	3.23			3.180	0.046

HARICOTS VERTS

Lab	Results					Mean	St Dev
1	0.218	0.210	0.216			0.215	0.004
2	0.09	0.09				0.090	0.000
3	0.217	0.227	0.207			0.217	0.010
5	0.17	0.15	0.16			0.160	0.010
7	0.192	0.196	0.189			0.192	0.004
8	0.18	0.20	0.21	0.19	0.20	0.196	0.011
9	0.0118	0.0187	0.0167			0.016	0.004
11	0.198	0.201	0.201			0.200	0.002
12	0.234	0.215	0.227	0.210	0.233	0.224	0.011
15	0.278	0.303	0.300			0.294	0.014
16	0.263	0.269	0.262			0.265	0.004
17	0.178	0.188	0.157			0.174	0.016

Table 34. Results of individual laboratories for VITAMIN B_2 (mg riboflavin/100 g dry weight)

MULTIVITAMIN MIXTURE

Lab	Results					Mean	St Dev
1	582	574				578.00	5.66
2	599					599.00	
3	585.928	571.100				578.51	10.49
4	575	605	622			600.67	23.80
5	542	558	536			545.33	11.37
7	620	480				550.00	99.00
8	566					566.00	
9	625.3	602.9				614.10	15.84
11	608.2	617.7				612.95	6.72
12	547.7	579.6	571.8	539.1		559.55	19.24
15	645.0	618.0	656.4			639.80	19.72
16	577	597				587.00	14.14
17	518	520	508	475		505.25	20.84

WHOLE MILK POWDER

Lab	Results					Mean	St Dev
1	1.32	1.24	1.16			1.240	0.080
2	0.79	0.80				0.795	0.007
3	1.362	1.396	1.399			1.386	0.021
4	1.15	1.26	1.30	1.21	1.70	1.324	0.218
5	1.32	1.33	1.25			1.300	0.044
7	1.38	1.28	1.54			1.400	0.131
8	1.19	1.20	1.15	1.20	1.16	1.180	0.023
9	1.21	0.87	1.07			1.050	0.171
11	1.737	1.707	1.760			1.735	0.027
12	1.40	1.45	1.34	1.37	1.34	1.380	0.046
15	1.970	1.948	1.903			1.940	0.034
16	2.559	2.491	2.556			2.535	0.038
17	1.46	1.48	1.45			1.463	0.015

Table 34. - continued

PORK MUSCLE

Lab	Results					Mean	St Dev
1	0.424	0.440	0.455			0.440	0.016
3	0.241	0.201	0.263			0.235	0.031
5	0.09	0.08	0.08			0.083	0.006
7	0.688	0.654	0.692			0.678	0.021
8	0.82	0.80	0.79	0.77	0.75	0.786	0.027
9	0.0943	0.0943	0.1020			0.097	0.004
11	0.801	0.822	0.804			0.809	0.011
12	0.112	0.113	0.133	0.120	0.121	0.120	0.008
15	0.881	0.899	0.842			0.874	0.029
16	1.116	1.169	1.175			1.153	0.032
17	0.248	0.260	0.234			0.247	0.013

HARICOTS VERTS

Lab	Results					Mean	St Dev
1	0.241	0.265	0.240			0.249	0.014
2	0.17	0.18				0.175	0.007
3	0.251	0.234	0.240			0.242	0.009
5	0.22	0.22	0.21			0.217	0.006
7	0.353	0.334	0.295			0.327	0.030
8	0.39	0.39	0.36	0.39	0.36	0.378	0.016
9	0.230	0.253	0.221			0.235	0.017
11	0.380	0.410	0.404			0.398	0.016
12	0.273	0.248	0.247	0.235	0.244	0.249	0.014
15	0.437	0.458	0.502			0.466	0.033
16	0.542	0.547	0.604			0.564	0.034
17	0.317	0.312	0.313			0.314	0.003

Table 35. Results of individual laboratories for TOTAL VITAMIN B_6 (mg pyridoxine/100 g dry weight)

MULTIVITAMIN MIXTURE

Lab	Results					Mean	St Dev
1	254.5	235.7				245.10	13.29
8	240					240.00	
9	276.5	260.5				268.50	11.31
11	223.9	230.6				227.25	4.74
12	288	273	283	287		282.75	6.85
15	257.5	287.8				272.65	21.43
16	273	272.7				272.85	0.21
18	245.4	236.6				241.00	6.22

WHOLE MILK POWDER

Lab	Results					Mean	St Dev
1	0.382	0.397	0.358			0.379	0.020
9	0.0658	0.0687	0.0646			0.066	0.002
11	0.357	0.357	0.353			0.356	0.002
12	0.278	0.251	0.270	0.333	0.311	0.289	0.033
15	0.376	0.368	0.390			0.378	0.011
16	0.333	0.396	0.414			0.381	0.043
18	0.2469	0.2334	0.2668	0.2598			

Table 35. - continued

PORK MUSCLE

Lab	Results					Mean	St Dev
1	1.38	1.35	1.41			1.380	0.030
8	0.94	0.96	0.95	0.94	0.96	0.950	0.010
9	0.139	0.135	0.199			0.158	0.036
11	1.837	1.837	1.860			1.845	0.013
12	0.755	0.700	0.778	0.726	0.740	0.740	0.029
15	1.622	1.665	1.62			1.636	0.025
16	1.524	1.948	1.734			1.735	0.212
18	0.89	0.90	0.88	0.87		0.885	0.013

HARICOTS VERTS

Lab	Results					Mean	St Dev
1	0.168	0.158	0.153			0.160	0.008
9	0.0423	0.0407	0.0467			0.043	0.003
11	0.240	0.240	0.239			0.240	0.001
12	0.070	0.060	0.055	0.058	0.080	0.065	0.010
15	0.225	0.217	0.216			0.219	0.005
16	0.299	0.323	0.316			0.313	0.012

Table 36. Results of individual laboratories for FOLIC ACID (mg /100 g dry weight)

MULTIVITAMIN MIXTURE

Lab	Results		Mean	St Dev
1	80.3	85.7	83.00	5.4

WHOLE MILK POWDER

Lab	Results			Mean	St Dev
1	0.0342	0.0328	0.0341	0.0337	0.0008

HARICOTS VERTS

Lab	Results			Mean	St Dev
1	0.115	0.113	0.113	0.114	0.0012

Table 37. Results of individual laboratories for VITAMIN B_{12} (ug cyanocobalamin/100 g dry weight)

MULTIVITAMIN MIXTURE

Lab	Results			Mean	St Dev
1	970	960		965.0	7.1
7	1040	930		985.0	77.8
16	809	866		837.5	40.3

WHOLE MILK POWDER

Lab	Results			Mean	St Dev
1	3.27	3.06	3.15	3.160	0.105
7	2.71	2.25	2.74	2.567	0.275
16	4.05	4.08	4.03	4.053	0.025

PORK MUSCLE

Lab	Results			Mean	St Dev
1	1.85	1.80	1.82	1.823	0.025
7	1.58	1.60	1.62	1.600	0.020
16	3.86	3.41	4.38	3.883	0.485

Table 38. Results of individual laboratories for VITAMIN C (mg /100g dry weight)

MULTIVITAMIN MIXTURE

Lab	Results					Mean	St Dev
1	23300	22900				23100.0	282.8
2	20439					20439.0	
3	20584.8	19233.9				19909.4	955.2
7	20300	20200				20250.0	70.7
9	17807	18670				18238.5	610.2
10	21700	21600				21650.0	70.7
11	19860	20418				20139.0	394.6
12	18670	18240	17450	20520		18720.0	1302.0
14	19100	19900				19500.0	565.7
15	19490	19190				19340.0	212.1
16	16480	16940				16710.0	325.3

HARICOTS VERTS

Lab	Results					Mean	St Dev
1	17.4	16.9	17.8			17.367	0.451
2	19.81	18.89	22.66			20.453	1.966
3	14.848	13.664	14.404			14.305	0.598
7	14					14.000	
9	343.6	386.1	338.4	394.4		365.625	28.714
10	14.0	16.2	16.1			15.433	1.242
11	15.76	15.7	15.55			15.670	0.108
12	13.3	12.1	12.7	14.2	12.4	12.940	0.832
14	15.94	16.15	15.80	16.43		16.080	0.274
15	15.70	15.70	15.50			15.633	0.115
16	14.35	13.98	14.23			14.187	0.189

Table 39. Results of individual laboratories for NIACIN (mg niacinamide/100 g dry weight)

MULTIVITAMIN MIXTURE

Lab	Results		Mean	St Dev
1	1398.4	1392.9	1395.65	3.89
3	1165.37	1137.39	1151.38	19.79
6	1370	1330	1350.00	28.28
7	1240	1210	1225.00	21.21
8	1260		1260.00	
11	1299.2	1288.4	1293.80	7.64
16	1668	1424	1546.00	172.53

WHOLE MILK POWDER

Lab	Results					Mean	St Dev
1	0.883	0.879	0.859			0.874	0.013
3	0.750	0.783	0.749			0.761	0.019
6	0.740	0.743	0.743			0.742	0.002
7	0.810	0.691	0.857			0.786	0.086
8	0.90	0.92	0.90	0.89	0.87	0.896	0.018
11	0.782	0.852	0.944			0.859	0.081
16	0.900	0.893	0.902			0.898	0.005

Table 39. - continued

PORK MUSCLE

Lab	Results					Mean	St Dev
1	19.0	19.7	19.9			19.533	0.473
2	10.84	11.26				11.050	0.297
3	26.757	28.400	26.610			27.256	0.994
6	23.5	23.3	23.4			23.400	0.100
7	24.2	25.1	26.4			25.233	1.106
8	24.8	24.6	24.8	23.9	24.0	24.420	0.438
16	25.267	24.862	26.793			25.641	1.018

HARICOTS VERTS

Lab	Results					Mean	St Dev
1	1.69	1.66	1.70			1.683	0.021
2	4.66	4.39				4.525	0.191
3	1.723	1.697	1.726			1.715	0.016
6	1.32	1.33	1.30			1.317	0.015
7	1.62	1.56	1.79			1.657	0.119
8	1.72	1.74	1.70	1.67	1.69	1.704	0.027
11	2.22	1.98	2.32			2.173	0.175
16	1.691	1.686	1.693			1.690	0.004

7. APPENDICES

APPENDIX 1

LIST OF PARTICIPANTS

Participant	Laboratory	City
Fernandez-Martin, F., Vidal, C.	Instituto del Frio, CSIC Instituto del Fermentaciones Industriales, CSIC	Madrid, Spain
Bitsch, R.	Universität Gesamthoch-schule Paderborn	Paderborn, Federal Republic of Germany
Lumley, I.	Laboratory of the Government Chemist	Teddington, United Kingdom
Leth, T., Jakobsen, J.	The National Food Agency of Denmark	Soborg, Denmark
Walter, P., Brawand,F.	Schweizerisches Vitaminin-Institut	Basel,Switzerland
Hollman, P.	RIKILT	Wageningen,The Netherlands
Favell, D.	Unilever Research	Bedford, United Kingdom
Mustranta, A.	VTT Food Research Laboratory	Espoo, Finland

LIST OF PARTICIPANTS - continued*

Participant	Laboratory	City
Schrijver, J., Van den Berg, H.	TNO-CIVO Institutes	Zeist, The Netherlands
Bognar, A.	Bundesforschungsanstalt für Ernährung	Stuttgart,Federal Republic of Germany
?	Servicio de Nutricion	Madrid, Spain
Palmer, R.	Leatherhead Food R.A.	Leatherhead,United Kingdom
Sheehy, T.	University College Cork	Cork,Ireland
Bilic, N.	Federal Dairy Research Institut	Liebefeld-Bern, Switzerland
Halen, B.	Swedish National Food Administration	Uppsala,Sweden
Bourgeois, C.	Produits Roche	Fontenay sousBois, France
Roomans, H.	Rijkskeuringsdienst van Waren	Maastricht, The Netherlands
Southgate, D., Finglas, P.	AFRC Institute of Food Research	Norwich, United Kingdom

* The order of the laboratories does not correspond to the laboratory numbers used in the text and figures.

APPENDIX 2

BCR REFERENCE MATERIALS FOR NUTRITIONAL ANALYSIS

First Intercomparison of Methods for Vitamins * Autumn 1989

Instructions and Reporting Forms

OBJECTIVE

The objective of this first study is to identify any problems which will have to be overcome before certification of a series of "food-matrix"/ reference materials for vitamins can be undertaken.

Participants are therefore asked to determine these vitamins in the preliminary materials provided (whole milk powder, lean pork muscle, haricots verts beans, and a multivitamin mixture) as accurately as possibly using the method that they consider most reliably in their hands.

As vitamin standards used as calibrants can be an important source of variation, errors originating from differences in vitamin standards will be estimated with the help of a Multivitamin Reference Mixture.

PROTOCOL

Please pay particular attention to:

- calibration of equipment and methods, and control of calibrants;
- ensuring that at least three separate determinations of each vitamin in each sample are made;
- reporting of results according to the specified units and the required number of

significant figures;
- provision of essential details of methods used, and where relevant, provision of representative chromatograms;
- requests for comments on suitability of methods for certification.

1. Materials provided

- 300 g (3 sachets) of Whole Milk Powder in a nitrogen flushed, laminate sachet in the form of a homogenized powder (spray-dried);
- 300 g (3 sachets) of Haricots Verts Beans in a nitrogen flushed, laminate sachet in the form of a homogenized powder (lyophilized);
- 150 g (3 sachets) of Lean Pork Muscle in a nitrogen flushed, laminate sachet in the form of a homogenized powder (lyophilized);
- 50 g (2 units) "BCR Multivitamin Reference Mixture", of known composition (see Appendix 3).

N.B.:Store materials in a refrigerator at 4°C until use. ALLOW SAMPLES TO WARM TO ROOM TEMPERATURE BEFORE OPENING AND SAMPLING. If sachets have been opened, provide an airtight packing before restorage.

2. Vitamins to be determined (where applicable to the material)

START with determination of the most LABILE vitamins (vitamin C, A, and B_2)

-vitamin A
-β-carotene
-vitamin E
-vitamin D_3
-vitamin B_1
-vitamin B_2
-vitamin B_6
-vitamin B_{12}
-vitamin C
-folic acid
-niacin

To ensure comparability of results, the loss of mass on drying at

atmospheric pressure for 4 hours at 103 ± 2°C is determined.

3. Methods of Analysis

The choice of method is left to the participant subject to the requirements of achieving the best level of accuracy and control of calibrants and calibration. Guidelines for the preparation of stock solutions of the BCR Multivitamin Reference Mixture are given in Appendix 4. To get the full benefit of this Mixture, please follow these guidelines closely.

4. Number of Determinations

- Food samples
  Although the samples have been shown to be homogeneous in one laboratory with respect to major nutrients and selected vitamins, this aspect must be further studied. Therefore, at least three separate determinations on three separately weighed sub-samples taken from at least two sachets will be made for each sample and vitamin.

- BCR Multivitamin Reference Mixture
  At least two separate determinations on two separately weighed sub-samples taken from two units will be made for each vitamin.

5. Reporting of Results

Results and details of the methods used should be reported on the reporting sheets provided.
Please ensure that results are clearly given, preferably typed.

- Results shall be expressed

  -on a dry-weight basis as determined by drying at atmospheric pressure for 4 hours at 103 ± 2°C.
  -in the units indicated on the reporting sheets, with and without a CORRECTION for the level found in the BCR Multivitamin Reference Mixture compared with the

theoretical level.

- For purposes of calculation only, please report results to at least 3 significant figures (more if necessary to avoid giving a series of identical values).

- Where relevant, copies of clearly labelled representative chromatograms should be given.

6. Reporting Date

Please return your results to:

P.J. Wagstaffe

BCR

DG XII

Commission of the European Communities

200 Rue de la Loi

B-1049 Brussels

by 21st November 1989.

APPENDIX 3

COMPOSITION OF BCR MULTIVITAMIN REFERENCE MIXTURE

- The Mixture contains vitamins A, E, D_3 and β-carotene in the form of beadlets made of gelatine, starch and sucrose.
- Vitamin B_{12} is adsorbed on dextrins.
- Other vitamins are added as the pure substances as specified beneath.

VITAMIN	THEORETICAL LEVEL (mg/ g)
Vitamin A: retinylacetate (M=328.5) 11000 IU =	3.784
β-carotene (M=536.8)	2
Vitamin E: α-tocopherolacetate (M= 472.7)	50
Vitamin D_3: cholecalciferol (M=384.6) 1300 IU =	0.0325
Vitamin B_1: thiamin chloride.HCl (M=337.3)	23
Vitamin B_2: riboflavin (M=376.4)	6
Vitamin B_6: pyridoxine.HCl (M=205.6)	3
Vitamin B_{12}: cyanocobalamin (M=1355)	0.008
Vitamin C: ascorbic acid (M=176.1)	200
Folic acid: pteroylmonoglutamic acid (M=441.4)	0.8
Niacin: nicotinamide (M=122.1)	13
Vitamin K_1	0.3
Biotin	0.10
Lactose	QSP

APPENDIX 4

GUIDELINES FOR THE PREPARATION OF STOCK SOLUTIONS OF THE BCR MULTIVITAMIN REFERENCE MIXTURE

Vitamin standards can be an important source of variation. Errors originating from differences in vitamin standards will be estimated with the help of the BCR Multivitamin Reference Mixture. To get the full benefit of this Mixture, it is necessary that all participants follow these guidelines closely. To ensure a representative sample of the BCR Multivitamin Reference Mixture a minimum sample weight for each vitamin is prescribed. It should be borne in mind that all work has to be done under the normal well-known conditions for the assay of vitamins in general, such as excluding daylight etc.

1 STOCK SOLUTIONS FOR THE FAT SOLUBLE VITAMINS

The procedure for the preparation of the stock solutions of vitamin A, vitamin D_3, vitamin E and β-carotene is the same (see below). These vitamins are present as powdered beadlets made of gelatin, starch and sucrose.

Theoretical level for one gram of the BCR Multivitamin Reference Mixture:

vitamin A (retinylacetate)	$11*10^3$ IU = 3.784 mg acetate
vitamin D3	1300 IU = 0.0325 mg cholecalciferol
α-tocopherylacetate	50 mg
β-carotene	2 mg

Weigh accurately at least one gram of the BCR Multivitamin Reference Mixture and transfer into a 500 ml volumetric flask. Add 50.0 ml of 0.02 mol/l hydrochloric acid, 100 mg pepsin (Merck cat. 7197), 200 mg trypsin (Merck cat. 8214) and heat to 50°C for 15 minutes with occasional agitation. Then immerse the flask in the ultrasonic bath for 3 minutes, add 400 ml absolute ethanol, cool to 20°C, again immerse in the ultrasonic bath for 3 minutes and fill up to the mark with absolute ethanol.

If necessary, centrifuge or filter through a 0.8 micron Millipore filter a part of this stock solution. Assay further as a standard solution for each vitamin by making an appropriate dilution.

N.B. Vitamin A and β-carotene are sensitive to acids, therefore it is necessary to carry out the determinations of vitamin A and β-carotene immediately after preparation of the stock solution.

It should be borne in mind that saponification of the stock solution may be necessary for vitamin A and vitamin E, depending on the method used for analysis of the samples.

2 STOCK SOLUTIONS FOR WATER SOLUBLE VITAMINS

It is allowed to use one stock solution for different vitamins, if the procedures for the individual vitamins are identical (see below). It should also be borne in mind that the vitamins in the stock solution have unequal stabilities.

Vitamin B_1

Theoretical level of thiaminchloride.HCl in the BCR Multivitamin Reference Mixture: 3 mg/g.

Weigh accurately at least one gram BCR Multivitamin Reference Mixture and transfer into a 500 ml volumetric flask. Add 1.0 g Titriplex III, 25 ml methanol and immerse in the ultrasonic bath for 5 minutes. Add 400 ml 0.01 mol/l hydrochloric acid and immerse again for 10 minutes in the ultrasonic bath. Shake for 5 minutes and fill up to the mark with 0.01 mol/l hydrochloric acid.

If necessary, centrifuge or filter through a 0.8 micron Millipore filter a part of this stock solution. Assay as standard solution by making an appropriate dilution.

Vitamin B_2

Theoretical level of riboflavin in the BCR Multivitamin Reference Mixture: 6 mg/g.

Weigh accurately at least one gram BCR Multivitamin Reference Mixture and transfer into a 500 ml volumetric flask. Add 450 ml 0.01 mol/l hydrochloric acid heated till 50-60°C. Immerse the flask for 5 minutes in the ultrasonic bath. Cool to 20°C and fill up to the mark with 0.01 mol/l hydrochloric acid.

If necessary, centrifuge or filter through a 0.8 micron Millipore filter a part of this stock solution. Assay as standard solution by making an appropriate dilution.

Vitamin B_6

Theoretical level of pyridoxine.HCl in the BCR Multivitamin Reference Mixture: 3 mg/g.

Weigh accurately at least one gram BCR Multivitamin Reference Mixture and transfer into a 500 ml volumetric flask. Add 1.0 g Titriplex III, 25 ml methanol and immerse in

the ultrasonic bath for 5 minutes. Add 400 ml 0.01 mol/l hydrochloric acid and immerse again for 10 minutes in the ultrasonic bath. Shake for 5 minutes and fill up to the mark with 0.01 mol/l hydrochloric acid.
If necessary, centrifuge or filter through a 0.8 micron Millipore filter a part of this stock solution. Assay as standard solution by making an appropriate dilution.

Vitamin B_{12}

Theoretical level of vitamin B_{12} in the BCR Multivitamin Reference Mixture:
0.008 mg/g.
Weigh accurately at least 10 grams BCR Multivitamin Reference Mixture and transfer into a 500 ml volumetric flask. Add 400 ml pyrosulfite- buffer (dissolve 10 g crystalline citric acid $C_6H_8O_7.H_2O$ + 20 g disodium hydrogen phosphate $Na_2HPO_4.2H_2O$ + 1 g sodium pyrosulfite $Na_2S_2O_5$ in water to 1000 ml). Allow to stand at room temperature for 15 minutes with occasional agitation, and fill up to the mark with water.
If necessary, centrifuge or filter through a 0.8 micron Millipore filter a part of this stock solution. Assay as standard solution by making an appropriate dilution.

Vitamin C

Theoretical level of ascorbic acid in the BCR Multivitamin Reference
Mixture: 200 mg/g.
Weigh accurately at least one gram BCR Multivitamin Reference Mixture and transfer into a 500 ml volumetric flask. Add 1.0 g Titriplex III, 25 ml methanol and immerse in the ultrasonic bath for 5 minutes. Add 400 ml 0.01 mol/l hydrochloric acid and immerse again for 10 minutes in the ultrasonic bath. Shake for 5 minutes and fill up to the mark with 0.01 mol/l hydrochloric acid.
If necessary, centrifuge or filter through a 0.8 micron Millipore filter a part of this stock solution. Assay as standard solution by making an appropriate dilution.

Folic acid

Theoretical level of folic acid in the BCR Multivitamin Reference
Mixture: 0.8 mg/g.
Weigh accurately at least one gram BCR Multivitamin Reference Mixture and transfer into a 500 ml volumetric flask. Add 400 ml water and raise the pH to 9 with ammonia solution (dilute 80 ml concentrated ammonia solution (approximately 25%(m/m) of NH_3) with water to 1000 ml). Allow to stand at room temperature for 15 minutes with

occasional agitation, and fill up to the mark with water.
If necessary, centrifuge or filter through a 0.8 micron Millipore filter a part of this stock solution. Assay as standard solution by making an appropriate dilution.

Niacin

Theoretical level of nicotinamide in the BCR Multivitamin Reference Mixture: 13 mg/g.
Weigh accurately at least 2.5 grams BCR Multivitamin Reference Mixture and transfer into a 500 ml volumetric flask. Add 1.0 g Titriplex III, 25 ml methanol and immerse in the ultrasonic bath for 5 minutes. Add 400 ml 0.01 mol/l hydrochloric acid and immerse again for 10 minutes in the ultrasonic bath. Shake for 5 minutes and fill up to the mark with 0.01 mol/l hydrochloric acid.
If necessary, centrifuge or filter through a 0.8 micron Millipore filter a part of this stock solution. Assay as standard solution by making an appropriate dilution.