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Improvements in the determination of vitamins in foods: method intercomparison studies and preparation of certified reference materials (CRMs)

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Accurate methods for the determination of vitamins in food are required for nutritional labelling, and for the production of food composition data for nutritional research on relationships between diet and health. The Standards, Measurement and Testing Programme of the European Commission is supporting a collaborative project involving 48 laboratories to improve their measurement in food. The research programme involves the use of method intercomparison studies to identify and control systematic errors, optimization of sample extraction and clean-up procedures, and the preparation of suitable food reference materials (RMs). Results are presented for the determination of vitamin D_3 by HPLC and folates by microbiological assay in various foods demonstrating improved agreement between laboratories and possible reasons for improvements. In addition, examples of the food RMs produced and their potential use are described.

INTRODUCTION

Accurate methods for the determination of vitamins in food are an essential requirement to meet EU food labelling directives, and for the production of detailed food composition data for nutritional research on relationships between diet and health. The European Commission, through its Standards, Measurement and Testing Programme, is supporting a collaborative project on this topic involving a consortium of 48 laboratories. Their analysis in foodstuffs is difficult for a number of reasons. These include the low concentrations of vitamins often found in complex organic matrices, which require vigorous extraction and clean-up procedures; different chemical structures for the same vitamin, often as bound forms in protein and coenzyme complexes; and the need to measure specific physiological forms.

The aim of this project is to improve the measurement of three fat-soluble vitamins (A, E and D), a range of carotenoids (α- and β-carotene, lutein, lycopene and zeaxanthin) and nine water-soluble vitamins (C, B_1, B_2, B_6 and B_{12}, folate, niacin, biotin and pantothenic acid) in foods. Once satisfactory agreement between laboratories using different methods has been obtained, certified values will then be assigned for each vitamin to the prepared food reference materials.

OUTLINE OF WORK PROGRAMME

The research programme involves four main themes. First, the use of interlaboratory studies involving microbiological, chemical and high-performance liquid chromatograhic (HPLC) techniques to identify and control systematic errors. This will lead to a better understanding of reasons for differences between methods for the same vitamin, and closer agreement in results overall. Secondly, work on the optimization of sample extraction and clean-up procedures for B-group vitamins, especially conditions for enzyme dephosphorylation and calibration. Thirdly, the preparation of suitably homogeneous and stable food reference materials (RMs) covering a range of matrices and vitamin

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concentrations. These RMs will be eventually certified for each vitamin and will be used for the quality control of analytical measurements performed for nutritional labelling and other purposes. Lastly, the results and experiences from the project are being used to assist in the establishment of standard methods of analysis by the European Committee for Standardization's (CEN) working group on *Food Analysis, Horizontal Methods, Vitamins* (CEN/TC 275/WG9).

Vitamin D

In 1992, the first intercomparison of HPLC methods for the determination of vitamin D_3 (cholecalciferol) in two samples (milk powder and margarine) was undertaken by 11 laboratories (Finglas *et al.*, 1994). Most of the procedures included the use of antioxidants (e.g. ascorbic acid) during saponification, a vitamin D_2 (internal standard) semi-preparative LC clean-up and a reverse-phase analytical system. The values for between-laboratory variation (11–16%) were generally good and well within the limits predicted for an analyte at this concentration. However, the values for the within-laboratory variation (11–16%) were poor, especially for the margarine sample. The results were discussed at an evaluation meeting and further suggestions for improvement were agreed upon as follows:

- the use of aqueous slurries for powdered milk samples to avoid the formation of emulsions during saponification and generally aid recoveries;
- the use of narrow band cuts during LC sample clean-up to minimize the inclusion of interfering compounds;
- the use of methanol for redissolving extracts prior to injection onto the analytical column;
- the use of small additions of hexane or chloroform to the mobile phase to improve the baseline resolution of vitamins D_2 and D_3.

A certification study was subsequently organized for the determination of vitamin D_3 in two candidate reference materials, margarine (RM 122) and milk powder (RM 421) based on the above criteria. Participants were requested to carry out a minimum of four replicates on two separate units of each RM on different days. The results are given in Table 1. Thus, there was an approximate 2–3-fold improvement in % CV_B and % CV_W for both RMs between the studies due to the recommendations made on methodology. These results, together with other data on vitamin D intercomparison studies, have being incorporated into the draft CEN method for vitamin D, which is currently being circulated to all CEN countries for comments.

Table 1. Comparison of vitamin D_3 HPLC results (μg/100 g) in two foods from first study (1992) and certification study (1994)

Study	Milk powder[1]	Margarine
First study (1992):		
No. of laboratories	11	11
Mean[2]	9.0136	11.5023
SE	0.4458	0.4393
CV_W (%)	9.2	12.8
CV_B (%)	15.9	10.9
Certification study (1994):		
No. of laboratories	12	12
Mean[3]	14.3246	12.4571
SE	0.2385	0.3163
CV_W (%)	4.9	6.5
CV_B (%)	5.2	8.4

[1]Different milk samples were used for each study.
[2]Duplicate determinations on two units of reference materials on different days.
[3]Four or five replicate determinations on two units of reference materials on different days.

Folates

At present the most widely used and accepted procedure for the determination of folates in foods is the microbiological assay (MA) using *Lactobacillus rhamnosus* (ATCC 7469). Despite the development of semi-automated procedures, including the microtitration plate format (Horne & Patterson, 1988), the microbiological approach is both time-consuming and demanding in execution, and can have a number of limitations, the most important of which is that it is difficult to obtain values for individual folates. The main stages in folate analysis and potential problem areas causing variability are summarized in Table 2.

Two objectives of this project were to investigate the main reasons for the poor agreement in results between laboratories using the MA, and to evaluate both the use of more rapid procedures, such as the radio-protein binding assay (RPBA, or 'radioassay kit'), and HPLC procedures, which give information on individual

Table 2. Summary of main stages in folate analysis and potential problem areas causing variability

Stage	Problem areas
Sample extraction and clean-up	Time/temperature (MA) Stability/interconversions (HPLC)
Deconjugation	Type deconjugase enzyme and optimal conditions for use (MA–HPLC)
End-method of determination:	
MA	Type organism, assay pH, quantitation
HPLC	Type detection/peak identification, calibration
RPBA	Assay pH/responses, calibration

MA, microbiological assay; HPLC, high-performance liquid chromarography; RPBA, radio-protein binding assay.

Table 3. Folate results (μg/100 g) in various samples obtained by microbiological assay (MA)

Samples	No. of laboratories	Method details[1]	Total folate[2]
First study (1990)[3]:			
Brussels sprouts	6	LR/CP (pH 6–6.2)	928 (592–1428)
Powder (lyophilized)	1	EH/CP (pH 6.2)	281
Second study (1992):			
Milk powder (infant formula)	5	LR/CP (pH 6.1–6.4)	42 (38–54)
Wholemeal flour	5		56 (48–65)
Yeast powder	6		3573 (2410–4470)
Milk powder (infant formula)	2	LR/CP (pH 6.7)	61
Wholemeal flour	2		66
Yeast powder	2		4620
Third study (1995):			
Milk powder (vitamin enriched)	6	LR/HK (pH 6.2)	122 (11–139)
Wholemeal flour	6		45 (25–55)
Pig's liver (lyophilized)	6		1555 (1040–2240)
Mixed vegetables (lyophilized)	5		317 (274–440)
Milk powder (vitamin enriched)	2	LR/HK (pH 6.7–6.8)	111 (109–113)
Wholemeal flour	2		40
Pig's liver (lyophilized)	2		1093 (872–1313)
Mixed vegetables (lyophilized)	2		241 (121–360)
Milk powder (vitamin enriched)	1	SF/HK (pH 6.2)	102
Wholemeal flour	1		80
Pig's liver (lyophilized)	1		1765
Mixed vegetables (lyophilized)	1		40

[1]LR, *Lactobacillus rhamnosus* (ATCC 7469); EH, *Enterococcus hirae*; SF, *Streptococcus faecalis* (ATCC 9790); CP, chicken pancreas and HK, hog kidney deconjugase enzymes.
[2]Mean (range).
[3]Finglas *et al.* (1993).

folates. In this paper the results are presented for MA only in Table 3.

In general, for samples with a high proportion of methyl-folate present (e.g. Brussels sprouts in Study 1, mixed vegetables in Study 3), the use of *E. hirae* or *S. faecalis* results in a significant underestimation of total folate by MA compared to *L. rhamnosus*. This can also be found to a lesser extent if a high pH (e.g. 6.7) is used for assay growth using *Lactobacillus rhamnosus* in these samples. There did not appear to be any significant differences between the hog kidney (HK) and human plasma (HP) deconjugase enzymes tested, although some of the in-house enzymes gave more variable results. A common HK enzyme was prepared from fresh material using the procedure of Gregory *et al.* (1984) and checked for activity prior to circulation to all laboratories for use. It is important to check the deconjugase activity of in-house enzyme preparations prior to use, especially for a new batch of material. For this a yeast powder can be used as it contains a high concentration of folates in the polyglutamate form.

Preparation of certified reference materials (CRMs) for vitamins

CRMs can be used to improve the quality of analytical measurements (accuracy, precision and comparability). In particular, they can be used to verify the accuracy of results, to monitor the performance of methods and demonstrate the equivalence between methods.

In this project a range of food RMs have been prepared covering the major food classes and have undergone extensive homogeneity and stability testing. A full list of RMs are given in Table 4. Both the short-term monitoring of selective vitamins at elevated temperatures, to test for possible degradation during shipment, and long-term stability testing have been undertaken. In general, satisfactory homogeneity and stability has been demonstrated for most vitamins and RMs tested. However, it has been difficult to distinguish between the long-term method repeatability of the methods used and possible instability resulting from vitamin degradation.

Table 4. Food reference materials prepared for vitamins

Reference materials(No.)	Form	Packaging/amount	Intended for:
Brussels sprouts(431)	Lyophilized powder	Sachets/25 g	C, B-group
Milk powder(421)	Vitamin enriched, spray-dried powder	Sachets/50 g	A, E, D, C and B-group
Margarine(122)	Canned product	Metal can, 200 g	A, E, D
Wholemeal flour(121)	Commercial flour	Sachets/50 g	B-group, C
Pig's liver(487)	Lyophilized powder	Glass bottles/25 g	A, E, C and B-group
Mixed vegetables(485)	Lyophilized powder	Sachets/25 g	Carotenoids, C, folate

Once improvements in methodology and acceptable agreement between laboratories using both the same and different type of method has been achieved, certified values will be assigned for each vitamin-RM. The first certification study covering vitamins A, E and D_3 in margarine (CRM 122) and milk powder (CRM 421), and vitamin C and niacin in CRM 421 and Brussels sprouts (CRM 431), has recently been completed (Finglas *et al.*, 1995). It is planned to have CRMs released in 1996. An additional factor for the uncertainty due to possible long-term instability in the overall uncertainty of the certified value has been included for each vitamin and CRM. The long-term stability will be further monitored at regular intervals in these CRMs to ensure that there are no significant changes in the certified values.

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