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Improvements in the determination of vitamins in food through intercomparisons and preparation of RMs for vitamin analysis within the BCR[1] programme

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Summary. In order to improve the methods for the determination of vitamins in food for nutritional purposes, the Commission's Community Bureau of Reference (BCR) has initiated a comprehensive research programme consisting of intercomparisons of methods to identify and eliminate sources of error and the preparation of reference materials (RMs). Six food RMs have been prepared to date including brussels sprouts, mixed vegetables and pigs' liver (all in the lyophilised form), vitamin enriched milk powder, wholemeal flour and margarine. The first five materials have been packaged into heat sealable, aluminium-laminate sachets under an inert atmosphere; margarine is a canned product. The initial homogeneity results have indicated no detectable signs of inhomogeneity for the vitamins/RMs investigated. Stability testing has monitored both short-term stability at elevated temperatures (+ 25 to 40°C, 8 weeks) and long-term stability −30 to + 20°C, 36 months). The former was used to evaluate the effect of adverse shipment conditions on vitamin stability. Vitamins C and B_1, two of the more labile vitamins, have been found to be stable for up to 4 weeks at + 25°C and 8 weeks at + 37°C in brussels sprouts (RM 431) and wholemeal flour (RM 122), respectively.

The results of long-term stability testing of vitamins C and B_1 in these RMs indicate there was no significant degradation of vitamin C in RM 431 for up to 24 months at − 18 and + 4°C when the data was expressed on the basis of the −30°C data (analytical control). Similarly, no significant degradation for vitamin B_1 in RM 122 was found at + 4 and + 20°C for up to 12 months, again after expressing the data on the basis of the analytical control (− 20°C). Once acceptable homogeneity and stability results have been found, certification studies for each vitamin/RM are planned.

1 Introduction

Vitamins are a large group of compounds which differ in their chemical composition, physiological action and nutritional importance in the diet. Increasing awareness by the consumer of the nutritional labelling of foods has placed an additional burden on the current techniques for vitamin analysis in foods. In order to improve the quality of vitamin analysis in food, the Commission's Community Bureau of Reference (BCR) initiated a research programme in 1989 primarily to meet the requirements of the Community legislation on the Nutritional Labelling of Foods (EEC Directive 90/946/EEC).

The project includes research into improvements in vitamin methodology, intercomparisons of methods between laboratories to identify and eliminate sources of error and the preparation of suitably homogeneous and stable reference materials (RMs) for food use.

As a first step of this programme, the Commission established a group of experts to review the status of vitamin analysis in food and to set priorities for action based on the nutritional importance and legislation requirements of individual vitamins. The expert committee concluded that all vitamins except for biotin, vitamin K and pantothenic acid ought to be studied and that an intercomparison of methods should initially be organised in order to assess the current "state-of-the-art" in vitamin analysis and identify problem areas.

2 First intercomparison of vitamin methods

Eighteen laboratories in Europe received three samples of dry food in the form of lyophilised pork muscle (RM 384), lyophilised Haricots verts beans (RM 383) and milk powder (RM 380). These materials were certified for the major nutrients and minerals [1, 2].

The homogeneity with respect to these nutrients was found to be satisfactory. In addition, the homogeneity for retinol, α-tocopherol, vitamins B_1 and B_2 was further studied and found to be adequate.

Each laboratory was requested to perform the analyses by its own methods and had to carry out at least three separate determinations of sub-samples taken from at least two of the sachets provided of the above samples. Results were

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Table 1. Indicative values (expressed in mg/100g dry mass) for vitamins in whole milk powder (RM 380), lyophilised pork muscle (RM 384) and haricots verts beans (RM 383)

RM	Vitamin	Method(s)[a]	No labs	Indicative value
Whole milk powder (RM 380)	Retinol	HPLC	12	0.267 ± 0.023
	α-Tocopherol	HPLC	11	0.603 ± 0.092
	Thiamin	HPLC/MA/F	8	0.320 ± 0.041
	Niacin	MA	7	0.831 ± 0.066
Lyophilised Pork Muscle (RM 384)	Thiamin	HPLC/MA/F	9	3.13 ± 0.29
	Niacin	MA	6	24.3 ± 2.64
Haricots verts beans (RM 383)	α-Tocopherol	HPLC	10	0.343 ± 0.056
	Vitamin C	HPLC/F	9	15.07 ± 1.33
	Thiamin	HPLC/F/MA	9	0.220 ± 0.038
	Niacin	MA	7	1.71 ± 0.025

[a] HPLC high performance liquid chromatography
MA microbiological assay
F fluorometric thiochrome assay

expressed in dry-mass as determined by a prescribed drying procedure. The results for retinol, α-tocopherol, β-carotene, vitamin C and niacin agreed for the samples and it was possible to propose indicative values for these vitamins (Table 1).

It was also clear that the extraction and enzyme hydrolysis steps for B-group vitamins in general needed further study. Few laboratories specialising in vitamin analysis were able to perform analyses of vitamin D, folate and vitamin B_{12} and further intercomparisons were planned here.

3 Preparation of reference materials for vitamins

3.1 Selection of RMs

In order to produce vitamin RMs it was important to consider three factors. Firstly to identify the major dietary sources of each vitamin so that any RM produced would be similar to the foods commonly analysed by the laboratories. Secondly the RMs should contain each vitamin at appropriate concentrations relative to the normal ranges found in foodstuffs. Thirdly it was important to produce a range of RMs representative of the major food types (meat, vegetables, cereal, dairy etc.) so that any specific interference problem present for a matrix type could be identified and a solution found. It was also essential to produce RMs in such a form that the vitamins were suitably stable and homogeneous, although this would be thoroughly investigated once the RM had been prepared. Six RMs have been prepared to date (Table 2).

3.2 Preparation stage

The different chemical and physical properties of the vitamins mean that they vary enormously in their degree of stability. In crystalline form they will retain their activity for several months or even years. However, when foods are processed, vitamins are subjected to a range of conditions that

Table 2. RMs prepared for vitamins (listed in descending order of importance for each RM)

RM No	Food	Vitamins intended for certification
431	Lyophilised brussels sprouts	C, niacin, B_1, B_2, folate, B_{12}
421	Vitamin enriched milk powder	A, E, D, C, β-carotene, folate, B_{12}
121	Margarine	A, E, D, β-carotene
122	Wholemeal flour	B_1, B_2, B_6, folate, niacin
-	Lyophilised pigs' liver	A, E, D, β-carotene, folate, B_{12}, C, B_1, B_2, B_6, niacin
485	Lyophilised mixed vegetables	Carotenoids, C, B_1, B_2, folate, B_{12}

can be detrimental to their stability such as light, oxygen, moisture, heat and pH. (A more detailed review of vitamin stability is given elsewhere [3]). The most sensitive vitamins are vitamin C, folate, thiamin and the carotenoids. Vitamins A and E are moderately stable, although interconversion of vitamers for these vitamins can take place on storage. Niacin and biotin are the most stable of all vitamins. Information on some of the vitamins, especially vitamin D, is incomplete.

In general, the approach made in the preparation of the RMs was to produce dry foods but taking care to control the conditions (time, temperature) of the lyophilisation stage in order to mimimize possible vitamin losses. In addition any mixing or blending of the material was kept to a minimum and wherever possible the material was stored at low temperatures in the dark. The final material was packaged into food-grade, aluminium-plastic sachets (200 × 170 mm). The excess air was expelled, and the sachets were nitrogen flushed prior to heat sealing with double seals. A typical scheme used for the preparation of the wholemeal flour (RM No 122) is Fig. 1.

Homogeneity checks for moisture, vitamin B_1 and niacin were performed prior to packaging on representative samples taken at regular intervals throughout the batch. If any inhomogeneity was detected, the material was thoroughly remixed and further tests performed.

Sachets were filled with 50 ± 0.1 g of the flour. Prior to sealing, the sachets were shaken slightly to allow the flour to settle evenly at the bottom of each sachet. Two sachets at a time were placed in the Multivac packaging machine, ensuring that the sealing head was free from dust, which prevented effective sealing. Each sachet was evacuated, flushed with nitrogen and then directly sealed with a double seal. Seal quality was checked, and each sachet labelled in sequential order. Three sachets from each days' packaging were retained for gas analysis. The average gas composition of these sachets was found to be by 99.5% N_2 and 0.33% O_2 by GLC analysis. A small proportion of sachets (< 10%) were slightly overfilled with nitrogen to varying degrees. These sachets were identified and not used in the stability trials. A total of 1082 sachets were stored at −20°C.

3.3 Homogeneity testing

The samples were prepared under conditions designed to ensure the highest achievable homogeneity. However

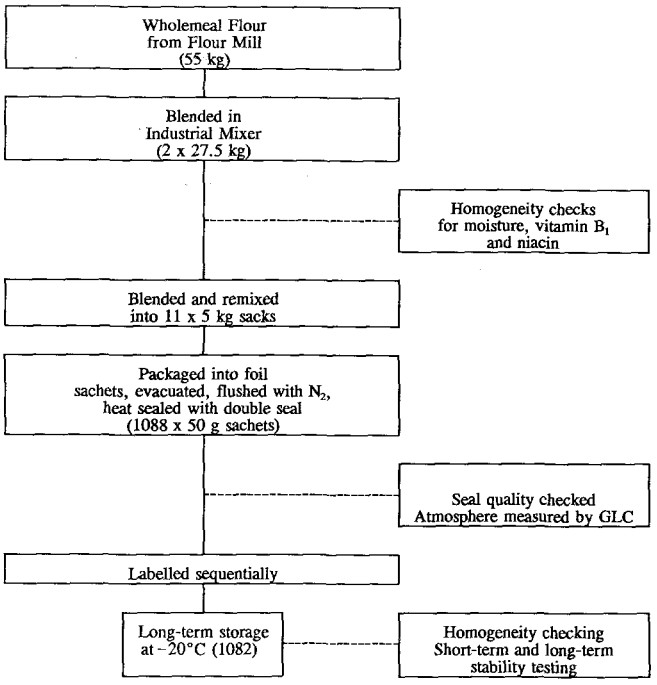

Fig. 1. Scheme for the preparation of the wholemeal flour RM

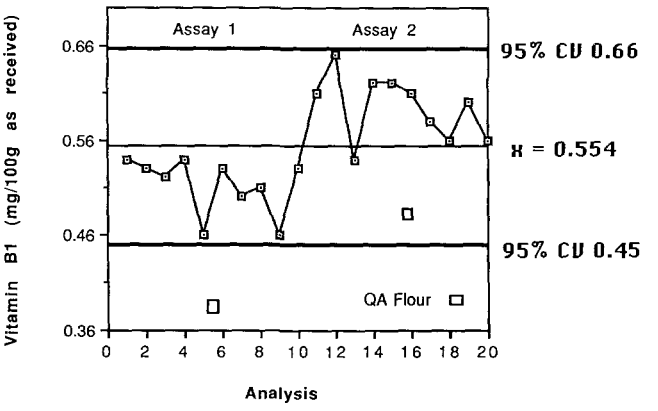

Fig. 2. The between-sachet homogeneity results for vitamin B_1 in wholemeal flour RM obtained from single analyses of twenty sachets taken throughout the packaging sequence (95% CV = 95% confidence value)

Table 3. Homogeneity results (mg/100g dry mass) for vitamin C in brussels sprouts (RM 431)

Variation	Mean	SD	% CV
Within-sachet	484	16	3.3
Between-sachet	503	18	3.6
Overall	499	19	3.8

demonstration of homogeneity depends very much on the repeatability of the vitamin analysis procedure employed. In the case of vitamin C in brussels sprouts (RM 431), both the within- and between-assay variation of the microfluorometric procedure used was low ($\leq 5\%$): thus it was possible to conclude from the values of CV_b and CV_w (Table 3) that the material was indeed acceptably homogeneous. The between-sachet coefficient of variation (CV_b) was obtained from the analysis of 20 sachets taken at regular intervals throughout the packaging sequence and the within-sachet coefficient of variation (CV_w), or method repeatability, was obtained from 10 replicate analyses of the same sachet.

In contrast the homogeneity results for vitamin B_1 (thiamin) in flour, which were obtained using a microbiological assay (MA), were more difficult to interpret. The precision of the MA used by this laboratory was estimated to be about 10% based on data for the QA flour sample assayed over several months. The within-assay precision, i.e. the variation within an assay performed by the same analyst was found to be between 5 and 8%. The results of the between-sachet variation for vitamin B_1 in flour is shown in Fig. 2. The within-assay repeatability for each of the assays performed on different days is at the expected level (about 6%). Similarly the overall CV_b (9.6%) is very close to the expected level (10%). Although there was a between-assay difference (Fig. 2), this was not found to be significant. This is also reflected in the data plotted for the QA flour sample. The homogeneity results are summarised in Table 4. If the between-sachet homogeneity data are corrected on the basis of the QA flour data, then the overal precision for the CV_b is reduced from 9.6% to 8.5%. Therefore, if all the data are taken into account, it may be concluded that RM 122 was homogeneous for vitamin B_1.

Table 4. Summary of homogeneity results for vitamin B_1 in flour (RM 122)

Variation	Mean	SD	% CV
Within-sachet	0.549	0.025	4.6 (normal: 5–8%)
Between-sachet			
Assay 1	0.512	0.030	5.9 (normal: 5–8%)
Assay 2	0.595	0.034	5.7 (normal: 5–8%)
Overall	0.554	0.053	9.6 (normal: 10%)
Between-sachet (corrected on QA)			
Assay 1	0.683	0.042	6.1 (normal: 5–8%)
Assay 2	0.605	0.034	5.6 (normal: 5–8%)
Overall	0.644	0.055	8.5 (normal: 10%)

3.4 Stability testing

The approach to the stability testing included both short-term stability at elevated temperatures (+ 25 to + 40°C) for 0–4 weeks and long-term stability at several temperatures (−30, −20, + 4 and + 20°C) for periods of up to 36 months with testing at 6 monthly intervals. The short-term stability was designed to evaluate the effect of adverse shipment conditions on the stability of the more labile vitamins.

The results for the short-term stability of vitamin C in RM 431 is shown in Fig. 3. After an initial rise and fall in the vitamin C content, there appears to be no further change in concentration between 7 and 28 days storage at + 25°C. This would indicate that the shipment of the RM was possible

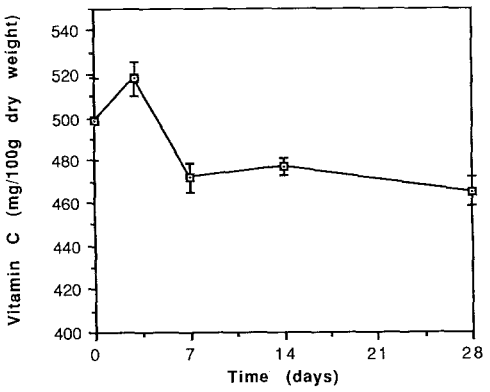

Fig. 3. The short-term stability results (mg/100 g dry weight) of vitamin C in lyophilised brussels sprouts RM.
—□— + 25 C

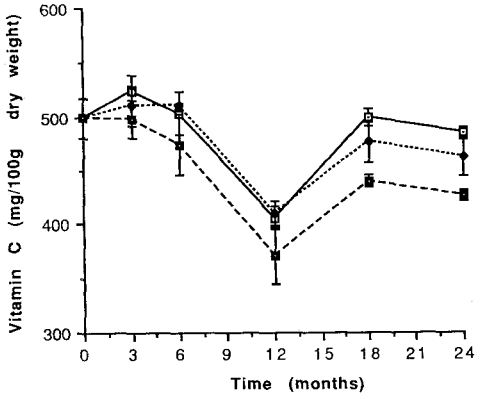

Fig. 4. The long-term stability results (mg/100 g dry weight) of vitamin C in lyophilised brussels sprouts RM.
—□— − 30 C; ···◆··· − 18 C; ---■--- + 4 C

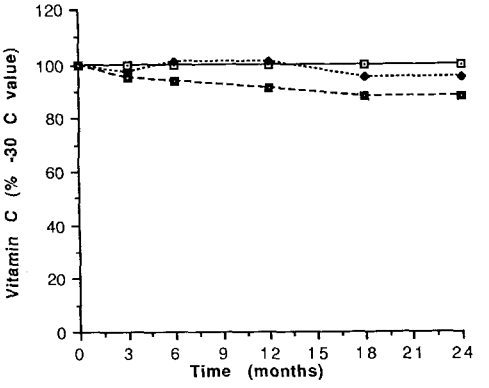

Fig. 5. The long-term stability results (mg/100 g dry weight) of vitamin C in lyophilised brussels sprouts RM expressed as a % of the − 30°C data (analytical control).
—□— − 30 C; ···◆··· − 18 (% − 30 C); ---■--- + 4 (% − 30 C)

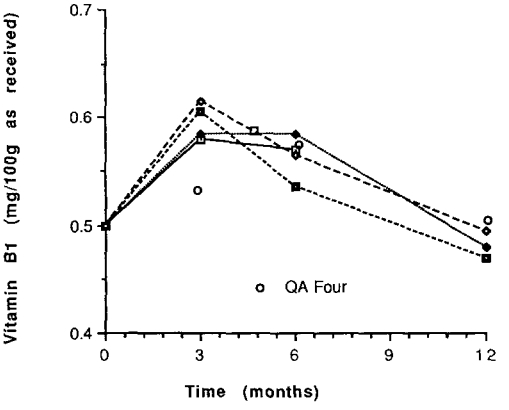

Fig. 6. The long-term stability results (mg/100 g as received) of vitamin B_1, in wholemeal flour RM
—□— − 70 C; —◆— − 20 C; ···■··· + 4 C; --○--- + 20 C

without any serious risk of vitamin C degradation. The results of the long-term stability are shown in Fig. 4. Although there appears to be a significant loss after 12 months in the vitamin C concentration, a closer examination of the data at 12 months reveals a problem in the calibration graph used. The analyses at this time were performed by a different analyst resulting in a different calibration line. Vitamin C levels at 18 and 24 months are both above the 12 month levels. In addition, the change in the vitamin C levels over the storage period is temperature independent indicating that variations are due to the long-term repeatability of the method. If the data is expressed as a % of the − 30°C data at each time point (assuming that vitamin C is stable at − 30°C for at least 24 months), then there is very little change in concentration at − 18 or + 4°C relative to the − 30°C value (Fig. 5). The vitamin C level at + 4°C after 24 months storage is lower compared to both the − 30 and − 18°C values but this was not found to be significant. Therefore it may be concluded that vitamin C is stable in RM 431 for 24 months at the three temperatures studied.

Similar results were obtained for both the short- and long-term stability of vitamin B_1 in wholemeal flour (RM 122). There was no significant degradation of the vitamin at + 37°C for up to 8 weeks. The long-term stability results are shown in Fig. 6. Again, the change in vitamin B_1 concentration on storage was attributed to variations in the long-term repeatability of the procedure and not any degradation of the vitamin. This is supported by the changes in the values for the QA flour sample. If the long-term stability results are expressed relative to the − 20°C data at each time point there is no change on vitamin B_1 levels on storage (Fig. 7).

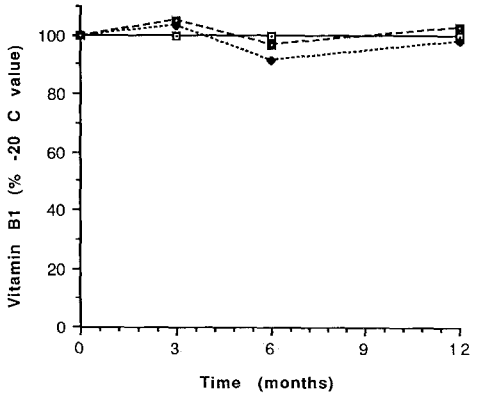

Fig. 7. The long-term stability results (mg/100 g as received) of vitamin B_1 in wholemeal flour RM expressed as a % of the − 20°C data (analytical control).
—□— − 20 C ···◆··· + 4 C (% − 20 C); ---■--- + 20 C (% − 20 C)

Certification exercises

Once the homogeneity and stability results have been found to be satisfactory, the certification studies for the various vitamins/RMs will be organised. It is planned to certify as many vitamins as possible in the six RMs prepared and it is anticipated that the first certification study will be organised in the latter half of 1992. The schedule will depend largely on the results of the various method improvement studies and the intercomparisons.

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