

FOOD STANDARDS AGENCY INFORMATION BULLETIN ON METHODS OF ANALYSIS AND SAMPLING FOR FOODSTUFFS

This Bulletin is issued by the Food Standards Agency to Public Analysts, to other analysts working in the food sector and to others with an interest in the sector. Its principal purpose is to act as an electronic consultation forum on methods of analysis and sampling for foodstuffs proposed for inclusion in EU Regulations and Directives, or on topics to be discussed in the organisation such as the Codex Alimentarius Commission. Other topics, e.g. forthcoming collaborative trials to validate specific methods of analysis, will be covered from time to time.

This Bulletin may be regarded as the successor to the MAFF Information Bulletin for Public Analysts on EEC Methods of Analysis and Sampling for Foodstuffs. **However, unlike that Bulletin, it will only be issued in electronic form.** It will be issued in pdf format downloadable from the FSA Website.

It should be regarded as somewhat less formal than the previous Bulletin. Comments are invited on any items included in the Bulletin, but only *via* a conventional email approach rather than through a Bulletin Board approach. It is hoped that this will not only elicit comments but also develop discussion between recipients; comments will therefore be copied to all Bulletin recipients.

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Collaborative Trial 148b: The Determination of Vitamins A, D and E in Vitamin Supplements

Information on the above collaborative trials, which were co-ordinated on the Agency's behalf by LGC, is given in this Bulletin: in particular, information on the trial format, methods of analysis used and the results obtained is given. Both trials were undertaken as part of FSA project E01057. Formal reports on the trial will eventually be submitted for publication in the scientific literature.

Comments on the collaborative trials would be appreciated.

Thank you.

The Determination of Thiamin, Riboflavin, Niacin, Vitamin B6 and Pantothenic acid in Vitamin Supplements.

Collaborative Study Report

December 2006

FA30/2006/111



*Setting standards
in analytical science*

The Determination of Thiamin, Riboflavin, Niacin, Vitamin B6 and Pantothenic acid in Vitamin Supplements – Collaborative Study Report

December 2006

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This Study was carried out as part of a project funded by the Food Standards Agency which aims to evaluate and improve methods for the determination of vitamins in Food Supplements.



*Setting standards
in analytical science*

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Summary

This collaborative Study was carried out as part of Project E01057- The development of fully validated procedures for the determination of vitamins in dietary supplements.

This project seeks to improve procedures and to provide guidance to enforcement and other analysts in the selection of appropriate methods of analysis when determining vitamins in food supplements.

Test procedures were reviewed and evaluated at LGC to determine their suitability for use with different supplement formulations. The study procedure was designed to be suitable for the analysis of selected B-group vitamins in a wide range of supplements. Following in-house evaluation, it was submitted for further validation by collaborative trial.

The study participants were mainly UK Public Analysts as these laboratories are primarily responsible for enforcement analysis in the UK and reliable methods are vital for this task.

Five test materials, representing a range of different supplement formulations were supplied to LGC by supplement manufacturer's and were distributed to the participating laboratories to be analysed in duplicate. Participants were asked to analyse the samples using the method supplied and to report the results to LGC. These results were then evaluated according to the IUPAC/ AOAC statistical approach.

The method was shown to be suitable for the determination of thiamin, niacin and vitamin B₆ in vitamin supplements. For riboflavin, the interlaboratory variability was too high. It should be noted however, that by the removal of only one or two additional data points, acceptable precision could be achieved by at least twelve laboratories in all but one of the samples. Some additional care is needed for this vitamin. The method is not generally suitable for the determination of pantothenic acid at these levels largely due to chromatographic difficulties although some participants could determine this vitamin also. Further optimisation may be required for the determination of riboflavin and pantothenic acid in some samples.

In general, the concentration of vitamins found in the study samples was in good agreement with the manufacturer's label claims after allowing for typical overages used in manufacture.

1. Introduction

A wide range of vitamin supplements is available to the consumer in the UK via retail outlets, mail order and the Internet. The majority of these products are produced to exacting specifications by reputable manufacturers but the quality of other products has often been questioned. It is expected that vitamin supplements contain the correct forms and amounts of vitamins that are declared on the product label and, in cases of dispute, it is important that accurate methods of analysis for the determination of vitamins in supplements are available.

In most cases, methods of analysis are available but these methods are not harmonised. Differences in results produced by these methods have led to disputes over the apparent content of tested samples. In addition, vitamin supplements are produced in a number of different forms and as new formulations are developed, new ingredients can cause severe analytical difficulties with existing procedures.

This report details a collaborative study of analytical methodology developed within the Food Standards Agency Project E01057 for the determination of thiamin, riboflavin, niacin, vitamin B6 and pantothenic acid in vitamin supplements.

2. Collaborative trial organisation and participation

Method to be tested

The method followed in the collaborative study involved extraction of the vitamins in citric acid with heat and ultra-sonication. The vitamins were then determined by reverse-phase HPLC with UV spectrometric detection. Details of the method used are given in Annex A.

Test materials

Five different commercial supplement formulations were used. These were chosen to provide a range of product types, ingredients, vitamins, minerals and analyte concentrations. These materials were taken from the bulk products during manufacture and were supplied direct from the manufacturing plant. LGC was supplied with the details of the specifications and formulations but these may be commercially sensitive and are not reported.

The materials were divided into samples of five tablets/capsules which were contained in screw-topped, polythene tubes. These were stored in a fridge, in the dark until dispatch. Collaborative trial samples were sent to participants labelled A–E. Participants were asked to analyse each sample in duplicate*. HPLC conditions were recommended but participants were allowed to use alternative columns and conditions if desired. Since all of the chosen laboratories were familiar with similar methods for vitamin analysis, no practice round was needed, but the study director was available for advice if required. The declared vitamin concentrations as would normally be shown on the product label were provided.

*(*Note: Since the samples were visually identifiable, it was not possible to provide “blind” duplicates, however each duplicate was treated as a separate sample.)*

Sample details are shown below:

Sample A

Coated, Multivitamin and Mineral Tablet

Inactive ingredients include calcium carbonate, dicalcium phosphate, sorbitol, magnesium stearate, microcrystalline cellulose and hydroxyl propyl methyl cellulose

Active Ingredients include Cr, Cu, K, I, Mg, Mn, Mo, Zn, & Se salts, B-group vitamins, vitamin C, vitamin K, vitamin A acetate, vitamin E & vitamin D₃

Sample B

Chewable Multivitamin Tablet with Iron and Calcium

Inactive ingredients include calcium carbonate, sorbitol, magnesium stearate, colours, sweeteners & flavourings.

Active ingredients include Ca, Fe, K & I salts, B-group vitamins, vitamin C, vitamin A acetate, vitamin E, & vitamin D₃.

Sample C

Chewable multivitamin and mineral tablet

Inactive ingredients include calcium carbonate, sugar, stearic acid, citric acid, magnesium stearate, silica, sweeteners & flavourings

Active ingredients include Ca & Fe salts, B-group vitamins, vitamin C, vitamin A acetate, vitamin E succinate, & vitamin D₃

Sample D

Multivitamin soft-gel capsule with cod liver oil

Inactive ingredients include gelatine.

Active ingredients include B-group vitamins, vitamin C, vitamin A, vitamin E acetate, vitamin D₃ & cod liver oil.

Sample E

Multivitamin and mineral soft-gel capsule with cod liver oil

Inactive ingredients include gelatine.

Active ingredients include Fe, Mg, Ca, Zn, Cu, I, Mn, Mo, K, P, B-group vitamins, vitamin C, inositol, choline, vitamin A, vitamin E acetate, vitamin D₂ & cod liver oil.

Homogeneity testing

Homogeneity and stability testing was undertaken by the manufacturer's as part of their normal manufacturing process. Since homogeneity and stability testing was undertaken by the manufacturers, no further testing was carried out by LGC.

Participants

Sixteen laboratories agreed to participate in the study although, for operational reasons, only 15 laboratories submitted results. Of these, ten were UK Public Analyst's laboratories from England, Scotland and Wales; three were UK private-sector analytical laboratories, one was a European State-funded food and feed control laboratory, and one (LGC) was the official UK, referee-analyst laboratory. A list of the participating laboratories is shown in the Acknowledgements section of this report.

Statistical analysis of results

The trial results were examined for evidence of individual systematic error ($p < 0.025$) using Cochran's and Grubbs' tests progressively, by procedures described in the internationally agreed Protocol for the Design, Conduct and Interpretation of Method-Performance Studies [1].

Calculations for repeatability (r) and reproducibility (R) as defined by that protocol were carried out on those results remaining after removal of outliers

When assessing a new method there is often no validated reference or statutory method with which to compare precision characteristics, hence it is useful to compare the precision data obtained from a collaborative trial with "predicted" levels of precision. These "predicted" levels are calculated from the Horwitz equation. Comparison of the trial results and the predicted levels give an indication as to whether the method is sufficiently precise for the level of analyte being measured [2].

Historically the Horwitz predicted value has been calculated from the Horwitz equation as shown in equation {1}

$$\{1\} \quad RSD_R = 2^{(1-0.5 \log C)}$$

where C = measured concentration of analyte expressed as a decimal (e.g. 1 g/100g = 0.01).

Thompson has recently described the use of a modified Horwitz function to predict levels of precision at $\mu\text{g}/\text{kg}$ and sub $\mu\text{g}/\text{kg}$ levels up to 120 $\mu\text{g}/\text{kg}$ [3]. The use of this function is shown to give an improved statistical representation at these levels.

For the purposes of this trial, samples analysed all contained vitamins at concentrations greater than 120 $\mu\text{g}/\text{kg}$ hence the Horwitz predicted values were calculated from the Horwitz equation {1}.

The HORRAT value [4] gives a comparison of the actual precision measured with the precision predicted by the Horwitz equation for a method measuring at that particular level of analyte. It is calculated as detailed in equation {2}.

$$\{2\} \quad Ho_R = RSD_R (\text{measured}) / RSD_R (\text{Horwitz})$$

A HORRAT value (Ho_R) of 1 usually indicates satisfactory inter-laboratory precision, whereas a value of >2 usually indicates unsatisfactory precision, i.e. one that is too variable for most analytical purposes or where the variation obtained is greater than that expected for the type of method employed.

Ho_r is also calculated using equation {3} and used to assess intra-laboratory precision,

$$\{3\} \quad Ho_r = RSD_r (\text{measured}) / RSD_r (\text{Horwitz})$$

This assumes the approximation that $r = 0.66R$ and that therefore, $RSD_r (\text{Horwitz}) = 0.66 RSD_R (\text{Horwitz})$. [2]

3. Results and Discussion

Results were submitted by fifteen laboratories. The laboratory numbers were randomly assigned at the start of the study and have been retained throughout. Consequently, where no results were returned, the laboratory number is missing. Tables 31 – 35 of Annex B show all of the data which was returned by the participants on a weight per tablet/capsule basis.

Raw data supplied by participants were in the form of mass of vitamin per tablet (or per capsule). For repeatability and reproducibility estimation, all values were converted to mass of vitamin per mass of dosage form (i.e. mg/g). Mass fractions were calculated using the mean tablet mass (derived from reported weighing data) shown in Table 36 of Annex B. Note that use of mean tablet mass can in principle increase apparent repeatability and reproducibility standard deviations if individual tablets differ significantly in mass.

Statistical evaluation and conclusions

Valid data was evaluated following the IUPAC Harmonised Protocol. This protocol requires automatic outlier rejection at the 97.5% level of confidence, with repeated outlier testing and rejection to a maximum of 2/9 (22%) of the valid data. Outlier tests were performed for outlying variance using Cochran's test, and for outlying means using the three Grubbs tests.

Data inspection, outlier identification and calculations

The results (in mg/g) and the calculated performance characteristics are given in Annex B; Tables 6 to 30 and the performance characteristics are summarised in Tables 1 to 5 of the Annex.

Outlier tests were carried out in conjunction with the repeatability and reproducibility calculations. Distribution plots were used to visually review individual data sets for serious departures from normality and to identify any extreme values prior to applying Cochran and Grubbs tests. Data identified by the protocol as outliers were removed before calculation of the performance characteristics. Precision values were compared to the expected Horwitz values at an appropriate level and HorRat values were calculated.

Non-compliant results

Laboratory 9 were unable to report any results within the required deadline.

No results were submitted for pantothenic acid by laboratory 1.

Laboratory 10 submitted a single pantothenic acid result for sample 10.

Outliers

Outliers identified by Grubbs or Cochran's tests were removed before calculation of the performance parameters. These are shown in Tables 6 – 30 in Annex B. In addition, for thiamin in sample D, Laboratory 12 was additionally identified as a Cochran's outlier after removal of initial outliers (See Annex B; Table 9). It was retained as the number of outliers would have exceeded the ($>2/9$ rule).

Precision Conclusions for each vitamin

Thiamin

The precision was acceptable (HorRat values <2) for all samples.

Niacin

The precision was acceptable (HorRat values <2) for all samples.

Vitamin B6

The precision was acceptable (HorRat values <2) for all samples.

Riboflavin

The repeatability ($RSD_r\%$) was acceptable for all samples with HorRat values <2 . The reproducibility ($RSD_R\%$) was acceptable for sample D but was too high for the remaining samples.

Further evaluation was carried out to look at the cause of this variability. Laboratory 14 had a tendency to produce low riboflavin results for all samples and could be removed for samples A – C. Laboratory 1 was also slightly high for sample A. Laboratories 3 and 5 showed poor duplicate agreement in sample E. Although these data were not statistical outliers, additional removal of these data for information, improved the HorRAT values for samples A – D to acceptable levels although the reproducibility variability for sample E was still too high. The data is shown in Table 37 in Annex B.

Pantothenic Acid

The repeatability ($RSD_r\%$) was acceptable for samples A, D & E but was high for samples B & C. The reproducibility ($RSD_R\%$) was too high for all samples.

Pantothenic acid has no specific chromophores in its structure and can only be detected at low wavelength ($\sim 210\text{nm}$). The sensitivity at this wavelength is poor and this region is prone to interferences from other matrix components or from solvents. Some participants were able to produce clean, measurable peaks but others were measuring misshapen blips on the baseline or shoulders on other peaks. The method may not be suitable for the detection of pantothenic acid at the concentrations used in the study without further concentration and or clean-up unless particular care is taken to optimise the separation and sensitivity for this analyte. Determination of pantothenic acid in pre-mixes or concentrates however, should be possible.

General Precision Conclusions

The Horrat values for all vitamins and samples are shown in Figures 6 & 7 in Annex B. The method appears suitable for the determination of thiamin, niacin, vitamin B₆ and in many cases, riboflavin although the variability of the latter was too great without additional removal of data points. Some additional care is needed for this vitamin. The method is not generally suitable for the determination of pantothenic acid at these levels largely due to chromatographic difficulties although some participants could determine this vitamin also. Further optimisation may be required for the determination of riboflavin and pantothenic acid in some samples.

Bias

During “in-house” validation, the method studied was used to determine water-soluble vitamins in a wide range of supplements and satisfactory recoveries were obtained for all vitamins. It was not possible to accurately determine the bias within the current study for the following reasons:

1. Samples were supplied by the manufacturers. It is common practice to add an additional amount of a vitamin to that declared on the label (an overage) to allow for degradation of the vitamins during the product lifetime. Although input data was supplied, the variation in the information supplied and the tolerances which applied were such that it was not always possible to calculate the actual value expected in each product. The samples should have at least the amount declared on the product label but may contain higher amount of vitamins within the permitted tolerance depending upon the age of the product.
2. Although homogeneity and stability was assessed by the supplier, no additional checks were made.

The bias has been assessed against the label claim information available and the results are shown in Figures 1–5 in Annex B. In general, the bias was acceptable with most of the vitamin results being at or above the claimed values. The riboflavin content was slightly low in samples A, C & E but the precision data was also variable for this vitamin. Vitamin B₆ was also slightly low in sample E.

4. Acknowledgements

This study is a result of a contract (Project E01057) between the Food Standards agency and LGC Ltd which aims to improve the reliability of methods for the determination of vitamins in food supplements. We would like to thank the FSA for their funding and also thank all staff involved in this project.

LGC would like to thank Peter Berry Ottaway of the Council for Responsible Nutrition (CRN) for his valuable help and advice throughout this project. We would also like to give our thanks to the vitamin supplement manufacturers who contributed valuable help, advice and methodological details to this project as a whole and to those that generously provided samples for analysis.

We also thank all of the following collaborators and their staff for their participation in this study:

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Mr A T Richards	Durham County Council
Dr G Stephenson	Somerset Scientific Services
Petr Cuhra	Czech Food & Feed State laboratory
Dr D J Campbell	West Yorkshire Analytical Services
Mr R A Stevens	Worcestershire County Council

5. References

- 1 Protocol for the Design, Conduct and Interpretation of Method-Performance Studies, Ed W. Horwitz, *Pure and Applied Chemistry*, 1995, **67**, (2), 331-343.
- 2 Horwitz, W., 1982, *Analytical Chemistry*, **54**, 67A-76A.
- 3 Thompson, M., 2000, Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing. *Analyst*, **125**, 385 - 386.
- 4 Peeler, J. T., Horwitz, W. and Albert, R., 1989, Precision parameters of standard methods of analysis of dairy products, *Journal of the Association of Official Analytical Chemists*, **72**, No 5, 784-806.

Annex A – Analytical method

The Determination of B-group Vitamins in Vitamin Supplements

1 Scope:

The method is suitable for the determination of Thiamin (B1), Riboflavin (B2), Niacin (B3), Pyridoxine (B6), and Pantothenic acid in multivitamin supplements. Its primary purpose is to enable the label declaration to be confirmed by analysis.

The method can also be used for the determination of vitamin B12 and/or Biotin in concentrates and pre-mixes. The concentration in finished products is generally too low to be determined by this procedure therefore these vitamins are excluded from this study. A separate extraction procedure is usually required for folic acid.

2 Principle:

The vitamins are extracted from the samples using an acidic extractant with heating and ultrasonication. The vitamins are then determined by reverse-phase HPLC with UV detection.

3 Apparatus

General laboratory glassware and equipment including the following items:

- 3.1 Ultrasonic bath
- 3.2 Water bath capable of maintaining 60°C
- 3.3 Hotplate or steam bath
- 3.4 Ball-mill (Optional)
- 3.5 Filter paper, general laboratory grade, 18cm diameter
- 3.6 0.45µm syringe filters
- 3.7 250ml volumetric flasks

4. Reagents

(Note¹: The product numbers given are those of Fisher Scientific UK and are given for information only. Other equivalent products can be used)

- 4.1 Citric acid, monohydrate (C6200/60)
- 4.2 Disodium EDTA (D0650/48)
- 4.3 Tween 80 (T4203/60)
- 4.4 Orthophosphoric acid (O/0500/PB08)
- 4.5 1-Octanol (O/0050/08)
- 4.6 Potassium dihydrogen phosphate (P4800/53)

- 4.7 Sodium heptane sulphonate (H0168/48)
- 4.8 Acetonitrile, HPLC grade
- 4.9 Sodium hydroxide, 1M solution. (J/07620/17). Dilute 1-10 to make 0.1M solution.
- 4.10 Hydrochloric acid, 1M solution. (J/4320/17). Dilute 1-10 to make 0.1M solution
- 4.11 Extraction buffer: (To make 5 litres)
- Weigh 250g citric acid into a 5L vessel. Using a measuring cylinder, add 3L of water and mix. Weigh 1g disodium EDTA into a 1-litre beaker. Add 500 ml of water and heat, with stirring on a hotplate or steam bath. Add 25ml of Tween 80 to the hot solution and stir until a clear solution is obtained. Add the hot EDTA/Tween solution to the citric acid solution followed by 1475 ml of water and mix.

Water used for reagent preparation should be of a quality suitable for HPLC.

5. Standards

5.1 Standard Substances

- 5.1.1 Thiamin hydrochloride, USP. (Sigma T4562)
- 5.1.2 Riboflavin, USP. (Sigma R7649)
- 5.1.3 Nicotinamide, USP. (Sigma N5535)
- 5.1.4 Pyridoxine hydrochloride, USP. (Sigma P4722)
- 5.1.5 Calcium Pantothenate, USP. (Sigma C8731)

Note²: The method can also be used for the determination of Nicotinic acid and Riboflavin phosphate. If these vitamins are to be determined, additional standards will be required as appropriate.

Note³. Equivalent products can be used.

5.2 Stock Solutions

- 5.2.1 Thiamin Stock Solution (~1000 ug/ml[#])

Weigh 127 ± 2 mg thiamin hydrochloride (5.1.1) into a 100ml volumetric flask. Add approximately 50ml of water and 5ml of 0.1M HCl. Dilute to volume with water and mix. Solution is stable for 10 days if stored in a refrigerator and out of the light.

{[#]calculated on the basis that 1mg Thiamin = 1.27mg Thiamin hydrochloride}

5.2.2 Riboflavin Stock Solution (~500 µg/ml)

Weigh 50 ± 0.5 mg riboflavin (5.1.2) into a 100ml flask. Add 10ml of 0.1M sodium hydroxide and swirl to dissolve. Immediately, add 15ml of 0.1M HCl and dilute to volume with water. Mix. Solution is stable for 10 days if stored in a refrigerator and out of light.

5.2.3 Nicotinamide Stock solution (~1000 ug/ml)

Weigh 100 ± 2 mg nicotinamide (5.1.3) into a 100ml volumetric flask. Dilute to volume with water and mix. Solution is stable for 10 days if stored in a refrigerator and out of light.

5.2.4 Pyridoxine Stock solution (~1000 µg/ml[#])

Weigh 122 ± 2 mg pyridoxine hydrochloride (5.1.4) into a 100ml volumetric flask. Add ~50ml of water and 5ml of 0.1M HCl. Dilute to volume with water and mix. Solution is stable for 10 days if stored in a refrigerator and out of the light.

{[#] calculated on the basis that 1mg of pyridoxine \equiv 1.22mg of pyridoxine hydrochloride}

5.2.5 Calcium Pantothenate (~1000 µg/ml[#])

Weigh 109 ± 2 mg of calcium pantothenate (5.1.5) into a 100ml volumetric flask. Dilute to volume with water and mix. Solution is stable for 10 days if stored in a refrigerator and out of the light.

{[#] calculated on the basis that 1mg of pantothenic acid \equiv 1.09 mg calcium pantothenate}.

5.3 Mixed, intermediate standard solution:

Prepare a mixed intermediate standard as follows:

Table 1: Preparation of Intermediate Standard Solution

Vitamin	Volume of Stock Std soln, ml	Final Volume ml	µg/ml
Thiamin	1	50	20
Riboflavin	2		20
Niacinamide	10		20
Pyridoxine	2		400
Calcium Pantothenate	5		100

The intermediate should be prepared on day of use and is stored in the refrigerator away from light. Dilutions are made with extraction buffer (4.11)

5.4 Calibration Standards

A range of calibration standards is prepared by diluting the intermediate standard (5.3) with extraction buffer (4.11) as in Table 2:

Table 2: Preparation of Calibration Standards

Volume of Std (5.3) / ml	Final volume / ml	B1 $\mu\text{g/ml}$	B2 $\mu\text{g/ml}$	B3 $\mu\text{g/ml}$	B6 $\mu\text{g/ml}$	Pantothenic acid $\mu\text{g/ml}$
5	10	10	10	100	20	50
2	10	4	4	40	8	20
1	10	2	2	20	4	10
1	20	1	1	10	2	5
1	50	0.4	0.4	4	0.8	2
1	100	0.2	0.2	2	0.4	1

6 HPLC

An HPLC system capable of providing a binary gradient at a flow-rate of 1ml/min is required. A programmable, multi-wavelength UV detector or photodiode array detector is ideally required although single wavelength detection and multiple runs can be used.

6.1 HPLC Mobile Phase

Mobile Phase A:

Weigh 10.2g Potassium dihydrogen phosphate (4.6) and 3.75g sodium heptane sulphonate (4.7) into a 2-litre beaker. Add 1500 ml of water and stir to dissolve. Using a pH meter, adjust the pH to 2.9 ± 0.1 using orthophosphoric acid.

Mobile Phase B:

Add 600ml of acetonitrile to 400ml of mobile phase A.

6.2 HPLC Conditions

Column: LichroCart, Superspher 60 RP Select B, 125mm x 4mm i.d. (Merck)

Flow rate: 1.0 ml/min

Column Temp: 30°C

Mobile phase: Gradient as below:

Table 3 HPLC Gradient

Time /min	%B
0	0
5	9
10	18
15	30
20	48
21	100
28	100
29	0
35	0

Note⁴: The gradient should be adjusted as necessary to obtain satisfactory resolution of the vitamins. Equivalent systems can be used. Satisfactory separations have also been obtained using Phenomenex Aqua or Luna C18(2) columns with acetonitrile/water gradients containing no ion-pair modifiers. The separation and peak symmetries were however not as good.

Autosampler: An autosampler with a cooled sample tray set to ~ 4°C is recommended if the chromatography is carried out overnight on automatic systems.

Detector:

A diode-array or programmable wavelength detector should be used if available. Single wavelength detection at 275nm can be used but for optimal sensitivity, the following wavelengths are recommended:

Table 4: Detection wavelengths

Vitamin	Wavelength (nm)
Calcium Pantothenate	205
Nicotinamide, Riboflavin, Thiamin, B12	260
Pyridoxine	290

7 Procedure

7.1 Sample Extraction

7.1.1 Tablets:

Weigh accurately not less than 20 tablets and grind to a fine powder in a ball mill. Calculate the mean tablet weight.

Weigh accurately, 1 ± 0.1 g of the ground sample into a 250ml volumetric flask. Add 150ml extracting solution (4.11), swirl to disperse the sample then place the flask in a water bath at 60°C for 10mins with frequent mixing. Transfer the flask to an ultrasonic bath and ultra-sonicate for 20min. Cool to ambient temperature, make up to volume with extraction buffer and mix. Filter through a general-purpose filter paper. Dilute the extract if necessary so that the expected concentrations of the vitamins of interest are within the calibration ranges used. Filter through a 0.45µm filter for HPLC if required.

Note 6: If a ball-mill is not available, the tablets should be ground in a pestle & mortar, taking care to ensure that the tablets are finely ground and homogeneous in appearance.

Note 7: Tablets containing carbonates or other disintegrants will effervesce. Allow the effervescence to subside before adding all of the extractant. A drop of octan-1-ol can be used to control frothing before making up to volume if required.

7.1.2 Capsules

Weigh not less than 20 capsules and calculate the average capsule weight.

Weigh 5 capsules into a 250ml volumetric flask. Add 150ml extracting solution (4.11), and place in a water bath at 60°C for 10mins with frequent mixing. (Check that the capsules have dissolved/dispersed. Heat and mix for an additional 10 minutes if required). Transfer the flask to an ultrasonic bath and ultra-sonicate for 20mins. Bulk to volume with extraction buffer & mix. Filter through a general-purpose filter paper. Dilute the extract if necessary so that the expected concentrations of the vitamins of interest are within the calibration range used. Filter through a 0.45µm filter for HPLC if required.

Note 8: Samples should be analysed at least in duplicate (10 capsules) to ensure that a representative sub-sample is obtained.

Note 9: Hard capsules containing powdered fill, can be emptied and treated as for tablets however, consideration should be given to the possibility of migration of analytes into the capsule shell.

7.1.3 Liquids, syrups and emulsions

Liquid supplements should be shaken well before use. Clear solutions can be diluted with extracting buffer and analysed directly by HPLC. Other liquids and emulsions should be treated as for tablets to ensure that the vitamins are fully extracted.

7.2 HPLC

Inject 20ul of the intermediate standard solution (5.3) onto the HPLC system and check that the chromatographic resolution of all of the vitamins and sensitivity is suitable for the purpose.

Inject each of the calibration standards in duplicate and determine the peak areas at the wavelength required (*See 6.2*). Identify the vitamins of interest by comparison of their retention times and, if available, their spectral profiles. Construct calibration curves for each of the vitamins of interest by plotting peak area against vitamin concentration in µg/ml.

Inject sample extracts in duplicate and calculate the vitamin concentration from the peak areas obtained at the relevant wavelength by interpolation from the appropriate calibration curve.

Calculate the vitamin concentration (C) for each vitamin of interest using the following equation:

$$\text{Vitamin content} = \frac{C * V * D * W_T}{W_S * 1000}$$

mg/Tablet

where:

- C = concentration of vitamin interpolated from the calibration curve in µg/ml.
- V = Final extract volume (normally 250ml)
- D = Dilution factor (if diluted)
- W_S = Weight of sample analysed
- W_T = Average tablet weight

OR

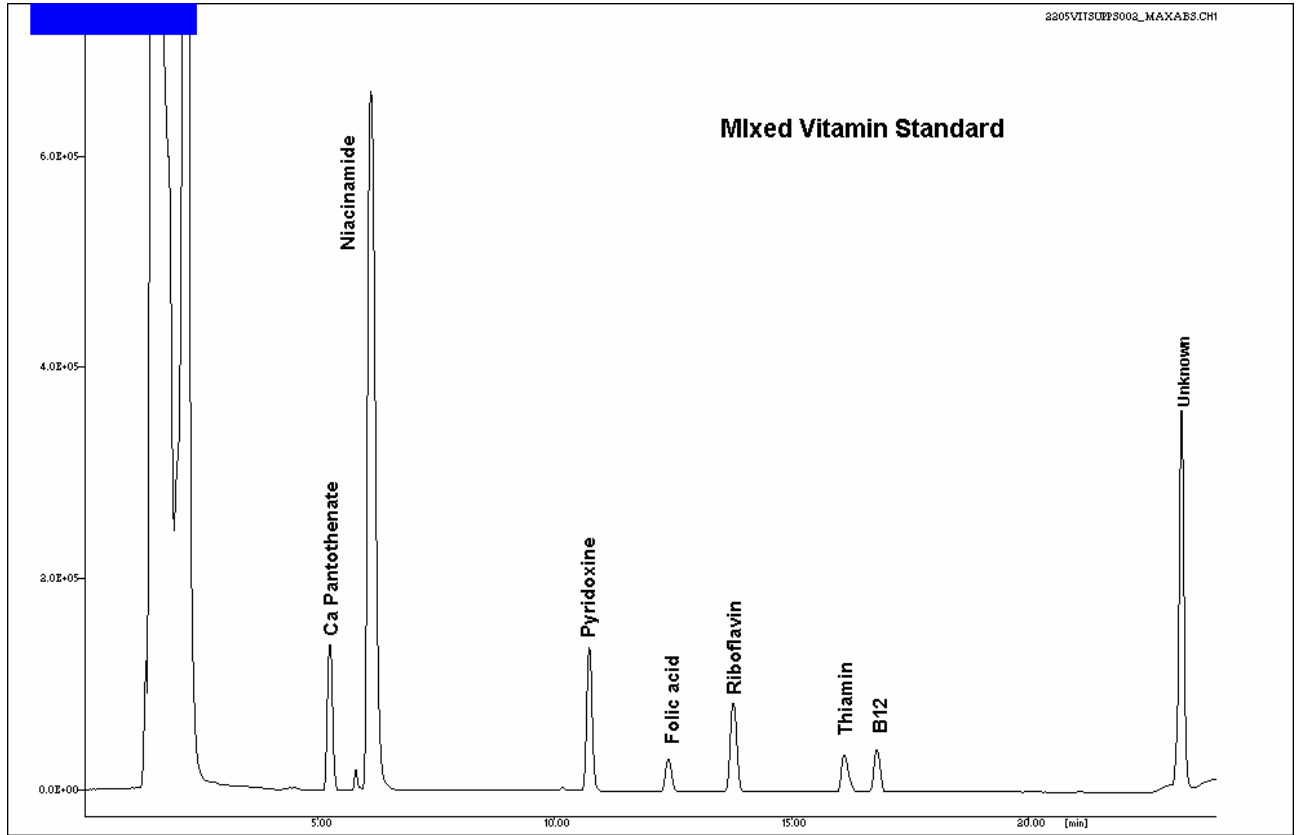
$$\text{Vitamin content} = \frac{C * V * D}{N * 1000}$$

mg/Capsule

where:

- C = concentration of vitamin interpolated from the calibration curve in µg/ml.
- V = Final extract volume (normally 250ml)
- D = Dilution factor (if diluted)
- N = Number of capsules analysed

Example Chromatogram



Precision data

The following, preliminary data was obtained by replicate analysis (n = 6) of a multivitamin tablet:

Tablet	Thiamin	Riboflavin	Nicotinamide	B6	Ca Pantothenate
Mean mg/Tablet	1.9	1.7	19.1	2.7	8.2
SD	0.17	0.10	1.15	0.18	0.54
r _{95%}	0.33	0.20	2.26	0.36	1.05
RSDr%	8.9	5.9	6.0	6.9	6.5

Detection Limit

The method is designed to be used for checking label declarations of vitamin supplements. The final concentrations of sample extracts are considerably higher than the instrumental detection limits with the exception of vitamin B12 and Biotin for which this method is not suitable.

Annex B: Results and Statistical evaluation Tables and Figures

Method Performance Characteristic	Coated, Multivitamin and Mineral Tablet	Chewable Multivitamin Tablet with Iron and Calcium	Chewable Multivitamin and Mineral Tablet	Multivitamin Soft-Gel Capsule with Cod Liver Oil	Multivitamin and Mineral Soft-Gel capsule with Cod Liver Oil
	Sample A	Sample B	Sample C	Sample D	Sample E
n	15	15	15	15	15
outliers	2	0	0	3	2
n _t	13	15	15	12	13
mean (mg/g)	1.02	0.52	1.78	1.27	4.06
r	0.12	0.08	0.13	0.05	0.17
S _t	0.042	0.029	0.05	0.019	0.060
RSD _t	4.1	5.5	2.7	1.5	1.5
Ho _t	1.1	1.3	0.8	0.4	0.5
R	0.17	0.12	0.28	0.07	0.73
S _R	0.061	0.044	0.101	0.024	0.260
RSD _R	6.0	8.6	5.7	1.9	6.4
Ho _R	1.1	1.4	1.1	0.4	1.4

Table 1: Summary of the performance characteristics for Thiamin

Method Performance Characteristic	Coated, Multivitamin and Mineral Tablet	Chewable Multivitamin Tablet with Iron and Calcium	Chewable Multivitamin and Mineral Tablet	Multivitamin Soft-Gel Capsule with Cod Liver Oil	Multivitamin and Mineral Soft-Gel capsule with Cod Liver Oil
	Sample A	Sample B	Sample C	Sample D	Sample E
n	15	15	15	15	15
outliers	1	0	2	0	0
n _t	14	15	13	15	15
mean (mg/g)	13.95	8.31	13.56	14.59	32.02
r	1.60	0.85	1.34	0.55	1.92
S _t	0.571	0.302	0.479	0.197	0.686
RSD _t	4.1	3.6	3.5	1.4	2.1
Ho _t	1.6	1.3	1.4	0.5	1.0
R	2.21	1.31	1.34	1.33	2.96
S _R	0.788	0.469	0.479	0.476	1.057
RSD _R	5.7	5.6	3.5	3.3	3.3
Ho _R	1.5	1.4	0.9	0.9	1.0

Table 2: Summary of the performance characteristics for Niacin

Method Performance Characteristic	Coated, Multivitamin and Mineral Tablet	Chewable Multivitamin Tablet with Iron and Calcium	Chewable Multivitamin and Mineral Tablet	Multivitamin Soft-Gel Capsule with Cod Liver Oil	Multivitamin and Mineral Soft-Gel capsule with Cod Liver Oil
	Sample A	Sample B	Sample C	Sample D	Sample E
n	15	15	15	15	15
outliers	0	1	1	0	1
n ₁	15	14	14	15	14
mean (mg/g)	1.50	0.68	1.39	1.66	13.33
r	0.21	0.09	0.13	0.13	0.70
S _r	0.073	0.032	0.047	0.046	0.249
RSD _r	4.9	4.7	3.4	2.8	1.9
Ho _r	1.4	1.2	1.0	0.8	0.7
R	0.34	0.13	0.18	0.17	1.09
S _R	0.123	0.048	0.065	0.059	0.388
RSD _R	8.2	7.1	4.7	3.6	2.9
Ho _R	1.5	1.2	0.9	0.7	0.8

Table 2: Summary of the performance characteristics for Vitamin B6

Method Performance Characteristic	Coated, Multivitamin and Mineral Tablet	Chewable Multivitamin Tablet with Iron and Calcium	Chewable Multivitamin and Mineral Tablet	Multivitamin Soft-Gel Capsule with Cod Liver Oil	Multivitamin and Mineral Soft-Gel capsule with Cod Liver Oil
	Sample A	Sample B	Sample C	Sample D	Sample E
n	15	15	15	15	15
outliers	1	1	1	3	0
n ₁	14	14	14	12	15
mean (mg/g)	0.86	0.62	1.66	1.23	2.18
r	0.10	0.04	0.19	0.11	0.43
S _r	0.036	0.014	0.067	0.039	0.153
RSD _r	4.2	2.3	4.1	3.1	7.0
Ho _r	1.1	0.6	1.7	0.9	2.1
R	0.39	0.23	0.64	0.17	1.07
S _R	0.141	0.083	0.227	0.059	0.381
RSD _R	16.3	13.5	13.7	4.8	17.5
Ho _R	2.8	2.2	2.6	0.9	3.5

Table 4: Summary of the performance characteristics for Riboflavin

Method Performance Characteristic	Coated, Multivitamin and Mineral Tablet	Chevable Multivitamin Tablet with Iron and Calcium	Chevable Multivitamin and Mineral Tablet	Multivitamin Soft-Gel Capsule with Cod Liver Oil	Multivitamin and Mineral Soft-Gel capsule with Cod Liver Oil
	Sample A	Sample B	Sample C	Sample D	Sample E
n	14	14	14	14	14
outliers	1	0	1	0	1
n ₁	13	14	13	14	13
mean (mg/g)	4.69	0.98	7.57	8.23	12.12
r	0.60	0.24	0.45	1.03	0.98
S _r	0.213	0.086	0.161	0.368	0.351
RSD _r	4.5	8.8	2.1	4.5	2.9
Ho _r	1.5	2.4	0.8	1.6	1.1
R	1.88	0.54	2.94	2.56	5.17
S _R	0.673	0.194	1.051	0.916	1.847
RSD _R	14.3	19.9	13.9	11.1	15.2
Ho _R	3.2	3.5	3.3	2.7	3.9

Table 5: Summary of the performance characteristics for Pantothenic acid

TITLE	B-vitamins Collaborative Trial		
EXPT_DESIGN	BLIND REPS: AOAC		
mean: n1, units	1.016	mg/g	
mean rep1...repn	1.019	1.014	
n, no of reps	15	2	
nc	0		
Outliers	2		
n1	13		
r	0.116		
sr	0.042		
RSDr, RSDrH0	4.087	3.724	
H0r	1.097		
R	0.172		
SR	0.061		
RSDR, RSDRH0	6.035	5.643	
H0R	1.069		
Critical Difference			
no. of replicates	2		
CrD95	0.090		
NON COMPLIANT	LAB ID	REP1	REP2
	1	1.08	0.95
Grubb's Reject	2	1.27	1.29
	3	0.95	0.88
	4	1.05	1.02
	5	1.02	1.10
	6	0.95	0.94
Grubb's Reject	7	1.32	1.32
	8	0.99	1.00
	10	1.09	1.16
	11	0.97	0.99
	12	1.02	0.95
	13	1.02	1.02
	14	1.02	1.02
	15	1.05	1.02
	16	1.02	1.11

Table 6: Precision characteristics for Thiamin in Sample A

TITLE	B-vitamins Collaborative Trial		
EXPT_DESIGN	BLIND REPS: AOAC		
mean: n1, units	0.516	mg/g	
mean rep1...repn	0.514	0.518	
n, no of reps	15	2	
nc	0		
Outliers	0		
n1	15		
r	0.080		
sr	0.029		
RSDr, RSDrH0	5.531	4.125	
H0r	1.341		
R	0.124		
SR	0.044		
RSDR, RSDRH0	8.614	6.249	
H0R	1.378		
Critical Difference			
no. of replicates			
CrD95	Enter no of reps above		
NON COMPLIANT	LAB ID	REP1	REP2
	1	0.51	0.54
	2	0.55	0.60
	3	0.40	0.50
	4	0.52	0.52
	5	0.50	0.50
	6	0.48	0.46
	7	0.60	0.60
	8	0.52	0.53
	10	0.53	0.52
	11	0.52	0.51
	12	0.60	0.50
	13	0.50	0.50
	14	0.50	0.50
	15	0.48	0.49
	16	0.47	0.48

Table 7: Precision characteristics for Thiamin in Sample B

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	1.776	mg/g
mean rep1...repn	1.794	1.757
n, no of reps	15	2
nc	0	
Outliers	2	
n1	13	
f	0.134	
sr	0.048	
RSDr, RSDrH0	2.694	3.424
H0r	0.787	
R	0.284	
SR	0.101	
RSDR, RSDRH0	5.702	5.188
H0R	1.099	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
Grubb's Reject	1	1.96 1.95
	2	2.15 2.13
	3	1.76 1.76
	4	1.84 1.81
	5	1.76 1.76
	6	1.66 1.66
	7	2.14 2.26
Grubb's Reject	8	1.80 1.81
	10	1.89 1.87
	11	1.71 1.69
	12	1.89 1.76
	13	1.76 1.76
	14	1.76 1.76
	15	1.89 1.74
	16	1.65 1.51

Table 8: Precision characteristics for Thiamin in Sample C

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	1.269	mg/g
mean rep1...repn	1.267	1.272
n, no of reps	15	2
nc	0	
Outliers	3	
n1	12	
f	0.053	
sr	0.019	
RSDr, RSDrH0	1.491	3.602
H0r	0.414	
R	0.068	
SR	0.024	
RSDR, RSDRH0	1.921	5.457
H0R	0.352	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
Cochran's Reject	1	1.31 1.30
	2	1.43 1.66
	3	1.26 1.26
	4	1.30 1.28
	5	1.26 1.26
Grubb's Reject	6	1.17 1.16
Grubb's Reject	7	1.63 1.63
	8	1.28 1.27
	10	1.30 1.28
	11	1.28 1.26
	12	1.19 1.26
	13	1.26 1.26
	14	1.26 1.26
	15	1.23 1.28
	16	1.27 1.28

Table 9: Precision characteristics for Thiamin in Sample D

Lab 12 also a Cochrans reject but retained as per 2/9 rule.

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	4.064	mg/g
mean rep1...repn	4.044	4.085
n, no of reps	15	2
nc	0	
Outliers	2	
n1	13	
f	0.169	
sr	0.060	
RSDr, RSDrH0	1.484	3.023
H0r	0.491	
R	0.727	
SR	0.260	
RSDR, RSDRH0	6.388	4.581
H0R	1.395	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
	1	4.37 4.34
	2	4.64 4.72
Cochran's Reject	3	4.23 3.79
	4	4.08 4.20
	5	3.94 3.94
	6	3.73 3.67
Grubb's Reject	7	5.39 5.54
	8	3.76 3.94
	10	4.15 4.26
	11	3.78 3.83
	12	3.94 3.94
	13	4.08 4.08
	14	3.94 4.08
	15	4.01 3.95
	16	4.15 4.15

Table 10: Precision characteristics for Thiamin in Sample E

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	13.951	mg/g
mean rep1...repn	14.002	13.899
n, no of reps	15	2
nc	0	
Outliers	1	
n1	14	
r	1.600	
sr	0.571	
RSDr, RSDrH0	4.096	2.511
H0r	1.631	
R	2.207	
SR	0.788	
RSDR, RSDRH0	5.650	3.804
H0R	1.485	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
	1	14.49 13.47
	2	15.15 15.01
	3	13.18 13.18
	4	14.35 14.35
	5	14.35 15.15
	6	14.10 14.17
	7	14.42 14.06
	8	14.30 14.30
Grubb's Reject	10	10.47 10.25
	11	14.27 13.76
	12	13.76 11.93
	13	13.98 13.98
	14	12.74 12.59
	15	14.27 14.07
	16	12.66 14.57

Table 11: Precision characteristics for Niacin in Sample A

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	8.314	mg/g
mean rep1...repn	8.216	8.412
n, no of reps	15	2
nc	0	
Outliers	0	
n1	15	
r	0.845	
sr	0.302	
RSDr, RSDrH0	3.631	2.714
H0r	1.338	
R	1.313	
SR	0.469	
RSDR, RSDRH0	5.639	4.113
H0R	1.371	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
	1	7.81 8.56
	2	7.64 7.79
	3	6.81 7.82
	4	8.23 8.22
	5	8.82 8.72
	6	8.43 8.33
	7	7.92 8.32
	8	8.72 8.86
	10	8.85 8.55
	11	8.82 8.60
	12	8.02 8.02
	13	7.92 8.72
	14	8.62 8.62
	15	8.26 8.28
	16	8.42 8.82

Table 12: Precision characteristics for Niacin in Sample B

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	13.558	mg/g
mean rep1...repn	13.508	13.607
n, no of reps	15	2
nc	0	
Outliers	2	
n1	13	
r	1.340	
sr	0.479	sr>sr
RSDr, RSDrH0	3.531	2.522
H0r	1.400	
R	1.340	set R=r
SR	0.479	set sr=sr
RSDR, RSDRH0	3.531	3.821
H0R	0.924	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
	1	13.4 13.4
	2	13.6 14.6
	3	13.1 12.8
	4	13.3 13.6
	5	13.3 13.3
	6	13.4 13.2
	7	13.3 14.0
	8	13.9 13.6
	10	11.7 12.6
	11	13.5 13.7
	12	13.3 15.0
	13	13.2 13.3
	14	12.0 12.0
	15	13.9 13.2
	16	14.2 13.2

Table 13: Precision characteristics for Niacin in Sample C

TITLE	B-vitamins Collaborative Trial		
	EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	14.587	mg/g	
mean rep1...repn	14.577	14.596	
n, no of reps	15	2	
nc	0		
Outliers	0		
n1	15		
r	0.552		
sr	0.197		
RSDr, RSDrH0	1.352	2.494	
H0r	0.542		
R	1.333		
SR	0.476		
RSDR, RSDRH0	3.263	3.779	
H0R	0.863		
Critical Difference			
no. of replicates			
CrD95	Enter no of reps above		
NON COMPLIANT	LAB ID	REP1	REP2
	1	13.3	13.9
	2	15.0	15.2
	3	14.9	14.5
	4	14.8	14.8
	5	14.5	14.7
	6	14.0	14.0
	7	14.9	14.7
	8	15.1	14.9
	10	15.1	14.8
	11	14.8	14.8
	12	13.7	14.0
	13	14.1	14.5
	14	14.9	14.8
	15	15.0	14.8
	16	14.5	14.7

Table 14: Precision characteristics for Niacin in Sample D

TITLE	B-vitamins Collaborative Trial		
	EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	32.023	mg/g	
mean rep1...repn	32.017	32.028	
n, no of reps	15	2	
nc	0		
Outliers	0		
n1	15		
r	1.921		
sr	0.686		
RSDr, RSDrH0	2.143	2.216	
H0r	0.967		
R	2.960		
SR	1.057		
RSDR, RSDRH0	3.301	3.357	
H0R	0.983		
Critical Difference			
no. of replicates			
CrD95	Enter no of reps above		
NON COMPLIANT	LAB ID	REP1	REP2
	1	30.1	29.9
	2	32.6	34.6
	3	32.1	31.3
	4	31.9	32.9
	5	32.1	32.5
	6	31.3	31.1
	7	32.4	30.9
	8	31.2	32.6
	10	34.5	32.8
	11	31.0	31.7
	12	31.5	30.8
	13	32.1	31.8
	14	32.7	32.7
	15	32.4	32.5
	16	32.5	32.4

Table 15: Precision characteristics for Niacin in Sample E

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	1.500	mg/g
mean rep1...repn	1.494	1.506
n, no of reps	15	2
nc	0	
Outliers	0	
n1	15	
r	0.206	
sr	0.073	
RSDr, RSDrH0	4.899	3.512
H0r	1.395	
R	0.343	
SR	0.123	
RSDR, RSDRH0	8.176	5.322
H0R	1.536	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
	1	1.44 1.33
	2	1.43 1.46
	3	1.39 1.32
	4	1.52 1.49
	5	1.54 1.68
	6	1.49 1.49
	7	1.76 1.76
	8	1.53 1.54
	10	1.58 1.59
	11	1.49 1.51
	12	1.39 1.17
	13	1.46 1.54
	14	1.46 1.54
	15	1.52 1.52
	16	1.41 1.65

Table 16: Precision characteristics for Vitamin B6 in Sample A

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	0.677	mg/g
mean rep1...repn	0.673	0.681
n, no of reps	15	2
nc	0	
Outliers	1	
n1	14	
r	0.089	
sr	0.032	
RSDr, RSDrH0	4.698	3.960
H0r	1.187	
R	0.134	
SR	0.048	
RSDR, RSDRH0	7.096	5.999
H0R	1.183	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
	1	0.60 0.71
	2	0.60 0.61
	3	0.50 0.70
	4	0.67 0.63
	5	0.70 0.70
	6	0.67 0.67
	7	0.70 0.80
	8	0.70 0.73
	10	0.67 0.64
	11	0.74 0.70
	12	0.60 0.60
	13	0.70 0.70
	14	0.70 0.70
	15	0.68 0.65
	16	0.67 0.67

Table 17: Precision characteristics for Vitamin B6 in Sample B

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	1.394	mg/g
mean rep1...repn	1.403	1.386
n, no of reps	15	2
nc	0	
Outliers	2	
n1	13	
f	0.133	
sr	0.047	
RSDr, RSDrH0	3.394	3.551
H0r	0.956	
R	0.182	
SR	0.065	
RSDR, RSDRH0	4.670	5.381
H0R	0.868	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
Cochran's Reject	1	1.33 1.64
	2	1.37 1.35
	3	1.38 1.38
	4	1.40 1.38
	5	1.38 1.38
	6	1.36 1.37
	7	1.64 1.64
Grubb's Reject	8	1.52 1.45
	10	1.40 1.42
	11	1.42 1.40
	12	1.26 1.26
	13	1.38 1.38
	14	1.38 1.51
	15	1.55 1.38
	16	1.43 1.35

Table 18: Precision characteristics for Vitamin B6 in Sample C

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	1.658	mg/g
mean rep1...repn	1.660	1.656
n, no of reps	15	2
nc	0	
Outliers	2	
n1	13	
r	0.129	
sr	0.046	
RSDr, RSDrH0	2.786	3.460
H0r	0.805	
R	0.165	
SR	0.059	
RSDR, RSDRH0	3.555	5.242
H0R	0.678	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
	1	1.66 1.63
	2	1.54 1.68
	3	1.71 1.56
	4	1.67 1.62
	5	1.63 1.63
	6	1.59 1.59
Grubb's Reject	7	2.00 1.93
	8	1.68 1.66
	10	1.68 1.63
	11	1.66 1.69
Grubb's Reject	12	1.41 1.48
	13	1.63 1.71
	14	1.63 1.63
	15	1.71 1.69
	16	1.77 1.81

Table 19: Precision characteristics for Vitamin B6 in Sample D

TITLE	B-vitamins Collaborative Trial		
	EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	13.331	mg/g	
mean rep1...repn	13.301	13.362	
n, no of reps	15	2	
nc	0		
Outliers	2		
n1	13		
r	0.698		
sr	0.249		
RSDr, RSDrH0	1.871	2.528	
H0r	0.740		
R	1.087		
SR	0.388		
RSDR, RSDRH0	2.912	3.831	
H0R	0.760		
Critical Difference no. of replicates			
CrD95	Enter no of reps above		
NON COMPLIANT	LAB ID	REP1	REP2
	1	13.3	13.0
	2	12.8	12.8
	3	13.4	12.7
	4	13.1	13.6
	5	13.3	13.6
	6	13.3	13.2
	7	16.5	17.2
Grubb's Reject	8	12.8	13.6
	10	13.4	13.4
	11	12.7	13.0
Grubb's Reject	12	12.0	11.7
	13	14.0	14.0
	14	13.3	13.4
	15	13.7	13.7
	16	13.8	13.8

Table 20: Precision characteristics for Vitamin B6 in Sample E

TITLE	B-vitamins Collaborative Trial		
	EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	4.691	mg/g	
mean rep1...repn	4.685	4.697	
n, no of reps	15	2	
nc	1		
Outliers	0		
n1	14		
r	0.597		
sr	0.213		
RSDr, RSDrH0	4.548	2.959	
H0r	1.537		
R	1.884		
SR	0.673		
RSDR, RSDRH0	14.346	4.483	
H0R	3.200		
Critical Difference no. of replicates			
CrD95	Enter no of reps above		
NON COMPLIANT	LAB ID	REP1	REP2
Non-Compliant	1	-	-
	2	5.20	5.51
	3	3.73	3.81
	4	4.55	4.37
	5	3.95	4.10
	6	4.74	4.69
	7	4.69	4.69
	8	4.14	4.15
	10	5.24	5.40
	11	4.88	4.84
	12	4.76	3.95
	13	4.90	4.76
	14	6.30	6.22
	15	4.80	4.95
	16	3.70	4.33

Table 21: Precision characteristics for Pantothenic acid in Sample A

TITLE	B-vitamins Collaborative Trial		
EXPT_DESIGN	BLIND REPS: AOAC		
mean: n1, units	0.975	mg/g	
mean rep1...repn	0.980	0.970	
n, no of reps	15	2	
nc	1		
Outliers	0		
n1	14		
r	0.241		
sr	0.086		
RSDr, RSDrH0	8.830	3.748	
H0r	2.356		
R	0.544		
SR	0.194		
RSDR, RSDRH0	19.922	5.678	
H0R	3.508		
Critical Difference			
no. of replicates			
CrD95	Enter no of reps above		
NON COMPLIANT	LAB ID	REP1	REP2
Non-Compliant	1	-	-
	2	0.99	0.97
	3	0.80	0.90
	4	0.65	0.70
	5	1.00	1.00
	6	0.57	0.62
	7	1.00	0.80
	8	1.06	1.05
	10	1.12	0.95
	11	1.16	1.10
	12	1.10	1.00
	13	0.90	1.10
	14	1.10	0.90
	15	0.93	1.08
	16	1.31	1.39

Table 22: Precision characteristics for Pantothenic acid in Sample B

TITLE	B-vitamins Collaborative Trial		
EXPT_DESIGN	BLIND REPS: AOAC		
mean: n1, units	7.574	mg/g	
mean rep1...repn	7.634	7.514	
n, no of reps	15	2	
nc	2		
Outliers	2		
n1	11		
r	0.451		
sr	0.161		
RSDr, RSDrH0	2.126	2.753	
H0r	0.772		
R	2.942		
SR	1.051		
RSDR, RSDRH0	13.873	4.171	
H0R	3.326		
Critical Difference			
no. of replicates			
CrD95	Enter no of reps above		
NON COMPLIANT	LAB ID	REP1	REP2
Non-Compliant	1	-	-
	2	8.165	8.202
	3	6.542	6.416
	4	7.120	7.284
	5	6.164	6.038
	6	7.372	9.536
	7	7.171	7.297
	8	6.781	6.466
	10		4.567
	11	7.473	7.422
	12	7.674	9.184
	13	9.309	8.806
	14	9.309	9.058
	15	8.605	8.605
	16	7.334	7.057

Table 23: Precision characteristics for Pantothenic acid in Sample C

TITLE	B-vitamins Collaborative Trial		
	EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	8.231	mg/g	
mean rep1...repn	8.136	8.326	
n, no of reps	15	2	
nc	1		
Outliers	0		
n1	14		
r	1.030		
sr	0.368		
RSDr, RSDrH0	4.469	2.719	
H0r	1.644		
R	2.564		
SR	0.916		
RSDR, RSDRH0	11.125	4.119	
H0R	2.701		
Critical Difference			
no. of replicates			
CrD95	Enter no of reps above		
NON COMPLIANT	LAB ID	REP1	REP2
Non-Compliant	1	8.9	9.7
	2	7.6	8.4
	3	7.3	7.3
	4	7.8	7.9
	5	10.0	10.0
	6	7.7	7.6
	7	6.9	6.7
	8	6.7	8.1
	10	8.1	8.2
	11	7.7	7.8
	12	8.7	8.8
	13	8.8	8.8
	14	8.8	9.2
	15	8.8	8.4
	16	9.0	8.7

Table 24: Precision characteristics for Pantothenic acid in Sample D

TITLE	B-vitamins Collaborative Trial		
	EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	12.121	mg/g	
mean rep1...repn	11.969	12.273	
n, no of reps	15	2	
nc	1		
Outliers	1		
n1	13		
r	0.984		
sr	0.351		
RSDr, RSDrH0	2.898	2.565	
H0r	1.130		
R	5.172		
SR	1.847		
RSDR, RSDRH0	15.240	3.886	
H0R	3.922		
Critical Difference			
no. of replicates			
CrD95	Enter no of reps above		
NON COMPLIANT	LAB ID	REP1	REP2
Non-Compliant	1	-	-
	2	13.0	13.0
	3	12.2	13.1
	4	11.1	11.8
	5	12.4	12.8
	6	8.3	8.2
	7	12.7	12.5
	8	9.9	10.7
	10	3.6	12.4
	11	9.7	10.0
	12	13.1	12.5
	13	13.7	14.1
	14	14.9	15.3
	15	13.3	13.7
	16	11.3	11.6

Table 25: Precision characteristics for Pantothenic acid in Sample E

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	0.861	mg/g
mean rep1...repn	0.862	0.861
n, no of reps	15	2
nc	0	
Outliers	1	
n1	14	
r	0.100	
sr	0.036	
RSDr, RSDrH0	4.154	3.818
H0r	1.088	
R	0.393	
SR	0.141	
RSDR, RSDRH0	16.310	5.785
H0R	2.819	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
	1	1.00 0.93
	2	1.13 1.17
	3	0.95 0.95
	4	0.86 0.80
	5	0.73 0.73
	6	0.73 0.72
	7	0.95 1.02
	8	0.88 0.86
	10	0.94 0.93
Cochran's Reject	11	0.67 0.90
	12	0.81 0.73
	13	0.88 0.88
	14	0.59 0.59
	15	0.81 0.81
	16	0.81 0.93

Table 26: Precision characteristics for Riboflavin in Sample A

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	0.615	mg/g
mean rep1...repn	0.612	0.618
n, no of reps	15	2
nc	0	
Outliers	1	
n1	14	
r	0.040	
sr	0.014	
RSDr, RSDrH0	2.319	4.017
H0r	0.577	
R	0.233	
SR	0.083	
RSDR, RSDRH0	13.518	6.087
H0R	2.221	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
	1	0.69 0.73
	2	0.66 0.68
	3	0.60 0.60
	4	0.67 0.68
	5	0.60 0.60
	6	0.54 0.56
	7	0.60 0.60
	8	0.67 0.69
	10	0.61 0.58
	11	0.65 0.68
	12	0.70 0.70
Cochran's Reject	13	0.50 0.60
	14	0.40 0.40
	15	0.63 0.60
	16	0.53 0.54

Table 27: Precision characteristics for Riboflavin in Sample B

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	1.659	mg/g
mean rep1...repn	1.660	1.659
n, no of reps	15	2
nc	0	
Outliers	1	
n1	14	
r	0.188	
sr	0.067	
RSDr, RSDrH0	4.053	3.459
H0r	1.172	
R	0.637	
SR	0.227	
RSDR, RSDRH0	13.703	5.242
H0R	2.614	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
Cochran's Reject	1	2.40 1.96
	2	1.90 2.09
	3	1.76 1.89
	4	1.75 1.76
	5	1.51 1.51
	6	1.51 1.48
	7	1.76 1.64
	8	1.55 1.56
	10	1.79 1.70
	11	1.49 1.55
	12	2.01 2.01
	13	1.51 1.64
	14	1.26 1.13
	15	1.90 1.80
	16	1.55 1.47

Table 28: Precision characteristics for Riboflavin in Sample C

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	1.226	mg/g
mean rep1...repn	1.221	1.231
n, no of reps	15	2
nc	0	
Outliers	3	
n1	12	
r	0.108	
sr	0.039	
RSDr, RSDrH0	3.149	3.621
H0r	0.870	
R	0.165	
SR	0.059	
RSDR, RSDRH0	4.806	5.486
H0R	0.876	
Critical Difference		
no. of replicates		
CrD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
Grubb's Reject	1	1.47 1.44
Cochran's Reject	2	1.28 1.67
	3	1.26 1.19
	4	1.29 1.30
	5	1.26 1.34
	6	1.16 1.17
	7	1.19 1.11
	8	1.18 1.28
	10	1.27 1.23
	11	1.45 1.46
	12	1.11 1.19
	13	1.26 1.26
	14	1.19 1.19
	15	1.23 1.27
	16	1.25 1.24

Table 29: Precision characteristics for Riboflavin in Sample D

TITLE	B-vitamins Collaborative Trial	
EXPT_DESIGN	BLIND REPS: AOAC	
mean: n1, units	2.176	mg/g
mean rep1...repn	2.203	2.150
n, no of reps	15	2
inc	0	
Outliers	0	
n1	15	
f	0.429	
sr	0.153	
RSDr, RSDrH0	7.045	3.321
H0r	2.121	
R	1.067	
SR	0.381	
RSDR, RSDRH0	17.508	5.032
H0R	3.479	
Critical Difference		
no. of replicates		
CfD95	Enter no of reps above	
NON COMPLIANT	LAB ID	REP1 REP2
	1	2.78 2.71
	2	2.48 2.64
	3	2.33 1.75
	4	2.06 2.04
	5	2.33 1.90
	6	1.84 1.59
	7	2.48 2.48
	8	1.68 1.57
	10	2.04 2.23
	11	2.13 2.27
	12	2.77 2.77
	13	2.04 2.04
	14	1.90 1.90
	15	1.71 1.81
	16	2.49 2.55

Table 30: Precision characteristics for Riboflavin in Sample E

Lab. Number	THIAMIN		NIACIN		VITAMIN B6		PANTOTHENIC ACID		RIBOFLAVIN	
	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2
1	1.47	1.30	19.80	18.40	1.97	1.82	-	-	1.37	1.27
2	1.74	1.76	20.7	20.51	1.95	2	7.1	7.53	1.54	1.6
3	1.3	1.2	18.0	18.0	1.9	1.8	5.1	5.2	1.3	1.3
4	1.43	1.40	19.60	19.60	2.07	2.04	6.22	5.97	1.17	1.09
5	1.4	1.5	19.6	20.7	2.1	2.3	5.4	5.6	1.0	1.0
6	1.30	1.29	19.26	19.35	2.04	2.03	6.48	6.40	1.00	0.98
7	1.8	1.8	19.7	19.2	2.4	2.4	6.4	6.4	1.3	1.4
8	1.35	1.36	19.53	19.53	2.09	2.10	5.65	5.67	1.20	1.18
10	1.49	1.58	14.3	14.0	2.16	2.17	7.16	7.38	1.29	1.27
11	1.33	1.35	19.49	18.80	2.04	2.06	6.67	6.61	0.92	1.23
12	1.4	1.3	18.8	16.3	1.9	1.6	6.5	5.4	1.1	1.0
13	1.4	1.4	19.1	19.1	2.0	2.1	6.7	6.5	1.2	1.2
14	1.4	1.4	17.4	17.2	2.0	2.1	8.6	8.5	0.8	0.8
15	1.43	1.40	19.49	19.22	2.08	2.07	6.56	6.76	1.11	1.10
16	1.40	1.52	17.3	19.9	1.92	2.26	5.06	5.91	1.11	1.27

Table 31: Analytical data submitted by each laboratory –Sample A (mg /Tablet)

Lab. Number	THIAMIN		NIACIN		VITAMIN B6		PANTOTHENIC ACID		RIBOFLAVIN	
	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2
1	0.51	0.54	7.79	8.54	0.60	0.71	N/A	N/A	0.69	0.73
2	0.55	0.6	7.62	7.77	0.6	0.61	0.99	0.97	0.66	0.68
3	0.4	0.5	6.8	7.8	0.5	0.7	0.8	0.9	0.6	0.6
4	0.52	0.52	8.21	8.20	0.67	0.63	0.65	0.70	0.67	0.68
5	0.5	0.5	8.8	8.7	0.7	0.7	1.0	1.0	0.6	0.6
6	0.48	0.46	8.41	8.31	0.67	0.67	0.57	0.62	0.54	0.56
7	0.6	0.6	7.9	8.3	0.7	0.8	1	0.8	0.6	0.6
8	0.52	0.53	8.70	8.84	0.70	0.73	1.06	1.05	0.67	0.69
10	0.53	0.52	8.83	8.53	0.67	0.64	1.12	0.95	0.61	0.58
11	0.52	0.51	8.80	8.58	0.74	0.70	1.16	1.10	0.65	0.68
12	0.6	0.5	8.0	8.0	0.6	0.6	1.1	1.0	0.7	0.7
13	0.5	0.5	7.9	8.7	0.7	0.7	0.9	1.1	0.5	0.6
14	0.5	0.5	8.6	8.6	0.7	0.7	1.1	0.9	0.4	0.4
15	0.48	0.49	8.24	8.26	0.68	0.65	0.93	1.08	0.63	0.60
16	0.47	0.48	8.40	8.80	0.67	0.67	1.31	1.39	0.53	0.54

Table 32: Analytical data submitted by each laboratory – Sample B (mg / Tablet)

Lab. Number	THIAMIN		NIACIN		VITAMIN B6		PANTOTHENIC ACID		RIBOFLAVIN	
	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2
1	1.56	1.55	10.65	10.62	1.06	1.3	N/A	N/A	1.91	1.56
2	1.71	1.69	10.8	11.61	1.09	1.07	6.49	6.52	1.51	1.66
3	1.4	1.4	10.4	10.2	1.1	1.1	5.2	5.1	1.4	1.5
4	1.46	1.44	10.60	10.80	1.11	1.10	5.66	5.79	1.39	1.40
5	1.4	1.4	10.6	10.6	1.1	1.1	4.9	4.8	1.2	1.2
6	1.32	1.32	10.64	10.49	1.08	1.09	5.86	7.58	1.20	1.18
7	1.7	1.8	10.6	11.1	1.3	1.3	5.7	5.8	1.4	1.3
8	1.43	1.44	11.08	10.81	1.21	1.15	5.39	5.14	1.23	1.24
10	1.50	1.49	9.31	9.98	1.11	1.13	NA	3.63	1.42	1.35
11	1.36	1.34	10.74	10.87	1.13	1.11	5.94	5.90	1.19	1.23
12	1.5	1.4	10.6	11.9	1.0	1.0	6.1	7.3	1.6	1.6
13	1.4	1.4	10.5	10.6	1.1	1.1	7.4	7.0	1.2	1.3
14	1.4	1.4	9.5	9.5	1.1	1.2	7.4	7.2	1.0	0.9
15	1.50	1.38	11.08	10.51	1.23	1.10	6.84	6.84	1.51	1.43
16	1.31	1.20	11.30	10.50	1.14	1.07	5.83	5.61	1.23	1.17

Table 33: Analytical data submitted by each laboratory – Sample C (mg /Tablet)

Lab. Number	THIAMIN		NIACIN		VITAMIN B6		PANTOTHENIC ACID		RIBOFLAVIN	
	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2
1	1.76	1.75	17.96	18.69	2.24	2.19	N/A	N/A	1.98	1.94
2	1.93	2.23	20.21	20.43	2.08	2.26	11.98	13.06	1.72	2.25
3	1.7	1.7	20.1	19.5	2.3	2.1	10.2	11.3	1.7	1.6
4	1.75	1.73	19.90	19.90	2.25	2.18	9.89	9.79	1.74	1.75
5	1.7	1.7	19.5	19.8	2.2	2.2	10.5	10.6	1.7	1.8
6	1.57	1.56	18.82	18.90	2.14	2.14	13.41	13.50	1.56	1.57
7	2.2	2.2	20.1	19.8	2.7	2.6	10.4	10.2	1.6	1.5
8	1.72	1.71	20.39	20.09	2.26	2.24	9.25	9.03	1.59	1.73
10	1.75	1.73	20.40	19.90	2.26	2.20	9.00	10.90	1.71	1.66
11	1.73	1.70	19.92	20.00	2.24	2.28	10.87	10.99	1.95	1.97
12	1.6	1.7	18.4	18.8	1.9	2.0	10.4	10.5	1.5	1.6
13	1.7	1.7	19.0	19.5	2.2	2.3	11.7	11.8	1.7	1.7
14	1.7	1.7	20.1	19.9	2.2	2.2	11.9	12.4	1.6	1.6
15	1.66	1.72	20.23	19.91	2.31	2.27	11.80	11.25	1.66	1.71
16	1.71	1.72	19.50	19.80	2.38	2.44	12.12	11.69	1.68	1.67

Table 34: Analytical data submitted by each laboratory – Sample D (mg / Capsule)

Lab. Number	THIAMIN		NIACIN		VITAMIN B6		PANTOTHENIC ACID		RIBOFLAVIN	
	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2
1	3.00	2.98	20.64	20.54	9.11	8.92	N/A	N/A	1.91	1.86
2	3.18	3.24	22.33	23.71	8.77	8.81	8.9	8.93	1.7	1.81
3	2.9	2.6	22.0	21.5	9.2	8.7	8.4	9.0	1.6	1.2
4	2.80	2.88	21.90	22.60	9.00	9.30	7.61	8.10	1.41	1.40
5	2.7	2.7	22.0	22.3	9.1	9.3	8.5	8.8	1.6	1.3
6	2.56	2.52	21.46	21.33	9.12	9.03	5.70	5.65	1.26	1.09
7	3.7	3.8	22.2	21.2	11.3	11.8	8.7	8.6	1.7	1.7
8	2.58	2.70	21.40	22.37	8.81	9.35	6.81	7.36	1.15	1.08
10	2.85	2.92	23.70	22.50	9.21	9.19	2.46	8.53	1.40	1.53
11	2.59	2.63	21.28	21.73	8.73	8.91	6.66	6.83	1.46	1.56
12	2.7	2.7	21.6	21.1	8.2	8.0	9.0	8.6	1.9	1.9
13	2.8	2.8	22.0	21.8	9.6	9.6	9.4	9.7	1.4	1.4
14	2.7	2.8	22.4	22.4	9.1	9.2	10.2	10.5	1.3	1.3
15	2.75	2.71	22.25	22.29	9.38	9.39	9.14	9.40	1.17	1.24
16	2.85	2.85	22.30	22.20	9.49	9.46	7.72	7.98	1.71	1.75

Table 35: Analytical data submitted by each laboratory – Sample E (mg / Capsule)

Sample	Dosage form	Wt per Tablet or Capsule (mg)
A	Tablet	1.3660
B	Tablet	0.9980
C	Tablet	0.7949
D	Capsule	1.3470
E	Capsule	0.6860

Table 36: Mean Tablet and Capsule masses

Sample	A	B	C	D	E
Ho_r	1.14	0.59	1.12	0.87	1.13
Ho_R	1.89	1.52	2.15	0.88	3.60

Table 37: HoRrat ratios for Riboflavin data with additional data removal

Key to Tables

Mean: n_1 , units	The mean of all the data used in the statistical analysis and units
Mean rep1...rep n	The mean of the first results set the mean of the n -th results set
n , no of reps	The total number of sets of data submitted, no of replicates
nc	Number of non compliant data entries
outliers	The number of results excluded from statistical analysis due to determination as outliers by either Cochran's or Grubbs' tests
n_1	The number of results used in statistical analysis
r	The repeatability limit (within laboratory variation) - the value below which the absolute difference between two single test results obtained with the same method on identical test material under the same conditions may be expected to lie with 95% probability
s_r	The standard deviation of the repeatability
RSD_r , RSD_rH0	The relative standard deviation of the repeatability ($S_r \times 100/MEAN$), the relative standard deviation of the repeatability of the Horwitz value.

H0 _r	The HORRAT value for repeatability is the observed RSD _r divided by the RSD _r value estimated from the Horwitz equation using the assumption $r = 0.66R$
R	The reproducibility limit (between-lab variation) - the value below which the absolute difference between two single test results obtained with the same method on the identical test material under different conditions may be expected to lie with 95% probability
S _R	The standard deviation of the reproducibility
RSD _R , RSD _{RH0}	The relative standard deviation of the reproducibility ($S_R \times 100/\text{MEAN}$) and the relative standard deviation of the reproducibility of the Horwitz value.
H0 _R	The HORRAT value for reproducibility is the observed RSD _R value divided by the RSD _R value calculated from the Horwitz equation
Cochran's	An outlying result as determined by Cochran's Test at $P < 0.01$ sample, not used in calculation of statistical parameters
Single Grubbs'	An outlying result as determined by single Grubbs' Test, $P < 0.05$ sample not used in calculation of statistical parameters
Double Grubbs	A pair outlying results as determined by double Grubbs' Test, $P < 0.05$ sample not used in calculation of statistical parameters

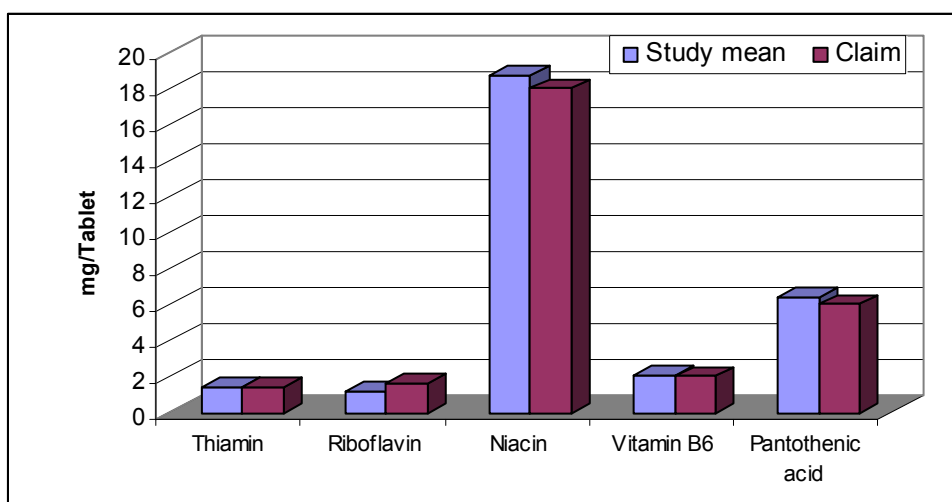


Figure 1 : Comparison of study mean to label claim – Sample A

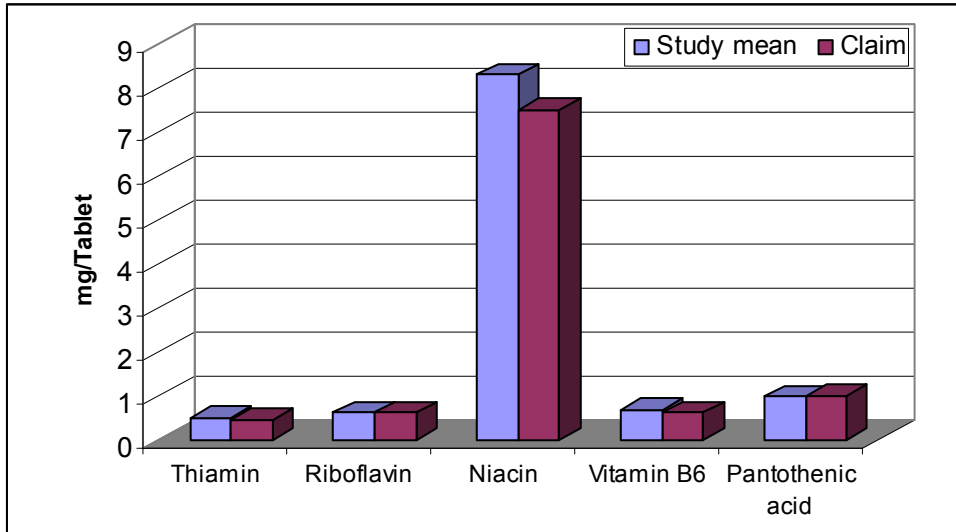


Figure 2 : Comparison of study mean to label claim – Sample B

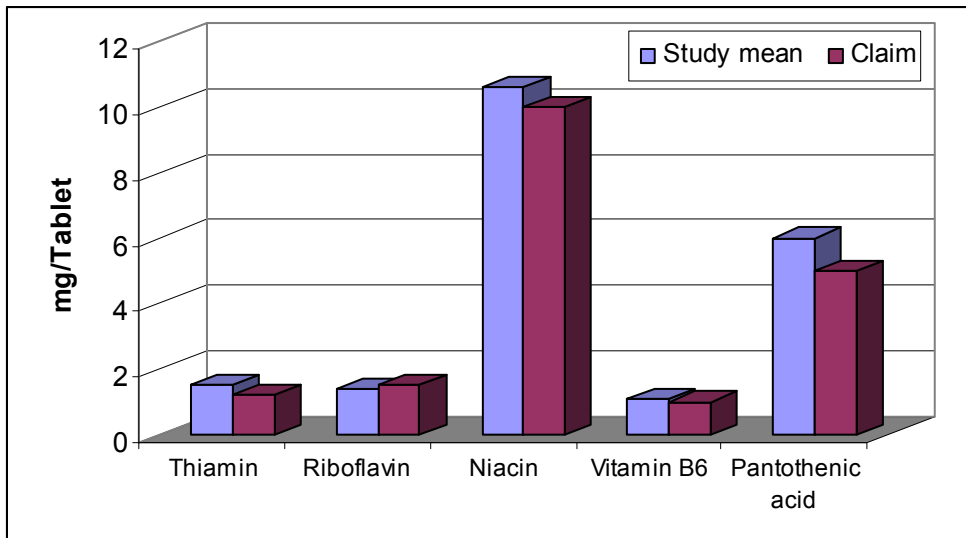


Figure 3 : Comparison of study mean to label claim – Sample C

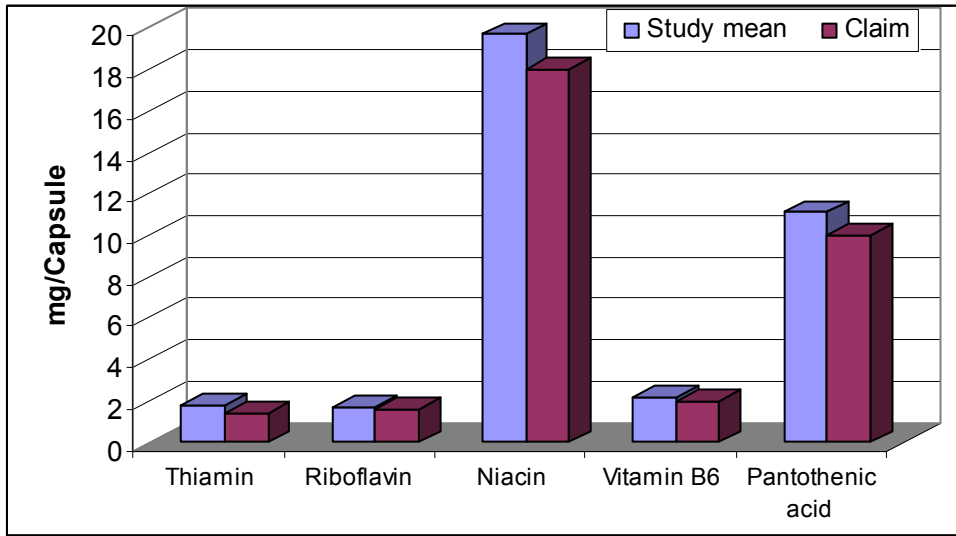


Figure 4 : Comparison of study mean to label claim – Sample D

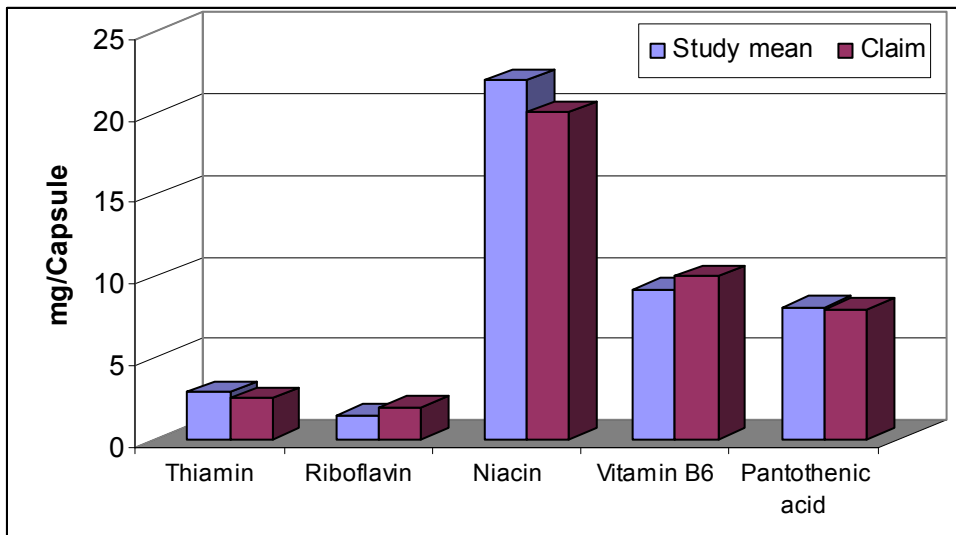


Figure 5 : Comparison of study mean to label claim – Sample E

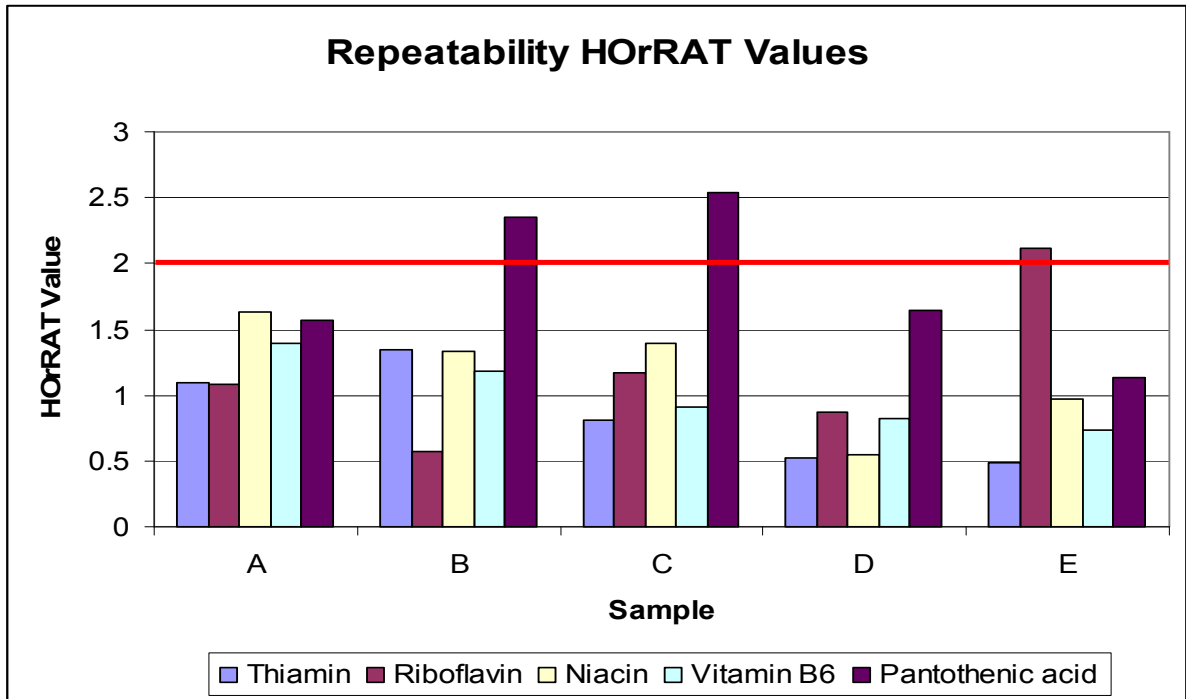


Figure 6: Summary of HorRat values for repeatability

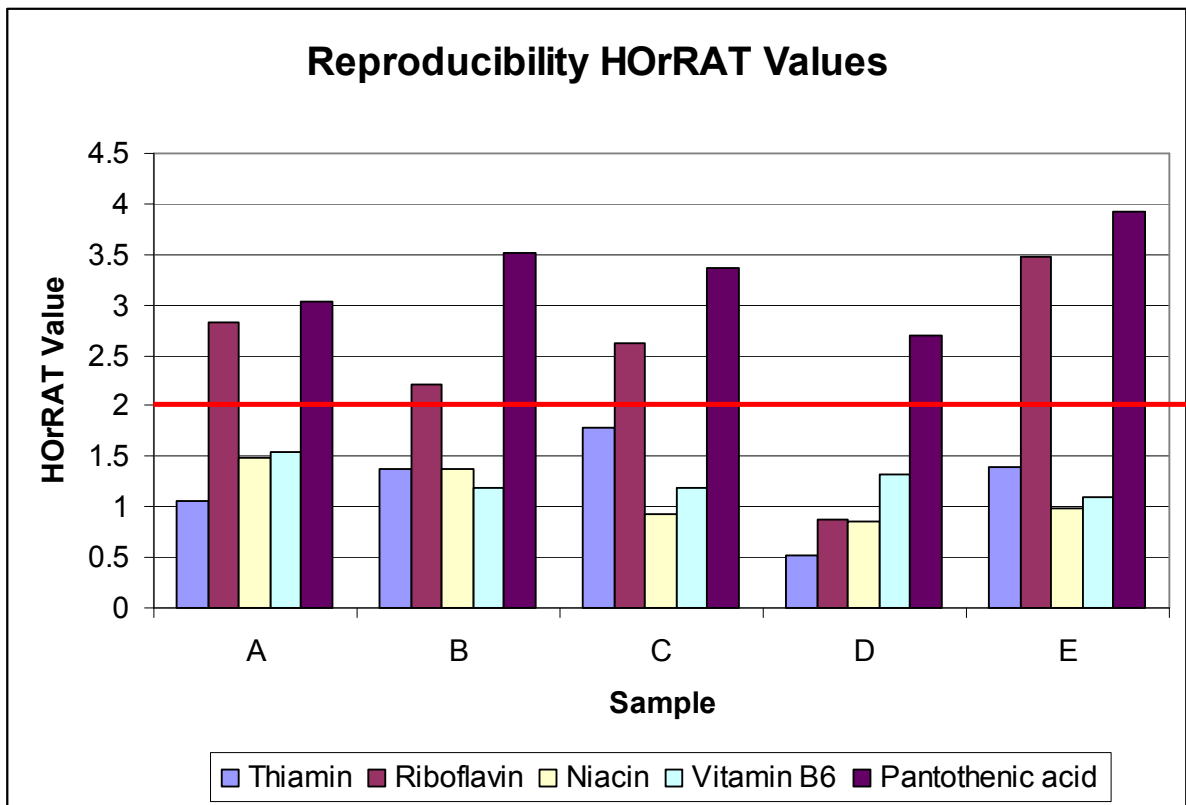


Figure 7: Summary of HorRat values for reproducibility

Annex C HPLC systems

HPLC columns and conditions were recommended but participants were allowed to vary these if system suitability was appropriate. The HPLC conditions used are shown in Table 1.

Table 1: HPLC conditions used by each participant

Lab No.	HPLC Used
1	As method
2	Lichrocart Lichrospher 100,RP18 5 μ , Gradient as method.
3	Supelco, Discovery C18 150 x 3 -5 μ , Gradient as method.
4	Licrocart, Licrospher 100 RP18, 5 μ ,125 x 4. Gradient as method.
5	As method but modified gradient used.
6	As method
7	Lichrocart, Lichrospher 100 250 x 3- 5 μ , modified gradient.
8	As method but modified gradient used.
10	As method but modified gradient used.
11	As method
12	As method but modified gradient used.
13	As method
14	N/A
15	Synergi 4 Hydro RP 80. Modified gradient
16	As method

The column and conditions suggested in the method protocol in Annex A were used unless otherwise specified. Several participants made modifications to the times or mobile phase concentrations to optimise the separation on their systems.

Annex D Standards and calibration

Source of standards

Standards were recommended but not supplied. The standards sources used are shown in Table 1:

Table 1: Standards used for the collaborative study

Lab No.	B1HCl	B2	Nicotinamide	B6 HCL	Ca Pantotheate
1	sigma usp	sigma usp	sigma usp	sigma usp	sigma usp
2	fluka	fluka	sigma	fluka	fluka
3	Acros	Acros	Acros	Acros	Acros
4	sigma	fluka	sigma	sigma	sigma
5	sigma usp	sigma usp	sigma usp	sigma usp	sigma usp
6	sigma usp	sigma usp	sigma usp	sigma usp	sigma usp
7	sigma	sigma	sigma	sigma	sigma
8	sigma	sigma	sigma	alldrich	sigma
10	sigma	sigma	sigma	sigma	sigma
11	sigma	sigma	sigma	sigma	sigma
12	sigma	sigma	sigma	sigma	sigma
13	sigma	sigma	sigma	sigma	sigma
14	N/A	N/A	N/A	N/A	N/A
15	qmx cert	sigmausp	sigmausp	qmx cert	sigmausp
16	sigma usp	sigma usp	sigma usp	sigma usp	sigma usp

All calibrations were linear and complied with the protocol. One participant used a higher calibration range and several said that the lowest standard was difficult to measure and unnecessary.

Annex E Participants Comments

The participants made the following comments:

Lab 1: No peak found for pantothenic acid. Some coelution between riboflavin and folic acid present.

Lab 2: Slightly higher calibration range used due to small peaks at low levels. Sample weight increased to 2g. Niacin and pantothenate calibrations not as good as others. Some coelution between B6 and folic acid.

Lab3: Five tablets/capsules seems too many for levels found. Suggested protocol duplication etc required long run-times with potential for degradation.

Lab 4: 30mins in sonic bath at 60°C with shaking used. Far UV Me CN used to allow measurement down to 205 for pantothenate. Uncooled autosampler used but seems OK.

Lab 6: Comments made re protocol Standard descriptions etc. Details for standard checking by UV would be beneficial.

Lab 7. No need to pre-mix solvent B. Used modified gradient with 100% MeCN as B to give equivalent gradient. Sample 1 difficult to grind coating – Some removed. Retention times not stable on his system.

Lab 8: Used a weight of powder equivalent to 5 tablets. Modified gradient to resolve riboflavin and folic acid. Some late eluting peaks could also carry over. Lowest standard not required.

Lab 10: Pantothenate and niacin not separated in sample c (1st injection only). Niacin peak split in sample E (1st injection only). Needed modified gradient despite specified column. Lowest standard not required.

Lab 12: Manual injections used. Retention times not stable (more equilibration?) Poor resolution between riboflavin and folic acid at 275nm.

Lab 13: Pantothenate difficult to measure. Possible interferent. Calibration did not go through zero. Some overlap between pantothenate and folic acid in second batch only.

Lab 15: Tablet A coating was hard to grind manually. No cooled autosampler but OK. 0.1M HCl & NaOH needed, not 1M as in method.... *(Modified {Ed..})*



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The Determination of Vitamins A, D & E in Vitamin Supplements

Collaborative Study Report

December 2006

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The Determination of Vitamins A, D & E in Vitamin Supplements – Collaborative Study Report

December 2006

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This Study was carried out as part of a project funded by the Food Standards Agency which aims to evaluate and improve methods for the determination of vitamins in Food Supplements.



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Summary

This collaborative Study was carried out as part of Project E01057- The development of fully validated procedures for the determination of vitamins in dietary supplements.

This project seeks to improve procedures and to provide guidance to enforcement and other analysts in the selection of appropriate methods of analysis when determining vitamins in food supplements.

Test procedures were reviewed and evaluated at LGC to determine their suitability for use with different supplement formulations. The study procedure was designed to be suitable for the analysis of the oil-soluble vitamins A, D and E in a wide range of supplements. Following in-house evaluation, it was submitted for further validation by collaborative trial.

The study participants were mainly UK Public Analysts as these laboratories are primarily responsible for enforcement analysis in the UK and reliable methods are vital for this task.

Six test materials, representing a range of different supplement formulations, were supplied to LGC by supplement manufacturer's and were distributed to the participating laboratories as blind duplicates. Participants were asked to analyse the samples using the method supplied and to report the results to LGC. These results were then evaluated according to the IUPAC/ AOAC statistical approach.

For Vitamin A, the method bias was acceptable for all but one sample. The repeatability was acceptable for two samples but was too variable for the remainder (HorRAT 2.0 -3.1). The reproducibility variation was also slightly high in all but one sample (HorRAT 2.0 – 2.7). Some improvement would be desirable.

For Vitamin D, the method bias was acceptable and the precision parameters were acceptable (HorRAT <2) except for Sample F where the repeatability HorRAT value was slightly high (2.1). Although the final data was acceptable, some data was rejected as invalid because of chromatographic interferences. A modified mobile phase is recommended to prevent this.

For Vitamin E, the bias was acceptable but the precision parameters were too variable. The HorRAT values were between ~1.7 - 3.5 for r and ~ 2.7 – 4.9 for R, indicating a significant inter-laboratory effect. The %RSD values for this analyte however, were similar to those for vitamins A & D₃ but since the predicted Horwitz value is concentration dependant, the HorRAT ratios are higher. Further work is required to examine the variability for Vitamin E.

The method was shown to be suitable for the determination of vitamins A, D & E in a range of supplements during in-house testing but the reported data for Vitamin A and to a greater extent, Vitamin E from the collaborative study was more variable than is desired. It is likely, that much of this variation may be due to lack of familiarity with the procedure coupled in some cases with chromatographic difficulties. The analyst must be aware of the potential for extraction and or chromatographic problems and in the usual context, the analyst would be guided by the label as to the analytes and ingredients present which would help to reduce the potential for analytical problems.

1. Introduction

A wide range of vitamin supplements are available to the consumer in the UK via retail outlets, mail order and the Internet. The majority of these products are produced to exacting specifications by reputable manufacturers but the quality of other products has often been questioned. It is expected that vitamin supplements contain the correct forms and amounts of vitamins that are declared on the product label and, in cases of dispute, it is important that accurate methods of analysis for the determination of vitamins in supplements are available

In most cases, methods of analysis are available but these methods are not harmonised. Differences in results produced by these methods have led to disputes over the apparent content of tested samples. In addition, vitamin supplements are produced in a number of different forms and as new formulations are developed, new ingredients could cause analytical problems with some existing procedures.

This report details a collaborative trial of analytical methodology developed within the Food Standards Agency Project E01057 for the determination of vitamins A, D and E in a range of vitamin supplements.

2. Collaborative trial organisation and participation

Method to be tested

The method followed in the collaborative study involved saponification of the dietary supplement in alcoholic potassium hydroxide solution and extraction of the vitamins into a solvent. Vitamin D₂ was added before saponification, as an internal standard for the determination of vitamin D₃. Vitamins A (expressed as *all-trans* retinol) and E (expressed as α -tocopherol) in the extracts were determined by reversed-phase HPLC with UV spectrometric detection. For vitamin D, interfering components from the extract were removed by semi-preparative, normal phase HPLC. Vitamin D was then determined by reversed-phase HPLC with UV spectrometric detection.

Details of the methodology employed are given in Annex A.

Test materials

Five different commercial supplement formulations were used. These were chosen to provide a range of different product types, ingredients, vitamins, minerals, and analyte concentrations. These materials were taken from the bulk products during manufacture and were supplied direct from the manufacturing plant. LGC was supplied with the details of the specifications and formulations but these may be commercially sensitive and are not reported. A sixth sample was purchased from a retail outlet.

Bulk materials were divided into individual samples of five tablets/capsules in screw-capped, polythene tubes which were stored in the dark, in a fridge until dispatch.

Collaborative trial samples were sent to participants as blind duplicates labelled, 1-12, to be analysed once only. HPLC conditions were recommended but participants were allowed

to use alternative columns and conditions if desired. Since all of the chosen laboratories were familiar with similar methods for vitamin analysis, no practice round was needed, but the study director was available for advice if required. Participants were not given any details of the test samples supplied even though label information would normally be available.

Sample details are shown below:

Sample A (1, 8) - Coated, Multivitamin and Mineral Tablet

Inactive ingredients included calcium carbonate, dicalcium phosphate, sorbitol, magnesium stearate, microcrystalline cellulose and hydroxyl propyl methyl cellulose

Active ingredients included Cr, Cu, K, I, Mg, Mn, Mo, Zn, & Se salts, B-group vitamins, vitamin C, vitamin K, vitamin A acetate, vitamin E & vitamin D₃

Sample B (2, 10) - Chewable Multivitamin Tablet with Iron and Calcium

Inactive ingredients included calcium carbonate, sorbitol, magnesium stearate, colours, sweeteners & flavourings.

Active ingredients included Ca, Fe, K & I salts, B-group vitamins, vitamin C, vitamin A acetate, vitamin E, & vitamin D₃.

Sample C (3, 7) - Chewable multivitamin and mineral tablet

Inactive ingredients included calcium carbonate, sugar, stearic acid, citric acid, magnesium stearate, silica, sweeteners & flavourings

Active ingredients included Ca & Fe salts, B-group vitamins, vitamin C, vitamin A acetate, vitamin E succinate, & vitamin D₃

Sample D (4, 12) - Multivitamin soft-gel capsule with cod liver oil

Inactive ingredients included gelatine.

Active ingredients included B-group vitamins, vitamin C, vitamin A, vitamin E acetate, vitamin D₃ & cod liver oil.

Sample E (5, 9) - Multivitamin and mineral soft-gel capsule with cod liver oil

Inactive ingredients included gelatine.

Active ingredients included Fe, Mg, Ca, Zn, Cu, I, Mn, Mo, K, P, B-group vitamins, vitamin C, inositol, choline, vitamin A, vitamin E acetate, vitamin D₂ & cod liver oil.

Sample F (6, 11) - Multivitamin Tablet

Inactive ingredients included dicalcium phosphate, microcrystalline cellulose, carboxy methyl cellulose, magnesium stearate, hydroxyl propyl methyl cellulose & glycerol.

Active ingredients include vitamin C, B-group vitamins, vitamin A acetate, vitamin E acetate & vitamin D₃.

Homogeneity testing

Homogeneity and product stability testing was undertaken by the manufacturer's as part of their normal manufacturing process. Since homogeneity and stability testing was undertaken by the manufacturers, no further testing was carried out by LGC.

Participants

Seventeen laboratories agreed to participate in the study although, for operational reasons, only 14 laboratories submitted results. Of these, ten were UK Public Analyst's laboratories from England, Scotland and Wales; two were UK private-sector analytical laboratories, one was a European State-funded food and feed control laboratory, and one (LGC) was the official UK, referee-analyst laboratory. A list of the participating laboratories is shown in the Acknowledgements section of this report.

Statistical analysis of results

The trial results were examined for evidence of individual systematic error ($p < 0.025$) using Cochran's and Grubbs' tests progressively, by procedures described in the internationally agreed Protocol for the Design, Conduct and Interpretation of Method-Performance Studies [1].

Calculations for repeatability (r) and reproducibility (R) as defined by that protocol were carried out on those results remaining after removal of outliers. The results are given in Annex B; Tables 4 to 20 and the performance characteristics are summarised in Tables 1 to 3 of the Annex.

When assessing a new method there is often no validated reference or statutory method with which to compare precision characteristics, hence it is useful to compare the precision data obtained from a collaborative trial with "predicted" levels of precision. These "predicted" levels are calculated from the Horwitz equation. Comparison of the trial results and the predicted levels give an indication as to whether the method is sufficiently precise for the level of analyte being measured [2].

Historically the Horwitz predicted value has been calculated from the Horwitz equation as shown in equation {1}

$$\{1\} \quad RSD_R = 2^{(1-0.5 \log C)}$$

where C = measured concentration of analyte expressed as a decimal (e.g. 1 g/100g = 0.01).

Thompson has recently described the use of a modified Horwitz function to predict levels of precision at $\mu\text{g}/\text{kg}$ and sub $\mu\text{g}/\text{kg}$ levels up to 120 $\mu\text{g}/\text{kg}$ [3]. The use of this function is shown to give an improved statistical representation at these levels.

For the purposes of this trial, samples analysed all contained vitamins at concentrations greater than 120 $\mu\text{g}/\text{kg}$ hence the Horwitz predicted values were calculated from the Horwitz equation {1}.

The HORRAT value [4] gives a comparison of the actual precision measured with the precision predicted by the Horwitz equation for a method measuring at that particular level of analyte. It is calculated as detailed in equation {2}.

$$\{2\} \quad H_{OR} = RSD_R (\text{measured}) / RSD_R (\text{Horwitz})$$

A HORRAT value (H_{oR}) of 1 usually indicates satisfactory inter-laboratory precision, whereas a value of >2 usually indicates unsatisfactory precision, i.e. one that is too variable for most analytical purposes or where the variation obtained is greater than that expected for the type of method employed.

H_{oF} is also calculated using equation {3} and used to assess intra-laboratory precision,

$$\{3\} \quad H_{oF} = RSD_f \text{ (measured)} / RSD_r \text{ (Horwitz)}$$

This assumes the approximation that $r = 0.66R$ and that therefore, $RSD_f \text{ (Horwitz)} = 0.66 RSD_R \text{ (Horwitz)}$. [2]

3. Results and Discussion

Results were submitted by fourteen laboratories. The laboratory numbers were randomly assigned at the start of the study and have been retained throughout. Consequently, where no results were returned, these laboratory numbers are missing. The sample numbers were decoded after the results had been returned. The data was audited and participants were invited to comment on any results which showed a large deviation from the remaining data-set. Tables 22–25 of Annex B show all of the valid data which was returned by the participants on a weight per tablet/capsule basis.

Raw data supplied by participants were in the form of mass of vitamin per tablet (or per capsule). For repeatability and reproducibility estimation, all values were converted to mass of vitamin per mass of dosage form (mg/g or $\mu\text{g/g}$). Mass fractions were calculated using the mean tablet mass (derived from reported weighing data) shown in Table 26 of Annex B. Note that use of mean tablet mass can in principle increase apparent repeatability and reproducibility standard deviations if individual tablets differ significantly in mass.

Statistical evaluation and conclusions

All results submitted were reviewed by the study co-ordinator and any gross discrepancies were discussed in general terms with the participant to ascertain whether there were any technical reasons for the differences observed. Where such reasons were identified, the data was rejected as invalid. Where no reasons could be found, data was retained for further analysis.

Valid data was evaluated following the IUPAC Harmonised Protocol. This protocol requires automatic outlier rejection at the 97.5% level of confidence, with repeated outlier testing and rejection to a maximum of 2/9 (22%) of the valid data. Outlier tests were performed for outlying variance using Cochran's test, and for outlying means using the three Grubbs tests. Additional outlier testing was performed for single observations for a single sample where visual inspection suggested an extreme value.

All data for Lab 17 was removed prior to processing as the laboratory did not use the analytical method under study.

Vitamin D_3 data for sample E were not processed, as the sample contained no vitamin D_3 . It was included in the study to show whether laboratories would detect the presence of vitamin D_2 in this sample.

Data inspection, outlier identification and calculations

In order to calculate the HorRat values, the results were converted to a mass/mass basis (e.g. $\mu\text{g/g}$) using the mean tablet or capsule weights shown in Table 26 of Annex B.

Outlier tests were carried out in conjunction with the repeatability and reproducibility calculations. Visual inspection via dot plots and distribution plots (examples are shown for Vitamin A in Figure 1 below) was used to review individual data sets for serious departures from normality and to identify any extreme values prior to applying Cochran and Grubbs tests on laboratory means. Data identified by the protocol as outliers were removed before calculation of the performance characteristics. In some cases, inspection or borderline outlier tests led to additional consideration and/or treatment for a particular set; these considerations are detailed below where treatment differed from the Protocol defaults or where additional information was obtained. Precision values were compared to the expected Horwitz values at an appropriate level and HorRat values were calculated. Summaries of the method performance data determined are given in Tables 1, 2 & 3 of Annex B.

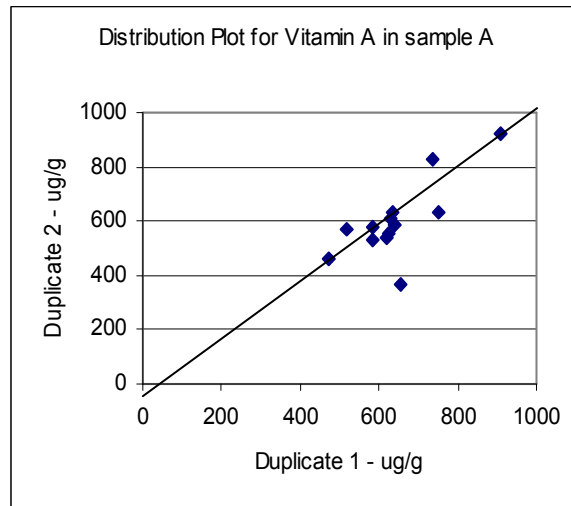
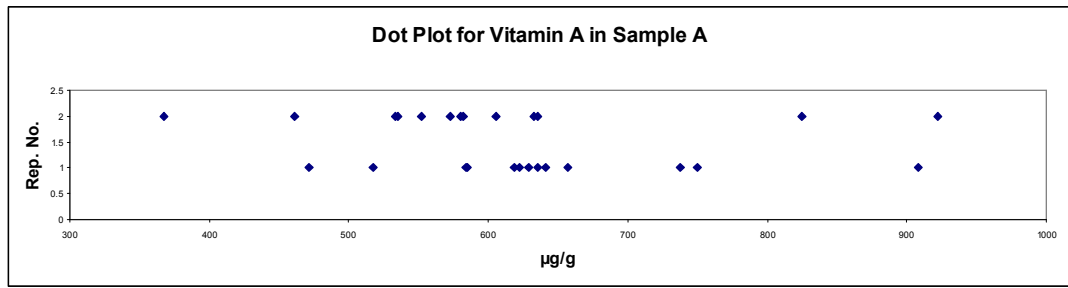


Figure 1: Dot Plot and Distribution Plot for Vitamin A in Sample A

Vitamin D

Sample E contained Vitamin D₂ instead of Vitamin D₃. It was included to check whether participants would detect the presence of vitamin D₂ as is sometimes added to vitamin supplements. The results reported for Vitamin D₃ in these samples are shown in Table 23 in Annex B.

Only three laboratories (one subsequently rejected as an in-house method was used) correctly indicated the presence of Vitamin D₂. Of the remainder, four laboratories reported vitamin D₃ concentrations greater than 1µg/g in at least one replicate whilst the others reported lower values. These false positive results are due to the presence of small (or in a few cases, larger) interfering peaks in the D₂/D₃ region of the chromatogram. This emphasises the importance of optimising the chromatography and checking for interfering peaks. Modification of the analytical HPLC mobile phase for vitamin D would be beneficial to minimise matrix interferences although in the normal course of events, the form of Vitamin D₂ added would be indicated on the sample label. The use of vitamins D₂ and D₃ together is not permitted.

The remaining vitamin D results for Lab 13 (the highest false positive) were reviewed and additionally outlier tested at the 95% level, with a view to removal given prior grounds for presuming technical error. In practice, no Lab 13 data were removed for this reason, although some were removed as 97.5% outliers following the normal IUPAC/AOAC criteria.

Vitamin D, Sample C

The initial outlier testing cycle identified Lab 10 as a Cochran outlier, and Lab 8 as a single low Grubbs outlier. After removal of these results (two of 9), a second cycle identified Lab 15 as a Grubbs outlier by comparison with the remaining laboratories. Lab 15 was, however, retained, as further rejections would exceed the maximum data rejection threshold of 22%.

Vitamin D Conclusions

The repeatability and reproducibility RSD's were similar in Samples A-E and the HorRat values were acceptable (<2). The Ho_R value for sample F was acceptable but was slightly higher than those for the remaining samples. The Ho_r value for this sample was slightly high.

It should be noted that a number of laboratories experienced difficulties with the analytical separation and several data points were rejected as invalid because of reported chromatographic interferences. In the author's experience, these difficulties can be largely avoided by selection of an alternative mobile phase (e.g. acetonitrile/methanol; 97/3) to extend the retention time of vitamins D₂ & D₃ and improve their resolution from other sample components.

Vitamin A

Vitamin A, Sample D

Table 12 shows Lab 8 as a Cochran outlier and Lab 7 as a Grubbs outlier. In fact, elimination of both by Grubbs test criteria would also be justified. The first cycle of outlier tests identified Lab 8 as a Cochran test outlier, but Labs 7 and 8 were additionally identified as paired Grubbs outliers in the initial cycle of outlier tests. Elimination of Lab 8

and retesting confirmed that Lab 7 remained a single Grubbs outlier (exceeding the 99% critical value as well as the AOAC/IUPAC 97.5% criterion).

Vitamin A, Sample E

Three laboratories were marked as outliers in the initial outlier test cycle: Lab 7 as a single Grubbs outlier, Labs 6 and 7 were marked as a low pair and Lab 7 and Lab 13 as an extreme high/low pair. The multiple test failures were all attributable to the extreme Lab 7 result: After eliminating Lab 7, a second outlier test cycle showed no outliers.

Vitamin A Conclusions

The within and between laboratory precision was generally too high in these samples. Hor values ranged between 1.6 and 3.1 and the HoR values ranged between 1.5 and 2.7. There did not seem to be a large interlaboratory effect but the within laboratory repeatability was higher than expected in some cases.

Vitamin E

Vitamin E, Sample D

Initial outlier testing identified Lab 7 as a single extreme outlier and Lab 7 and Lab 13 as a low pair. On retesting after eliminating Lab 7 only, Lab 13 was not identified as an outlier, even at the less stringent 95% level. Lab 13 was accordingly retained.

Vitamin E Sample F

Visual inspection showed that one of the Lab 5 results for sample F was unusually low, at 14.70 mg/g. Outlier testing for the individual point at the 97.5% level showed the item as borderline (Grubbs 1 test statistic 28.5 against a critical value of 33.8); therefore the point was retained.

Vitamin E Conclusions

The repeatability and reproducibility parameters for all of the samples were larger than expected for analyte concentrations at this level as the HoRrat values were unacceptable (>2) in almost all cases. The %RSD's for vitamin E were similar to those for the other vitamins despite the higher level of the target analyte present which suggests that the variability may not be concentration dependant in this case. Further work is needed to investigate the cause of the variability.

General Precision Conclusions

Reviewing the reproducibility data, the reproducibility standard deviations are broadly similar and not strongly dependent on analyte level. Since the Horwitz ratio is concentration-dependent, the vitamin E data shows larger Horwitz ratios (HorRAT), while some of the lower concentration vitamins appear to perform well against the Horwitz criteria.

There is no clear relationship between repeatability and reproducibility for the different vitamins (Fig 2). Although the lower s_R/s_r ratios tend to be for the low-concentration samples in this data set, particularly vitamin D, there is no statistically significant trend, and s_R/s_r is above 2 for at least some of the results for each vitamin. Between-laboratory effects are therefore substantial for at least some examples of all vitamins, if (for this data set) more often dominant for vitamin E.

The reason for the larger variability for the determination of vitamin E is unclear and requires further evaluation. It is likely that at least some of the variability in the data for all vitamins is due to lack of familiarity with the analytical method and the precision would be expected to improve if further rounds were undertaken.

During “in-house” validation, the method studied was used to determine the oil-soluble vitamins in a range of different supplements and satisfactory recovery and precision was obtained.

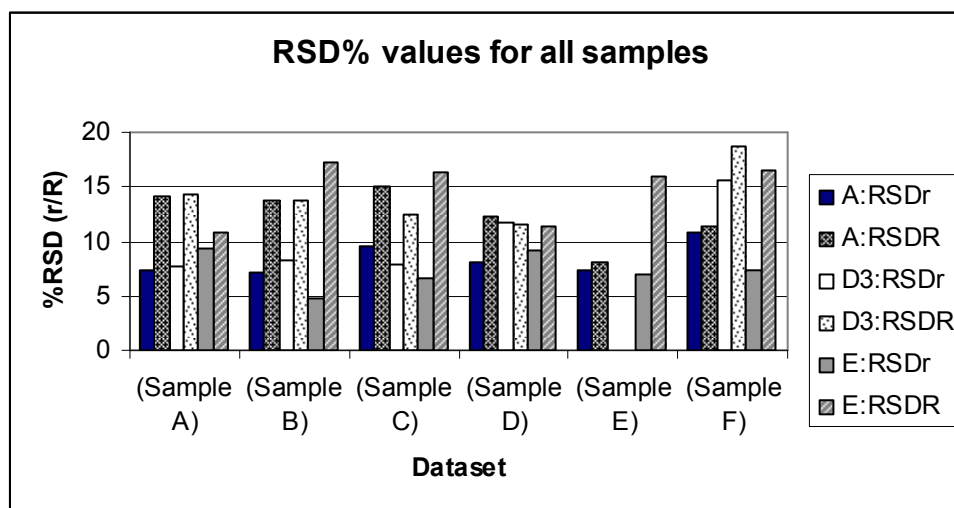


Figure 2: RSDr & RSDR values for all samples and vitamins

Bias

It was not possible to accurately determine the bias within the current study for the following reasons:

1. Samples were supplied by the manufacturers (except for one sample which was purchased from a retail outlet). Although in some cases, input data was supplied, the variation in the information supplied and the tolerances which applied were such that it was not always possible to calculate the actual value expected in each product. It is common practice to add an additional amount of a vitamin to that declared on the label (an overage) to allow for degradation of the vitamins during the product lifetime. The samples should have at least the amount declared on the product label but may contain a higher amount of vitamins within the permitted tolerance depending upon the age of the product.
2. The samples had been held in a fridge at LGC for several months before being used in this study. Although there should be little change under these storage conditions and all of the samples were stored under the same conditions, no additional stability testing was undertaken before distribution.

The bias was therefore assessed against the label claims provided.

For vitamin A, the study mean was slightly higher than or equal to the claimed values as expected, for all samples other than sample F which gave a slightly low result (84%).

For Vitamin D₃, the study means were higher than, or equal to the claimed values although sample F was again, slightly low (92%).

For vitamin E, the study means were higher than or equal to the claimed values for all samples.

4. Acknowledgements

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Nigel Payne	Pattinson Scientific Services
Chris Heide	Somerset Scientific Services
Frank Hollywood	Staffordshire County Laboratory & Scientific Services
Duncan Campbell	West Yorkshire Scientific Services
Steve Revett	Direct Laboratories

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Annex A – Analytical Method

The determination of Vitamins A, D & E in food supplements

1 Purpose

The method was developed for the determination of oil soluble vitamins in food supplements as part of a study commissioned by the Food Standards Agency. The protocol has been validated by collaborative study as described in this report.

2 Scope

This SOP specifies a method for the determination of vitamins A, D and E in vitamin supplements by high performance liquid chromatography.

It is applicable to the determination of the following:

Vitamin A as all trans-retinol

Vitamin E as α -tocopherol,

Vitamin D as Ergocalciferol (D2) and Cholecalciferol (D3)

Vitamin A isomers are not separated.

Vitamin E isomers may be separated on some HPLC systems but their determination is not described.

Vitamin esters are hydrolysed to the corresponding alcohols and are determined as alcohols.

Vitamin D3 is the most common form of vitamin D used in supplements but Vitamin D2 can sometimes be used. Either form can be determined using this procedure providing that they are not both present.

3 Principle of Analysis

The dietary supplement is saponified in alcoholic potassium hydroxide solution and the vitamins are extracted into a solvent. Vitamin D2 is added before saponification, as an internal standard for the determination of vitamin D3 (*or vice versa*). Vitamins A and E in the extract are determined by reverse phase HPLC with UV spectrometric detection. For vitamin D, interfering components from the extract are removed by semi-preparative, normal phase HPLC. Vitamin D is then determined by reverse phase HPLC with UV spectrometric detection.

4 Apparatus

- 4.1 General laboratory glassware and equipment including the following:
- 4.2 Variable wavelength, UV spectrophotometer and 1 cm path length quartz cells
- 4.3 Balance, analytical. 4 decimal place.
- 4.4 Heating mantle, fitted with water cooled reflux condensers.

- 4.5 Turbovap or rotary evaporator, operating at 35- 40°C.
- 4.6 Saponification flasks: 150 ml round-bottomed.
- 4.7 500 ml separating funnels
- 4.8 250ml separating funnels.
- 4.9 Automatic pipettes.
- 4.10 Fraction collection tubes, glass
- 4.11 Filter paper, glass fibre (GF/A)
- 4.12 HPLC Equipment
 - 4.12.1 Semi-Preparative HPLC system (for vitamin D only)
 Consisting of a pump (1.5ml/min), sample injection device (150µl), column (Partisil 5 PAC e.g. Jones chromatography Ltd, 25cm x 4mm id), UV detector (265nm), a means of collecting a defined portion of the column eluent (fraction collector) and a data handling device. The column shown is suitable but other alternatives may be used if the suitability criteria are fulfilled.
 - 4.12.2 Analytical HPLC system
 Consisting of a pump (1ml/min), sample injection device (10 – 50µl), column (Zorbax ODS e.g. Jones chromatography Ltd, 25cm x 4mm id), programmable-wavelength UV or photo-diode array detector and a data handling device. The wavelength should be set to 325nm for retinol, 292nm for alpha tocopherol and 265nm for Vitamin D.

NB/ The column and conditions shown are suitable but other alternatives may be used if the suitability criteria are fulfilled.

5 Reagents

- 5.1 Potassium hydroxide
- 5.2 Potassium hydroxide solution (50% w/v): Weigh 100 ± 0.1 g potassium hydroxide into a 250ml Duran bottle or similar vessel, using a top pan balance. Add 100ml of water and stir until dissolved whilst cooling in cold water. The solution can be stored in a fridge for up to one month.
- 5.3 Absolute ethanol
- 5.4 Butylated Hydroxyl Toluene (BHT)
- 5.5 0.2% BHT solution in ethanol: Weigh 1 ± 0.05 g BHT into a 500ml volumetric flask. Add about 100ml of ethanol and swirl until completely dissolved. Make up to 500ml with ethanol. Store in a fridge for up to one month.
- 5.6 Ethyl acetate, HPLC or glass distilled grade.
- 5.7 Petroleum Spirit 40/60, HPLC or glass distilled grade.
- 5.8 Extraction solvent: Mix 20 volumes of ethyl acetate with 80 volumes petroleum spirit (40/60). (NB: 200 ml required per sample)
- 5.9 Methanol, HPLC or equivalent grade.
- 5.10 Hexane, HPLC or equivalent grade.
- 5.11 Propan-2-ol, HPLC or equivalent grade.

- 5.12 Acetonitrile, HPLC or equivalent grade.
- 5.13 Dichloromethane, HPLC or equivalent grade.
- 5.14 Sodium chloride, GPR
- 5.15 Sodium chloride solution (10% w/v): Dissolve 100g of sodium chloride in 1litre of water.
- 5.16 Sodium sulphate, granular
- 5.17 HPLC Mobile phases
 - 5.17.1 Semi-preparative system (For Vitamin D only)

Hexane / Propan-2-ol; 99/1 v/v: Using a measuring cylinder, add 990 ml of Hexane and 10ml of Propan-2-ol to a one litre reservoir bottle. Mix and de-gas before use.
 - 5.17.2 Analytical System

Acetonitrile / Dichloromethane / Methanol 70/20/10 v/v/v. Using a measuring cylinder, add 700ml Acetonitrile, 200ml Dichloromethane and 100ml Methanol to a one litre reservoir bottle. Mix and de-gas before use.

NB/ Alternative mobile phases may be used if the suitability criteria are fulfilled.

6 Standard substances

6.1 Standard Materials

- 6.1.1 Cholecalciferol (Vitamin D3), crystalline pure.

This shall be of the highest purity obtainable (> 98%) {e.g. Sigma Ltd, cat no. C9774 or USP}. Store in accordance with manufacturer's instructions.
- 6.1.2 Ergocalciferol (Vitamin D2) crystalline pure.

This shall be of the highest purity obtainable (> 98 %) {e.g. Sigma Ltd, cat no. E1007 or USP} Store in accordance with manufacturer's instructions.
- 6.1.3 All-trans retinol.

Crystalline, synthetic all-trans retinol, {e.g. Sigma, No R-7632, in sealed 25mg ampoules.} Store in accordance with manufacturer's instructions.
- 6.1.4 dl- α -Tocopherol.

dl- α -Tocopherol, synthetic, (>95%) {e.g. Sigma, Cat. No. T3251}. Store in accordance with manufacturer's instructions.

6.2 Stock solutions

- 6.2.1 Vitamin D2 stock solution 1mg/ml

Weigh accurately, about 100 mg of Vitamin D₂ into a 100ml volumetric flask using an analytical balance. Make to volume with absolute ethanol. This solution contains approximately 1mg/ml of vitamin D₂ and is stable for 1 month if stored in a freezer.

6.2.2 Vitamin D3 stock solution 1mg/ml

Weigh accurately, about 100 mg of Vitamin D₃ into a 100ml volumetric flask using an analytical balance. Make to volume with absolute ethanol. This solution contains approximately 1mg/ml of vitamin D₃ and is stable for 1 month if stored in a freezer.

6.2.3 All-trans retinol stock solution 1mg/ml (nom by wt.)

Dissolve approximately 25mg of all-trans retinol in 25ml of ethanol and store in an amber coated, 25ml volumetric flask prior to use. Store in a freezer for up to one month but check by UV before use.

6.2.4 Vitamin E stock solution 1mg/ml.

Dissolve approximately 50mg of α-tocopherol in 50ml of methanol in an amber-coated volumetric flask. Stable for one month if stored in a freezer.

6.3 Concentration test standards.

The actual concentration of each standard should be determined by UV spectrometry of the following standard solutions:

6.3.1 Vitamin D2 concentration test solution (10µg/ml).

Pipette 1 ml of vitamin D2 stock solution (6.2.1) into a 100ml volumetric flask and make to volume with ethanol. Measure the absorbance of this solution in a 1cm quartz cell at 265nm using ethanol as reference. Calculate the concentration of D2 using the following equation:

$$\frac{A_{265} \times 10^4}{475} \mu\text{g/ml}$$

Where A_{265} is the absorption value of the vitamin D2 solution at 265nm and 475 is the $E_{1\text{cm}}^{1\%}$ value (Source: Pharmaceutical Codex, 11th ed., 1979.) Prepare on day of use.

6.3.2 Vitamin D3 concentration test solution (10µg/ml)

Pipette 1 ml of vitamin D3 stock solution (6.2.2) into a 100ml volumetric flask and make to volume with ethanol. Measure the absorbance of this solution in a 1cm quartz cell at 265nm using ethanol as reference. Calculate the concentration of D3 using the following equation:

$$\frac{A_{265} \times 10^4}{480} \mu\text{g/ml}$$

Where A_{265} is the absorption value of the vitamin D3 solution at 265nm and 480 is the $E_{1\text{cm}}^{1\%}$ value (Source: Pharmaceutical Codex, 11th ed., 1979.) Prepare on day of use.

6.3.3 All-trans retinol concentration test solution (~ 5µg/ml nom.*)

Dilute 1ml of all-trans retinol stock solution (6.2.3) to 200ml with ethanol. Measure the absorbance of this solution in a 1cm quartz cell at 325nm using ethanol as reference. Calculate the mass concentration using the following equation:

$$\frac{A_{325} \times 10^4}{1830} \mu\text{g/ml}$$

where A₃₂₅ is the absorption value of the all-trans retinol solution at 325nm and 1830 is the E_{1%1cm} value. Check the stock solution by spectrophotometry each day.

**Note 1: Commercial sources of all-trans retinol, typically contain only 75 % (or less) of their nominal value when determined by UV spectrometry.*

Note 2: The absorbance maximum should be between 325 – 326nm. New and stored standards of retinol should be checked for the presence of degradation analogs by determining the absorbance at 300nm, 325nm, 350nm & 370nm with ethanol as reference. The standard is suitable if the ratios do not exceed the following:

Table 1: Vitamin A Absorption ratios

λ_{nm}	A/A_{325}
300	0.602
350	0.452
370	0.093

6.3.4 Vitamin E concentration test standard (~100µg/ml)

Dilute 5ml of α-tocopherol stock solution (6.2.4) to 50ml with methanol. Measure the absorbance of this solution in a 1cm quartz cell at 292nm using methanol as reference. Calculate the mass concentration using the following equation:

$$\frac{A_{292} \times 10^4}{75.8} \quad \mu\text{g /ml}$$

where A₂₉₂ is the absorption value of the α-tocopherol solution at 292nm and 75.8 is the E_{1%1cm} value.

6.4 Working Standards

6.4.1 Vitamin D2 and vitamin D3 Semi-Preparative Standard. (~ 4µg/ml Vitamin D)

Pipette 200µl of vitamin D2 stock standard (6.2.1) and vitamin D3 stock standard (6.2.2) into a 100ml volumetric flask. Make to volume with hexane and mix. Prepare on day of use.

6.4.2 Vitamin D3 Analytical Standard (0.4µg/ml, 1µg/ml, 2µg/ml).

Note: The Vitamin D concentration is calculated using the internal standard added to the sample however, the following standards are required to demonstrate that the D2/D3 response is linear.

Pipette 2, 5 and 10ml of vitamin D3 concentration test solution (6.3.1) into three 50ml volumetric flasks. Add 5ml vitamin D2 concentration test solution (6.3.2) and 100µl of 0.2% BHT solution to each flask and make to volume with analytical mobile phase. Prepare on day of use.

Note: If the supplement contains Vitamin D2, D2 & D3 should be reversed.

- 6.4.3 All-trans retinol intermediate standard (~45µg/ml*).
 Pipette 3ml of all-trans retinol stock standard (6.2.3) into a 50ml volumetric flask. Make to volume with methanol. Prepare on day of use.
 (* Concentration assumes ~75% Purity by UV spectrometry)
- 6.4.4 Mixed Vitamin A & E Calibration Standards
 Mix aliquots of the vitamin A intermediate standard (6.4.3) and the vitamin E stock solution (6.2.4) and dilute to volume with analytical mobile phase as shown in Table 2. Prepare on day of use.

Table 2: Vitamin A & E Calibration Standards

	Std 1	Std 2	Std 3	Std 4
Vitamin A Intermediate/ml	0.5	2	5	10
Vitamin E Stock /ml	0.5	2	5	10
0.2% BHT / (µl)/	100	100	100	100
Final Volume/ ml	50	50	50	50
Vitamin A µg/ml	0.45	1.8	4.5	9
Vitamin E µg/ml	10	40	100	200

7 System suitability

7.1 Semi preparative system

Inject 150ul of the semi-preparative standard solution onto the system. There should be no separation between the D2 and D3 peaks. These should elute as a single peak (normally between 10-15 minutes). The retention time of further injected portions must be constant.

7.2 Analytical system

7.2.1 Vitamins A & E

The system is suitable if all-trans retinol and alpha tocopherol are resolved from each other and are free from interferences from other co-extractants of the samples analysed.

7.2.2 Vitamin D

There should be greater than >95% resolution between Vitamin D2 and D3. These must also be free from interferences from other co-extractants of the samples analysed.

8 Procedure

8.1 General

Oil soluble vitamins are sensitive to UV radiation and to atmospheric oxidation. It is therefore necessary to protect against exposure to light during analysis and to use antioxidants to protect sample extracts.

8.2 Preparation of the test sample

This method has been validated for concentrations of vitamins at about 5 x the RDA. (A (4 mg), E (40 mg) and D (25µg)). For most supplements, this is equivalent to 5 capsules, 5 tablets or 2 ml of oil. Further validation may be required for samples containing much higher concentrations of vitamins.

8.2.1 Tablets:

Weigh at least 5 tablets and calculate the mean tablet weight. The tablets should be finely ground on the day of analysis. If hand grinding is used, care is needed to ensure a homogeneous powder, as some tablet coatings can be very hard.

8.2.2 Capsules:

Analyse whole to ensure that there is no migration into the shells.

8.2.3 Oils and suspensions:

Shake well and analyse directly. Do not use more than 2ml of oil.

8.3 Extraction

Weigh an amount of ground tablet equivalent to 5 tablets, or add 5 capsules or 2ml of oil into a 150ml round flask. Add ~ 0.5g of ascorbic acid and 30 ml of 0.2%BHT in ethanol (5.5). Add the vitamin D₂ internal standard (the amount of internal standard added should be approximately equal to the amount of vitamin D in the sample used, e.g. 2.5ml of D₂ Concentration Test solution (6.3.2) = 25ug. If D₂ is declared in the sample, D₃ is used as the internal standard).

Add 5 ml of 50% KOH solution (5.2) and swirl to ensure that the sample is dispersed. Reflux on a heating mantle for 30 minutes, regularly swirling the flask (Capsules should dissolve in the first ten minutes). Allow to cool slightly and transfer the extract to a 250ml separating funnel whilst still warm. Dissolve any residue in the flask with 30ml of water and transfer to the funnel. Rinse the flask with another 30 ml of water and add to the funnel. Cool under cold running water to ambient temperature.

Rinse the saponification flask with 100 ml ethyl acetate / petroleum ether (5.8) and add to the funnel. Shake gently and release any vapour pressure by carefully opening the tap with the flask inverted. Close the tap and shake vigorously for 1 minute. Allow the phases to separate.

Run the lower, aqueous layer into a second 250ml separating funnel, and the upper, organic layer into a 500ml separating funnel.

Add 50ml of ethyl acetate / petroleum ether (5.8) to the aqueous layer and shake as above for 1 minute. Allow the layers to separate and transfer the aqueous

layer to the original 250ml funnel. Add the organic (upper) layer to the 500ml funnel.

Add 50ml of acetate / petroleum ether (5.8) to the aqueous layer and shake as above for 1 minute. Allow the layers to separate and discard the aqueous layer. Add the organic (upper) layer to the 500ml funnel.

Wash the combined organic layers with 3 x 100ml of 10% salt solution (5.15), followed by 3 x 100 ml of water (6 washes in all) For each wash, release the vapour pressure and shake the funnel gently. Do not shake too vigorously to prevent the formation of emulsions, especially for the early washes). Discard the aqueous (lower) layer each time.

Filter the washed extract through some sodium sulphate contained in a fast filter paper into a 200ml volumetric flask. Rinse the funnels with a little ethyl acetate / petroleum ether (5.8) and add to the filter funnel. Make to volume with ethyl acetate / petroleum ether (5.8).

Note: If emulsions are formed, they can usually be broken by adding a little saturated salt solution and swirling.

Note: Some capsules may leave a white suspension in the organic layer. This is normally retained on the sodium sulphate filter bed.

Note: Occasionally, a capsule will contain an ingredient that can cause a sticky gel to block the funnel outlet. If this occurs, transfer the funnel contents to a second flask via the neck leaving the gel in the first flask. Continue the extraction.

8.4 Preparation of HPLC extracts

For Vitamin A and E: Transfer an appropriate volume (e.g. 3ml) of the sample extract to a turbovap tube or rotary evaporator flask and evaporate to dryness. Re-dissolve in a suitable volume of analytical mobile phase (e.g. 10 ml). The volumes should be chosen so that the analyte concentration in the injected solution lies within the calibration ranges for vitamins A & E.

For vitamin D: Filter the remaining sample extract through a GF/A filter paper into a turbovap tube or rotary evaporator flask. Evaporate to dryness and re-dissolve in 4ml hexane. Filter the hexane extract through a 0.45µm syringe filter if necessary.

8.5 Analytical HPLC for Vitamin A and E

Inject 20 µl aliquots (or a suitable volume) of the analytical standards onto the HPLC column and measure the peak areas. Construct a calibration line. Chromatograph the sample extracts under the same conditions and determine the vitamin concentrations in the extracts by interpolation.

8.6 Semi-preparative HPLC for Vitamin D

Inject 150ul of the vitamin D standard onto the semi-preparative HPLC system until the retention time of the standard is constant. Inject a 150 µl aliquot of the concentrated sample extract onto the semi-preparative system. Collect the eluent which elutes within two minutes either side of the D2/D3 retention time

(Total collection time = 4 mins). Evaporate the solvent to dryness and dissolve the residue in 300µl of analytical mobile phase.

8.7 Analytical HPLC for Vitamin D

Inject 50µl aliquots of the analytical standards solution onto the HPLC column and measure the peak areas of the D2 and D3 peaks. Calculate the response factor at each level (see Calculations). Chromatograph the sample extracts under the same conditions, identify the D2 and D3 peaks from their retention times, and measure their peak areas.

8.8 Calculation

8.8.1 Vitamin A:

The vitamin A content for tablets is given by:

$$\frac{C * V * D * T}{W} \quad \mu\text{g / Tablet expressed as all-trans retinol}$$

Where C = µg/ml of all-trans retinol in the injected solution

V = volume of extract used (200ml)

D = dilution of sample extract for HPLC

W = Wt of sample taken

T = Mean Tablet weight

The vitamin A content for capsules is given by:

$$\frac{C * V * D}{N} \quad \mu\text{g/capsule}$$

Where N is the number of capsules used.

The vitamin A content for oils & suspensions is given by:

$$\frac{C * V * D}{W} \quad \mu\text{g/g}$$

Where W is the weight of oil/suspension used.

If it is necessary to calculate the result as µg/ml, determine the weight of a known (e.g. 100 ml) volume of oil and multiply the above results by the value obtained in g/ml.

8.8.2 Vitamin E:

The vitamin E content for tablets is given by:

$$\frac{C * V * D * T}{W * 1000} \quad \text{mg / Tablet expressed as dl- alpha tocopherol}$$

Where C = µg/ml of α-tocopherol in the injected solution

V = volume of extract used (200ml)

D = dilution of sample extract for HPLC

W = Wt of sample taken

T = Mean Tablet weight

The vitamin E content for capsules is given by:

$$\frac{C * V * D}{N * 1000} \quad \text{mg/capsule}$$

Where N is the number of capsules used.

The vitamin E content for oils & suspensions is given by:

$$\frac{C * V * D}{W * 1000} \quad \text{mg/g}$$

Where W is the weight of oil used

If it is necessary to calculate the result as mg/ml, determine the weight of a known (e.g. 100 ml) volume of oil and multiply the above results by the value obtained in g/ml.

8.8.3 Vitamin D

The vitamin D content for tablets is given by:

$$\text{Vitamin D3} = \frac{A_{D3} \times \text{IS} \times \text{Rf} \times T}{A_{D2} \times W} \quad \mu\text{g/Tablet}$$

Where A_{D3} = Area of the D3 peak

A_{D2} = Area of the D2 peak

IS = Weight of internal standard (D2) added to the sample in µg.

W = Weight of the sample taken

T = Mean Tablet Weight in grams.

$$\text{Rf} = \text{Response Factor} = \frac{A_{D2} \times C_{D3}}{A_{D3} \times C_{D2}}$$

Where A_{D2} , A_{D3} , C_{D2} and C_{D3} are the areas and concentrations of vitamins D₂ and D₃ in the analytical standards in µg/ml. The response factor should be similar at each concentration level and should be approximately equal to 1. The mean response factor should be used.

The vitamin D content for capsules is given by:

$$\text{Vitamin D3} = \frac{A_{D3} \times IS \times Rf}{A_{D2} \times N} \quad \mu\text{g/Capsule}$$

where N = Number of capsules used.

The vitamin D content for oils & suspensions is given by:

$$\text{Vitamin D3} = \frac{A_{D3} \times IS \times Rf}{A_{D2} \times W} \quad \mu\text{g/capsule}$$

where N is the number of capsules used.

9 Safety

- 9.1 Potassium hydroxide is caustic and gets hot when dissolved in water. Ensure that glassware is sound and wear eye protection when handling solutions. In the event of splashes in the eye, irrigate the affected eye immediately with water and seek medical attention. In case of contact with skin, wash with water immediately.
- 9.2 The solvents used for extraction are toxic and highly flammable. Precautions must be taken to ensure that all sources of ignition are excluded from the working area. Avoid breathing the vapour. Extraction should be carried out in a fume cupboard. Filter papers, waste solutions etc should be allowed to stand in a fume cupboard until the solvent has evaporated or disposed of in a manner suitable for flammable solvents. Waste solvent (hydrocarbons, alcohols, ethers and acetone) must be disposed of in an approved manner.
- 9.3 Solvent pressure can build up during the extraction phases. The pressure should be released at the beginning of each extraction/wash step. When venting, the outlet of the funnel should be pointed away from the face and into a fume cupboard. Venting should be done slowly and carefully to avoid ejection of solvent.

Annex B: Results and Statistical evaluation Tables and Figures

Table 1: Method Performance Results for Vitamin A

Method Performance Characteristic	Coated Multivitamin and Mineral Tablet (Sample A)	Chewable Multivitamin Tablet with Iron and Calcium (Sample B)	Chewable Multivitamin and Mineral Tablet (Sample C)	Multivitamin Soft-Gel Capsule with Cod Liver Oil (Sample D)	Multivitamin and Mineral Soft-Gel Capsule with Cod Liver Oil (Sample E)	Multivitamin Tablet (Sample F)
n	13	12	13	13	12	13
outliers	2	0	0	2	1	0
n ₁	11	12	13	11	11	13
mean	605	362	1244	552	1167	1570
r	123	72	331	125	238	477
s _r	44.1	25.8	118.3	44.8	85.1	170.3
RSD _r	7.3	7.1	9.5	8.1	7.3	10.8
Ho _r	1.8	1.6	2.6	2.0	2.0	3.1
R	240	139	523	190	265	500
S _R	85.5	49.7	186.8	67.8	94.7	178.6
RSD _R	14.1	13.7	15.0	12.3	8.1	11.4
Ho _R	2.3	2.1	2.7	2.0	1.5	2.2

Table 2: Method Performance Results for Vitamin D

Method Performance Characteristic	Coated Multivitamin and Mineral Tablet (Sample A)	Chewable Multivitamin Tablet with Iron and Calcium (Sample B)	Chewable Multivitamin and Mineral Tablet (Sample C)	Multivitamin Soft-Gel Capsule with Cod Liver Oil (Sample D)	Multivitamin Tablet (Sample F)
n	11	8	10	10	10
outliers	1	0	2	2	0
n ₁	10	8	8	8	10
mean	4.48	2.70	5.97	4.10	10.9
r	0.96	0.63	1.30	1.35	4.8
s _r	0.343	0.225	0.462	0.481	1.70
RSD _f	7.7	8.3	7.8	11.7	15.6
H _{0f}	0.9	0.9	1.0	1.4	2.1
R	1.80	1.03	2.08	1.32	5.7
S _R	0.643	0.369	0.741	0.470	2.03
RSD _R	14.4	13.7	12.4	11.5	18.8
H _{0R}	1.1	1.0	1.0	0.9	1.7

Table 3: Method Performance Results for Vitamin E

Method Performance Characteristic	Coated Multivitamin and Mineral Tablet (Sample A)	Chewable Multivitamin Tablet with Iron and Calcium (Sample B)	Chewable Multivitamin and Mineral Tablet (Sample C)	Multivitamin Soft-Gel Capsule with Cod Liver Oil (Sample D)	Multivitamin and Mineral Soft-Gel Capsule with Cod Liver Oil (Sample E)	Multivitamin Tablet (Sample F)
n	13	12	13	13	12	13
outliers	1	1	0	1	0	0
n ₁	12	11	13	12	12	13
mean	9.15	6.20	7.22	9.20	37.67	29.03
r	2.42	0.84	1.33	2.37	7.25	6.0
s _r	0.860	0.301	0.476	0.845	2.59	2.13
RSD _r	9.4	4.8	6.6	9.2	6.9	7.3
Ho _r	3.5	1.7	2.4	3.4	3.2	3.2
R	2.79	2.99	3.32	2.92	16.9	13.5
S _R	0.995	1.069	1.18	1.044	6.029	4.823
RSD _R	10.9	17.2	16.4	11.3	16.0	16.5
Ho _R	2.7	4.0	3.9	2.8	4.9	4.8

Table 4: Results and Statistical Evaluation for Vitamin D3 ($\mu\text{g/g}$) in Coated, Multivitamin and Mineral Tablets

Laboratory	Sample 1	Sample 8
1	4.27	4.52
5	4.87	4.78
6	4.13	3.68
8 [C]	4.87	0.61
9	4.14	4.87
10	4.24	3.56
11	4.72	3.99
13	3.94	3.60
14	4.56	4.85
15	4.88	4.29
16	5.85	5.81
Overall Mean	4.48	
r	0.96	
s_r	0.343	
RSD_r	7.7	
HO_r	0.9	
R	1.80	
s_R	0.643	
RSD_R	14.4	
HO_R	1.1	

Table 5: Results and Statistical Evaluation for Vitamin D3 ($\mu\text{g/g}$) in Chewable Multivitamin Tablets with Iron and Calcium

Laboratory	Sample 2	Sample 10
1	2.68	2.80
5	2.51	2.26
6	2.70	2.35
8	2.50	1.91
9	1.76	Invalid
10	3.01	2.60
11	2.73	2.91
13	2.82	2.76
14	3.53	Invalid
15	2.67	Invalid
16	3.47	3.21
Overall Mean	2.70	
r	0.63	
s_r	0.225	
RSD_r	8.3	
HO_r	0.9	
R	1.03	
s_R	0.369	
RSD_R	13.7	
HO_R	1.0	

Table 6: Results and Statistical Evaluation for Vitamin D3 ($\mu\text{g/g}$) in Chewable Multivitamin and Mineral Tablets

Laboratory	Sample 3	Sample 7
1	6.68	6.39
5	5.56	5.43
6	5.48	5.58
8 [SG]	2.78	1.42
9	6.42	6.47
10 [C]	3.76	9.30
11	5.12	4.94
13	Invalid	Invalid
14	6.33	6.47
15	6.28	4.61
16	6.52	7.20
Overall Mean	5.97	
r	1.30	
s_r	0.462	
RSD_r	7.8	
HO_r	1.0	
R	2.08	
s_R	0.741	
RSD_R	12.4	
HO_R	1.0	

Table 7: Results and Statistical Evaluation for Vitamin D3 ($\mu\text{g/g}$) Multivitamin Soft-Gel Capsules with Cod Liver Oil

Laboratory	Sample 4	Sample 12
1	4.18	3.99
5	3.89	3.97
6	4.32	3.41
8[C]	4.04	0.99
9	3.36	Invalid
10	3.87	3.33
11	3.81	4.89
13[SG]	6.60	7.17
14	3.81	4.78
15	4.35	3.74
16	4.75	4.54
Overall Mean	4.10	
r	1.35	
s_r	0.481	
RSD_r	11.7	
HO_r	1.4	
R	1.32	
s_R	0.470	
RSD_R	11.5	
HO_R	0.9	

Table 8: Results and Statistical Evaluation for Vitamin D3 ($\mu\text{g/g}$) in Multivitamin Tablets

Laboratory	Sample 6	Sample 11
1	11.34	11.48
5	7.22	9.50
6	13.43	11.53
8	6.80	11.53
10	9.24	12.91
11	11.67	11.97
13	9.60	9.83
14	10.47	10.66
15	11.97	8.63
16	14.63	13.41
Overall Mean	10.89	
r	4.75	
s_r	1.696	
RSD_r	15.6	
Ho_r	2.1	
R	5.68	
s_R	2.028	
RSD_R	18.8	
Ho_R	1.7	

Table 9: Results and Statistical Evaluation for Vitamin A ($\mu\text{g/g}$) in Coated, Multivitamin and Mineral Tablets

Laboratory	Sample 1	Sample 8
1	750	635
3	641	582
5	619	536
6	471	462
7 [C]	657	367
8	585	580
9	584	533
10	623	552
11	737	825
13 [DG]	909	922
14	629	606
15	635	633
16	517	573
Overall Mean	605	
r	123	
s_r	44.1	
RSD_r	7.3	
Ho_r	1.8	
R	240	
s_R	85.5	
RSD_R	14.1	
Ho_R	2.3	

Table 10: Results and Statistical Evaluation for Vitamin A ($\mu\text{g/g}$) in Chewable Multivitamin Tablets with Iron and Calcium

Laboratory	Sample 2	Sample 10
1	318	315
3	389	335
5	396	349
6	349	341
7	320	254
8	347	330
9	312	305
10	424	371
11	442	432
13	454	407
14	358	Invalid
15	355	377
16	371	393
Overall Mean	362	
r	72	
s_r	25.8	
RSD_r	7.1	
Ho_r	1.6	
R	139	
s_R	49.7	
RSD_R	13.7	
Ho_R	2.1	

Table 11: Results and Statistical Evaluation for Vitamin A ($\mu\text{g/g}$) in Chewable Multivitamin and Mineral Tablets

Laboratory	Sample 3	Sample 7
1	1324	1100
3	1377	1207
5	1475	1292
6	1373	1160
7	747	931
8	1289	1361
9	992	1162
10	885	1238
11	1494	1396
13	1271	1238
14	1242	1274
15	1353	1322
16	1408	1428
Overall Mean	1244	
r	331	
s_r	118.3	
RSD_r	9.5	
HO_r	2.6	
R	523	
s_R	186.8	
RSD_R	15.0	
HO_R	2.7	

Table 12: Results and Statistical Evaluation for Vitamin A ($\mu\text{g/g}$) in Multivitamin Soft-Gel Capsules with Cod Liver Oil

Laboratory	Sample 4	Sample 12
1	569	496
3	632	657
5	659	530
6	430	480
7 [SG]	317	171
8 [C]	964	629
9	546	441
10	513	567
11	660	654
13	499	562
14	540	513
15	548	562
16	546	535
Overall Mean	552	
r	125	
s_r	44.8	
RSD_r	8.1	
Ho_r	2.0	
R	190	
s_R	67.8	
RSD_R	12.3	
Ho_R	2.0	

Table 13: Results and Statistical Evaluation for Vitamin A ($\mu\text{g/g}$) in Multivitamin and Mineral Soft-Gel Capsules with Cod Liver Oil

Laboratory	Sample 5	Sample 9
1	1175	1116
3	1238	1080
5	1382	1126
6	1056	1079
7 [SG]	640	765
8	1205	1209
9	1056	1070
10	1195	1264
11	1083	1311
13	1320	1244
14	1071	Invalid
15	1103	1140
16	1127	1100
Overall Mean	1167	
r	238	
s_r	85.1	
RSD_r	7.3	
Ho_r	2.0	
R	265	
s_R	94.7	
RSD_R	8.1	
Ho_R	1.5	

Table 14: Results and Statistical Evaluation for Vitamin A ($\mu\text{g/g}$) in Multivitamin Tablets

Laboratory	Sample 6	Sample 11
1	1607	1447
3	1658	1753
5	1762	1409
6	1454	1430
7	1484	1155
8	1680	1503
9	1378	1383
10	1564	1722
11	1567	1703
13	1842	1797
14	1929	1308
15	1614	1510
16	1524	1640
Overall Mean	1570	
r	477	
s_r	170.3	
RSD_r	10.8	
Ho_r	3.1	
R	500	
s_R	178.6	
RSD_R	11.4	
Ho_R	2.2	

Table 15: Results and Statistical Evaluation for Vitamin E (mg/g) in Coated, Multivitamin and Mineral Tablets

Laboratory	Sample 1	Sample 8
1	10.11	9.25
3	8.02	7.77
5	11.79	8.74
6	9.39	8.65
7	10.62	8.84
8	7.94	8.05
9 [C]	19.46	9.44
10	7.97	7.96
11	8.80	9.74
13	9.07	9.23
14	9.08	10.21
15	10.15	9.20
16	9.01	9.88
Overall Mean	9.15	
r	2.42	
s _r	0.860	
RSD _r	9.4	
Ho _r	3.5	
R	2.79	
s _R	0.995	
RSD _R	10.9	
Ho _R	2.7	

Table 16: Results and Statistical Evaluation for Vitamin E (mg/g) in Chewable Multivitamin Tablets with Iron and Calcium

Laboratory	Sample 2	Sample 10
1	5.98	6.30
3	4.57	4.43
5 [C]	7.66	4.97
6	7.09	7.38
7	6.82	7.01
8	5.44	4.85
9	6.62	6.54
10	6.55	5.50
11	5.55	5.82
13	5.49	5.17
14	7.22	Invalid
15	6.55	6.32
16	8.10	8.38
Overall Mean	6.20	
r	0.84	
s _r	0.301	
RSD _r	4.8	
Ho _r	1.7	
R	2.99	
s _R	1.069	
RSD _R	17.2	
Ho _R	4.0	

Table 17: Results and Statistical Evaluation for Vitamin E (mg/g) in Chewable Multivitamin and Mineral Tablets

Laboratory	Sample 3	Sample 7
1	7.09	6.29
3	6.28	6.25
5	9.13	7.76
6	8.19	7.25
7	7.52	6.80
8	6.55	7.13
9	8.02	7.57
10	6.99	6.08
11	6.60	6.29
13	5.07	4.94
14	7.49	8.08
15	7.47	7.62
16	9.45	9.77
Overall Mean	7.22	
r	1.33	
s _r	0.476	
RSD _r	6.6	
Ho _r	2.4	
R	3.32	
s _R	1.18	
RSD _R	16.4	
Ho _R	3.9	

Table 18: Results and Statistical Evaluation for Vitamin E (mg/g) in Multivitamin Soft-Gel Capsules with Cod Liver Oil

Laboratory	Sample 4	Sample 12
1	9.27	8.46
3	8.52	8.58
5	11.65	9.32
6	7.52	8.58
7 [SG]	5.27	3.00
8	10.91	9.09
9	10.03	7.93
10	9.13	9.23
11	8.69	8.36
13	7.28	8.69
14	9.81	9.76
15	9.50	9.74
16	10.48	10.30
Overall Mean	9.20	
r	2.37	
s _r	0.845	
RSD _r	9.2	
Ho _r	3.4	
R	2.92	
s _R	1.044	
RSD _R	11.3	
Ho _R	2.8	

Table 19: Results and Statistical Evaluation for Vitamin E (mg/g) in Multivitamin and Mineral Soft-Gel Capsules with Cod Liver Oil

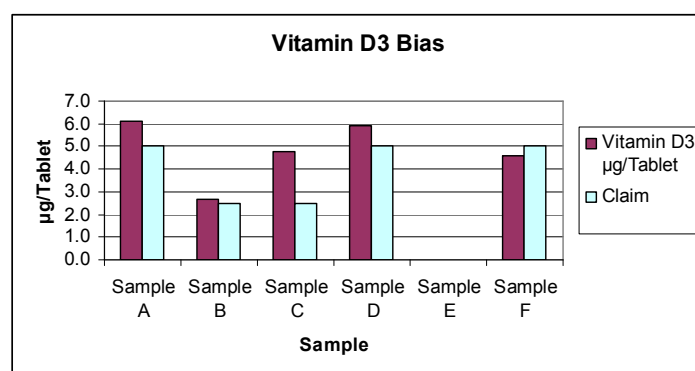
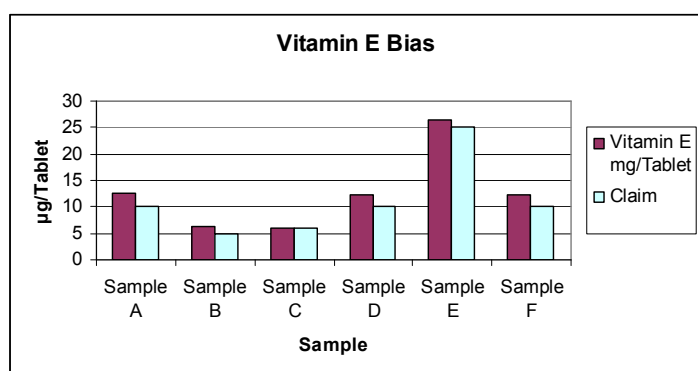
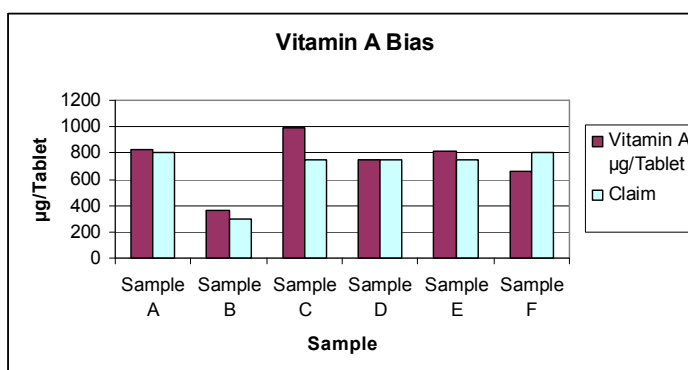
Laboratory	Sample 5	Sample 9
1	40.05	36.93
3	32.63	33.42
5	49.04	41.66
6	41.44	43.17
7	20.88	27.03
8	34.83	37.40
9	43.10	40.38
10	37.26	36.97
11	29.72	35.68
13	38.45	36.86
14	40.58	Invalid
15	40.14	39.06
16	44.66	43.21
Overall Mean	37.67	
r	7.25	
s _r	2.590	
RSD _r	6.9	
Ho _r	3.2	
R	16.9	
s _R	6.029	
RSD _R	16.0	
Ho _R	4.9	

Table 20: Results and Statistical Evaluation for Vitamin E (mg/g) in Multivitamin Tablets

Laboratory	Sample 6	Sample 11
1	30.96	31.50
3	27.08	28.28
5	14.70	21.12
6	33.17	32.72
7	29.95	24.32
8	28.84	25.71
9	34.04	32.51
10	25.62	27.83
11	24.32	27.85
13	29.29	29.36
14	31.64	31.92
15	33.55	30.02
16	36.49	37.38
Overall Mean	29.03	
r	6.0	
s _r	2.13	
RSD _r	7.3	
Ho _r	3.2	
R	13.5	
s _R	4.823	
RSD _R	16.5	
Ho _R	4.8	

Table 21: Comparison of mean study values against label claim values

Analyte	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F
Vitamin A µg/Tablet	827	361	993	744	812	663
Claim	800	300	750	750	750	800
Vitamin D₃ µg/Tablet	6.1	2.7	4.8	5.9	-	4.6
Claim	5	2.5	2.5	5	-	5
Vitamin E µg/Tablet	12.5	6.2	5.8	12.4	26.3	12.3
Claim	10	5	6	10	25	10



Figs 1 – 3: Comparison of Study means against label claim values

Table 22: Vitamin D3 results returned from the collaborative study

Sample	A		B		C		D		F	
	1	8	2	10	3	7	4	12	6	11
Participant number	µg/Tablet		µg/Tablet		µg/Tablet		µg/Capsule		µg/Tablet	
1	5.84	6.18	2.67	2.79	5.33	5.1	5.64	5.38	4.82	4.88
5	6.66	6.54	2.5	2.25	4.44	4.33	5.25	5.35	3.07	4.04
6	5.64	5.03	2.69	2.34	4.37	4.45	5.83	4.59	5.71	4.9
8	6.66	0.84	2.49	1.91	2.22	1.13	5.44	1.34	2.89	4.9
9	5.66	6.66	1.76	Invalid	5.12	5.16	4.53	Invalid	4.73	Invalid
10	5.8	4.86	3	2.59	3	7.42	5.22	4.49	3.93	5.49
11	6.45	5.45	2.72	2.9	4.09	3.94	5.14	6.59	4.96	5.09
13	5.39	4.92	2.81	2.75	No result	No result	8.9	9.67	4.08	4.18
14	6.23	6.63	3.52	Invalid	5.05	5.16	5.14	6.45	4.45	4.53
15	6.67	5.86	2.66	No result	5.01	3.68	5.86	5.04	5.09	3.67
16	7.99	7.94	3.46	3.2	5.2	5.75	6.4	6.12	6.22	5.7

Notes for Table 22

Labs 2, 4, 12	No data returned
Lab 3	All Vitamin D data rejected as all results high and no agreement between duplicates for any sample. Chromatographic interference suspected.
Lab 7	All Vitamin D data rejected as invalid. Initial data all high and variable. 2 nd dataset submitted after query was all very low and variable. Data rejected due to chromatographic and or calculation problems.
Lab 9	Interferences were noted by the participant with samples 10, 11, & 12. Since these values were extreme, they were rejected as invalid.
Lab 13	No results submitted for samples 3 & 7 due to analytical error.
Lab 14	Value for sample 10 rejected as result very low and no correlation between paired chromatograms for this sample. Chromatography suspect.
Lab 15	No result submitted for sample 10 due to interferences
Lab 17	All results rejected as protocol not followed. – In-house procedure used.

Table 23: Vitamin D3 results reported for Sample E (5 & 9)

Sample	E	
	5	9
Participant number	µg/Capsule	
1	1.01	1.09
5	0.34	0
6	0.04	0.06
8	0.22	0.17
9	1.31	2.16
10	0	0
11	0.28	0.2
13	3.13	2.58
14	4.34	Invalid
15	0.53	4.26
16	0	0

Table 24: Vitamin A results from Collaborative Study (expressed as All-trans retinol)

Sample	A		B		C		D		E		F	
	1	8	2	10	3	7	4	12	5	9	6	11
Participant number	µg/Tablet		µg/Tablet		µg/Tablet		µg/Capsule		µg/Capsule		µg/Tablet	
1	1025	868	317	314	1057	878	767	669	817	776	683	615
3	876	796	388	334	1099	963	852	885	861	751	705	745
5	846	732	395	348	1177	1031	889	714	961	783	749	599
6	644	631	348	340	1096	926	580	647	734	750	618	608
7	898	502	319	253	596	743	427	231	445	532	631	491
8	800	793	346	329	1029	1086	1300	848	838	841	714	639
9	798	729	311	304	792	927	736	595	734	744	586	588
10	851	755	423	370	706	988	691	764	831	879	665	732
11	1008	1128	441	431	1192	1114	890	881	753	912	666	724
13	1242	1260	453	406	1014	988	672	758	918	865	783	764
14	860	828	357	Invalid	991	1017	728	692	745	Invalid	820	556
15	868	865	354	376	1080	1055	739	757	767	793	686	642
16	707	783	370	392	1124	1140	736	721	784	765	648	697

Labs 2, 4 & 12 did not submit data

Lab 14 - values for samples 9 & 10 rejected as the results for these samples appeared to have been reversed for both Vitamins A & E.

Lab 17- Data rejected as invalid as an in-house method was used.

Table 25: Vitamin E results from Collaborative Study (expressed as alpha tocopherol)

Sample	A		B		C		D		E		F	
	1	8	2	10	3	7	4	12	5	9	6	11
Participant number	mg/Tablet		mg/Tablet		mg/Tablet		mg/Capsule		mg/Capsule		mg/Tablet	
1	13.82	12.65	5.97	6.28	5.66	5.02	12.49	11.40	27.85	25.68	13.16	13.39
3	10.96	10.62	4.56	4.42	5.01	4.99	11.49	11.57	22.69	23.24	11.51	12.02
5	16.12	11.94	7.64	4.96	7.29	6.19	15.70	12.56	34.10	28.97	6.25	8.98
6	12.84	11.83	7.07	7.36	6.54	5.79	10.14	11.56	28.82	30.02	14.10	13.91
7	14.52	12.08	6.80	6.99	6.00	5.43	7.11	4.05	14.52	18.80	12.73	10.34
8	10.85	11.00	5.43	4.84	5.23	5.69	14.71	12.25	24.22	26.01	12.26	10.93
9	26.60	12.91	6.60	6.52	6.40	6.04	13.52	10.69	29.97	28.08	14.47	13.82
10	10.90	10.88	6.53	5.49	5.58	4.85	12.31	12.44	25.91	25.71	10.89	11.83
11	12.03	13.32	5.54	5.81	5.27	5.02	11.71	11.27	20.67	24.81	10.34	11.84
13	12.40	12.62	5.48	5.16	4.05	3.94	9.81	11.71	26.74	25.63	12.45	12.48
14	12.41	13.95	7.20	Invalid	5.98	6.45	13.23	13.15	28.22	Invalid	13.45	13.57
15	13.88	12.57	6.53	6.30	5.96	6.08	12.81	13.13	27.91	27.16	14.26	12.76
16	12.32	13.50	8.08	8.36	7.54	7.80	14.13	13.88	31.06	30.05	15.51	15.89

Labs 2, 4 & 12 did not submit data

Lab 14 values for samples 9 & 10 rejected as the results for these samples appeared to have been reversed for both Vitamins A & E.

Lab 17 Results rejected as in-house method used.

Table 26: Mean Tablet and capsule masses

Sample	Identifiers	Dosage form	Mean tablet mass (g)
Sample A:	1/8	Tablet	1.3669
Sample B:	2/10	Tablet	0.9976
Sample C:	3/7	Tablet	0.7981
Sample D:	4/12	Capsule	1.3480
Sample E:	5/9	Capsule	0.6954
Sample F:	6/11	Tablet	0.4251

Key to tables

mean	the mean of all the data used in the statistical analysis
n	total number of sets of data submitted
outliers	number of results excluded from statistical analysis due to determination as outliers by either Cochran's or Grubbs' tests
n_1	number of results used in statistical analysis
nc	a non compliant result (e.g. protocol or method not followed)
c	an outlying result as determined by Cochran's Test at $P < 0.01$ level, not used in calculation of statistical parameters
gs	an outlying result as determined by single Grubbs' Test, $P < 0.025$ level not used in calculation of statistical parameters
gd	an outlying result as determined by double Grubbs' Test, not used in calculation of statistical parameters
r	repeatability limit (within laboratory variation) - the value below which the absolute difference between two single test results obtained with the same method on identical test material under the same conditions may be expected to lie with 95% probability
s_r	the standard deviation of the repeatability
RSD_r	the relative standard deviation of the repeatability ($S_r \times 100/MEAN$).
Ho_r	the HORRAT value for repeatability is the observed RSD_r divided by the RSD_r value estimated from the Horwitz equation using the assumption $r = 0.66R$
R	reproducibility limit (between-lab variation) - the value below which the absolute difference between two single test results obtained with the same method on the identical test material under different conditions may be expected to lie with 95% probability
S_R	the standard deviation of the reproducibility
RSD_R	the relative standard deviation of the reproducibility ($S_R \times 100/MEAN$)
Ho_R	the HORRAT value for reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

Annex C – HPLC Systems Used

HPLC columns and conditions were recommended but participants were allowed to vary these if system suitability was appropriate. The conditions used by each participant are shown in Table 1.

Table 1: HPLC conditions used for Vitamins A, D & E

Participant Number	Vitamin D Semi-Prep	Vitamin D Analytical	Vitamins A & E Analytical
1	Partisil 5 PAC, 250x4.6; Amb, Hex/Propan-2-ol; 99/1, 1.0ml/min, UV265, Manual F/C	Zorbax ODS, 250 x 4.6, 25°C, MeCN/DCM/MeOH;70/20/10,1.0ml/min, UV265	Zorbax ODS, 250 x 4.6, 25°C, MeCN/DCM/MeOH;70/20/10, 0.8ml/min, UV292/325
3	Partisil PAC, 250x4.6; 20°C, Hex/Propan-2-ol; 99/1, 1.5ml/min, UV265, Manual F/C	Synergi Hydro C18, 250 x 4.6, 20°C, MeCN/DCM/MeOH;70/20/10,1.0ml/min, PDA265	Phenomenex Gemini C18, 250x4.6, 20°C, MeCN/DCM/MeOH;70/20/10,1.0ml/min, DAD 292 & 325
5	Hichrom Partisil 5 PAC, 250x4.6; Amb, Hex/Propan-2-ol; 99/1, 1.5ml/min, PDA 265, Manual F/C	Zorbax ODS, 250 x 4.6, 30°C, MeCN/DCM/MeOH;70/20/10,1.0ml/min, UV265	Zorbax ODS, 250 x 4.6, 30°C, MeCN/DCM/MeOH;70/20/10,1.0ml/min, PDA292/325
6	Merck Lichrosorb Si 60, 250 x 4.6-5µ,30°C, Hex/Propan-2-ol; 99/1, 1.5ml/min, DAD 266, Manual F/C	Agilent Hypersil BDS C18, 100 x 4-3µ, 30°C, MeCN/MeOH/H ₂ O; 94/3/3,1.0ml/min, DAD 266	Agilent Hypersil BDS C18, 100 x 4-3µ, 30°C, MeCN/MeOH/H ₂ O; 94/3/3,1.0ml/min, DAD 294,326
7	Partisil, 250x4.6; Amb, Hex/Propan-2-ol; 99/1, 1.5ml/min, UV265, Manual F/C	Spherisorb 5 ODS2, 250 x 4.6, Amb, MeCN/DCM/MeOH;70/20/10,1.0ml/min, UV265	Spherisorb 5 ODS2, 250 x 4.6, Amb, MeCN/DCM/MeOH;70/20/10,1.0ml/min, UV292/325
8	Partisil 5 PAC, 250x4.6; Amb, Hex/IPA; 99/1, 1.5ml/min, UV 265, Manual F/C	Waters Spherisorb 5 ODS1, 250 x 4.6, Amb, MeCN/DCM/MeOH;70/20/10,1.0ml/min, DAD265	Waters Spherisorb 5 ODS1, 250 x 4.6, Amb, MeCN/DCM/MeOH;70/20/10,1.0ml/min, DAD292/325

Table 1 (continued) : HPLC conditions used for Vitamins A, D & E

9	Partisil PAC, 5µ, 250x4; Amb, Hex/Propan-2-ol; 99/1, 1.5ml/min, UV265, Manual F/C	Zorbax ODS, 5µ, 250 x 4.6, Amb, MeCN/DCM/MeOH;70/20/10,1.0ml/min, PDA 265	Zorbax ODS, 5µ, 250 x 4.6, Amb, MeCN/DCM/MeOH;70/20/10,1.0ml/min, PDA 292,325
10	Partisil PAC, 250x4.6; Amb, Hex/Propan-2-ol; 99/1, 1.5ml/min, UV265, Manual F/C	Alltech, Allsphere ODS1,250 x 4.6, Amb, MeCN/DCM/MeOH;70/20/10,1.0ml/min, DAD265	Alltech, Allsphere ODS1,250 x 4.6, Amb, MeCN/DCM/MeOH;70/20/10,1.0ml/min, DAD292,325
11	Partisil PAC, 250x4.6; Amb, Hex/Propan-2-ol; 99/1, 1.5ml/min, UV265, Manual F/C	Zorbax ODS, 250 x 4.6, 25°C, MeCN/DCM/MeOH;70/20/10,1.0ml/min, UV265	Zorbax ODS, 250 x 4.6, 25°C, MeCN/DCM/MeOH;70/20/10,1.0ml/min, UV292/325
13	Watrex, Biospher Si100, 250x4.6,5µ, Amb, Hex/propan-2-ol, 99/1,1ml/min,PDA 265,manual	Supelco, Discovery C18, 250x4.6,5µ,Amb, MeCN/DCM/MeOH;70/20/10,1.0ml/min, DAD 265	Merck, Lichrosorb 100 RP-18, 250x4µ,Amb, MeCN/DCM/MeOH;70/20/10,1.0ml/min, DAD 292/325
14	Not supplied	Not supplied	Not supplied
15	Partisil 5 PAC, 250x4.6; Amb, Hex/IPA; 99/1, 1.5ml/min, UV 265, Manual F/C	Zorbax ODS, 250 x 4.6, 25°C, MeCN/DCM/MeOH;70/20/10,1.0ml/min, UV265	Zorbax ODS, 250 x 4.6, 25°C, MeCN/DCM/MeOH;70/20/10,1.0ml/min, UV292/325
16	Partisil 5 PAC, 250x4.6; Amb, Hex/IPA; 99/1, 1.5ml/min, UV 265, Auto F/C	Zorbax ODS, 250 x 4.6, 30°C, MeCN/DCM/MeOH;70/20/10,1.0ml/min, PDA 265	Zorbax ODS, 250 x 4.6, 30°C, MeCN/DCM/MeOH;70/20/10,1.0ml/min, PDA 292,325 MeCN/MeOH 97/3 used for Vit D
17	Hichrom Hypersil H5 250 x 4.6, 5µ, Hexane/THF/IPA, 98:1:1, 1ml/min, UV 265, AUTO	Vydac, Protein and Peptide C18, 250x4.6,5µ,40°C MeOH/Water, 1ml/min,UV265	A// Hypersil ODS, 250x4.6,5µ,40°C,MeOH/Water, 97/3, 1.5ml/min, UV 325 (A), Flu 284/334 (E)

Annex D - Standards and Calibration

Standards were recommended but not supplied. The standards used are shown below:

Participant number	Vit A Std	Vit E Std	Vit D2 Std	Vit D3 Std
1	Sigma R7632	Sigma T3251	Sigma E1007	Sigma C9774
3	Sigma R7632	Sigma T3251	Acros 42263	Sigma C9774
5	Sigma R7632	Sigma T3251	Sigma E9007	Sigma C1357
6	Sigma R7632	Fluka 95240	Fluka 95220	Fluka 95230
7	Sigma R7632	Sigma T3251	USP 1239005	Sigma C9774
8	Sigma R7632	Sigma T3251	Sigma E1007	Sigma C9774
9	Sigma R7632	Sigma T3251	Sigma E1007	Sigma C9774
10	Sigma R7632	Sigma T3251	Sigma E1007	Sigma C9774
11	Sigma R7632	Sigma T3251	Sigma E-5750	Sigma C1357
13	Sigma R7632	Fluka 95240	Sigma E-5750	Sigma C9756
14	Not supplied	Not supplied	Not supplied	Not supplied
15	Sigma R7632	Sigma T3251	Sigma E1007	Sigma C9774
16	Fluka 95144	Sigma T3251	LGC Promochem 1239005 USP	LGC Promochem 13100 USP
17	Sigma R3250	Fluka 95240	Acros 422630050	Acros 421070010

Concentration checks

There was considerable variation in the absorbances returned for the concentration check standards, especially for vitamin A. This emphasises the need to check the concentration spectrophotometrically as the purity of the stock standards may vary.

Most vitamin D response factors were between 0.90 - 1.04 but two laboratories had response factors between 0.7 - 0.8.

Calibration

All calibrations were linear and complied with the protocol.

Annex E – Participant comments

All but one of the participants followed the protocol and had no problems in following the instructions provided. The remaining laboratory (17) chose to use their in-house procedure which was similar but sufficiently different to exclude their results from the study. There were some delays in obtaining suitable HPLC columns or standards.

Several laboratories reported minor variations to the chromatography conditions (e.g. injection volumes etc) which the study coordinator accepted as being within allowable parameters for the protocol

A number of laboratories reported interfering peaks in the Vitamin D analysis.

Lab 1 commented that the absorbances used for checking the purity of the Vitamin A standard were slightly higher than specification.

Lab 9. The concentration of Vitamin D2 Internal standard used was half that specified due to a dilution error. The A300/A325 purity check ratio limit was exceeded for the vitamin A standard (2 sources tried). Standards and samples were kept in a fridge for up to 4 days before analysis. Little change in absorbance for the standards was noted.

Lab 10 purchased a new, vitamin A standard but stock solutions prepared from this failed the purity check procedure. An older standard already held was therefore used.

Lab 13 used a larger aliquot of vitamin A stock solution to prepare his intermediate standard as his absorbance reading was lower than expected. They also expressed some reservations as to the quality of the chromatograms for Vitamin D but did not have time to modify the HPLC conditions further. In fact, although other peaks were present in the chromatograms, the results produced were acceptable

Lab 14 had some interference with the Vitamin D peaks in samples 2 & 12. Peaks were integrated by dropping a perpendicular to the baseline.

Lab 15 reported some potentially interfering peaks in some vitamin D traces. Sample 10 in particular was unresolved and was not reported. Although he stated that these peaks could be resolved on their “in-house” system (Spherisorb ODS2, MeOH/H₂O : 95/5), results using these conditions were not reported.

Lab 16 reported interferences with vitamin D for some samples using the recommended conditions. Results for vitamin D were obtained using a modified mobile phase. (MeCN / MeOH 97/3).

Lab 17 used an in-house procedure as this was their normal procedure and set-up. Although the procedure used was similar and produced similar results, the extraction and analysis both varied from the protocol; therefore the results were rejected by the coordinator.



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