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The development and evaluation of secondary food reference materials for the determination of cholesterol, fatty acids and selected water-soluble vitamins in foods

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The suitability of a number of 'off-the-shelf' food items for use as secondary reference materials for incorporation into routine work batches to enhance quality assurance has been evaluated over a 5 month period. The commodities were canned ham (tested for cholesterol and fatty acids), wheat germ (tested for fatty acids, thiamine, riboflavin and niacin), two fish oils (tested for fatty acids) and powdered orange drink vitamin C supplement (tested for ascorbic acid). Foods were purchased locally and analysed using recognized methods. Seven replicate analyses were performed initially on each food to establish homogeneity, with subsequent analyses in triplicate. Overall mean results (±2 s.d.) for the 5 month study were as follows: cholesterol in canned ham, 65±7.2 mg/100 g; thiamine in wheatgerm, 1.97±0.26 mg/100 g; riboflavin in wheatgerm, 0.27±0.17 mg/100 g; niacin in wheatgerm, 7.1±0.9 mg/100 g; ascorbic acid in vitamin C supplement, 718±70 mg/100 g; C18:0, C18:1 and C18:2 in wheatgerm, 0.72±0.11, 15.2±0.5 and 57.7±1.4% total fatty acids respectively; C18:0, C18:1 and C18:3 in canned ham, 12.5±0.6, 44.7±1.4 and 0.57±0.12% total fatty acids respectively; C18:0, C20:5 and C22:6 in cod liver oil, 2.4±0.1, 12.6±1.2, and 5.6±0.8% total fatty acids respectively; C18:0, C20:5 and C22:6 in ω3 fish oil, 3.7±0.2, 19.2±0.5, and 12.8±1.4% total fatty acids, respectively. Statistical data were obtained from the multiple analyses, and based on the data generated, the foods tested were shown to have satisfactory homogeneity and stability for use as secondary reference materials over an extended period. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Reference materials are finding increasing application in quality control procedures, where they play a role in establishing the accuracy of analytical data. It is equally important to establish and monitor analytical precision, which is normally done by undertaking replicate analysis of the sample or by use of a suitable control sample in the analysis batch. According to Taylor (1987), routine use of control samples and control charts to establish 'statistical control' of the method minimizes the value of, and requirement for, duplicate analyses. In addition, reference materials can be used to compare and evaluate methods.

While it would be desirable to include certified or standard reference materials (CRMs and SRMs, respectively) in every analytical batch, they are often expensive, and the cost increases when used frequently. Another major problem with their use is that materials containing the analyte of interest in an appropriate matrix may not be available. To address this, a wider variety of reference materials needs to be developed (Greenfield & Southgate, 1992).

A cheap and simple way to develop such materials would be to use readily available 'off-the-shelf' items. Like CRMs and SRMs, these items should be homogeneous, easily transportable, stable over an extended period, and ideally (though usually impractical), they should be similar in nature and level of analyte to the actual samples being analysed.

The present work describes the analysis of a variety of 'off-the-shelf' foods to test the stability of cholesterol,

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fatty acids, thiamine, riboflavin, niacin and ascorbic acid over a 5 month period, with a view to incorporating them as secondary reference materials in appropriate routine work batches to enhance quality assurance. It should be emphasized that it was not the intention to produce CRMs or SRMs because of the large number of analyses required (both chemical and statistical) to certify properly such materials.

MATERIALS AND METHODS

Materials

Ideally, reference materials should be homogeneous, easily transportable, stable over an extended period, and contain a measurable level of analyte. The foods that were chosen for the study were thought to possess the above attributes. All foods were purchased from local retail outlets, and prepared as described below. Wheatgerm was chosen for thiamine, riboflavin, niacin and fatty acid profile analyses, canned ham for cholesterol and fatty acid profile, powdered orange drink Vitamin C supplement for ascorbic acid and two fish oils for fatty acid profile. Four products were chosen for the fatty acid profile study because of the wide variety of samples that are actually analysed in our laboratory. Each of the samples had different levels of the fatty acids, with the two fish oils containing appreciable levels of the polyunsaturated fatty acids C20:5 and C22:6. For convenience, three particular fatty acids were chosen for each commodity. For wheatgerm, C18:0, C18:1 and C18:2 were chosen; for canned meat, C18:0, C18:1 and C18:3; and for the fish oils, C18:0, C20:5 and C22:6. The fatty acids in wheatgerm and canned meat were chosen to represent differing levels of percentage total fatty acids, while two polyunsaturated acids were included for the fish oils, since these are more likely to deteriorate with time than saturates or monounsaturates.

Statistical methods

Sample homogeneity was assessed using %CV data from the initial seven replicate analyses. Stability of the analytes over the 5 month period was assessed by hypothesis testing of the slope of the line of best fit (Weiss & Hassett, 1982). Analytes for which the slope could not be distinguished statistically from zero ($\alpha = 0.05$) were considered stable over the 5 months.

Reagents

Cholesterol, cholestane, dithiothreitol, sodium deoxycholate, thiamine hydrochloride, riboflavin, alpha-amylase, nicotinic acid (niacin), cyanogen bromide and eicosapentaenoic and lignoceric acid methyl esters were obtained from Sigma Chemical Co., St Louis, MO 63178, USA. Other fatty acid methyl esters were obtained from Nu Chek Prep Inc., Elysian, MN 56028, USA. Clarase enzyme was obtained from Miles Laboratories, Sydney, Australia. Pyridine was obtained from Ajax Chemicals, Sydney, Australia. D-Erythorbic acid was obtained from Fluka AG, Switzerland. Chloroform, L-ascorbic acid, orthophosphoric acid, hexane sulphonic acid sodium salt, heptane sulphonic acid sodium salt and sulphanilic acid were obtained from BDH Chemicals Pty Ltd, Kilsyth, Victoria, Australia. All other chemicals and solvents were A.R. grade or HPLC grade and used without further purification.

Sample preparation

Wheatgerm
The contents of the 500 g packet of raw wheatgerm were milled to a fine powder and transferred to 5 ml glass vials each containing approximately 1 g. These vials were sealed and stored at −18°C until required.

Canned ham
Several 125 g cans of processed ham were purchased. One can was opened when required, and the contents mixed thoroughly before analysis.

Vitamin C supplement
Several 500 g jars of the supplement were purchased. The contents of one jar were milled to a fine powder and transferred to 6 ml glass vials each containing approximately 5 g. The vials were sealed and stored in a dark cupboard until required.

Fish oils
The 200 ml of cod liver oil and 50 capsules of 'ω3' fish oil (each capsule containing 1 g of oil) were both contained in amber glass bottles and stored at 4°C until required. The cod liver oil was mixed thoroughly before analysis. One capsule of the 'ω3' fish oil was used per analysis.

Thiamine and riboflavin

Sample treatment
An AOAC (1980*a*) official method was used. Approximately 1 g of wheatgerm was weighed accurately into a 50 ml graduated polypropylene centrifuge tube, then 17.5 ml of 0.1 N H_2SO_4 was added. The tubes were capped and shaken vigorously for 1 min. Following this, the tubes were placed in a boiling water bath for half-an-hour, with shaking at 10 min intervals. The tubes then were cooled to less than 40°C in an ice bath, and 2.5 ml of 2% aqueous clarase or alpha-amylase (found to be equivalent in this application) was added. After mixing, the digest was heated at 55°C for 1 h, with intermittent shaking, and then cooled, made to 25 ml with water, and mixed. Tubes were then centrifuged at 2500 rpm for 15 min, the supernatant filtered through a 0.8 μm nylon filter disc, then analysed by HPLC.

HPLC-thiamine

A reverse phase ion pair system based on that used by Wimalasiri & Wills (1985) was used. Equipment consisted of two model 501 HPLC pumps (one for mobile phase, the other for oxidant), a model 712 WISP, and model 470 fluorescence detector (Waters Chromatography, Milford, MA 01757, USA), with a 3.9×300 mm steel column containing C18 Spherisorb ODS2 packing (Phase Separations Ltd, Queensferry, Clwyd CH5 2LR, UK). A C18 precolumn (Waters) was used to protect the analytical column. The mobile phase was 0.005 M hexane sulphonic acid (sodium salt) in 85% methanol/water. Then 1 ml of glacial acetic acid was added per 500 ml, and the pH adjusted to 6.0 with 5% aqueous sodium bicarbonate. The mobile phase was filtered and degassed before use. For post-column oxidation of thiamine to thiochrome, a solution of 0.33 g of potassium ferricyanide and 15 g of sodium hydroxide per litre of water was prepared fresh and stored in an amber glass container. Flow rates for the mobile phase and oxidant were 1.3 and 1.0 millilitres/min, respectively. Detector settings were as follows: excitation: 360 nm; emission: 435 nm; sensitivity: 16; gain: ×100; time constant: 4. Peak heights were used in the calculation.

Standards were prepared by weighing 20.0 mg of thiamine hydrochloride into a 200 ml volumetric flask, then dissolving and making to volume with 20% ethanol in water. Working standards (0.1–1.0 μg/ml) were prepared from aliquots of this standard, which were subjected to the same extraction procedure as the samples.

HPLC-riboflavin

The same equipment as for thiamine was used, except that no oxidant was required. The mobile phase was 0.005 M heptane sulphonic acid (sodium salt) in 45% methanol/water, filtered and degassed before use. Detector settings were as follows: excitation: 360 nm; emission: 525 nm; sensitivity: 16; gain: ×100; time constant: 1.5. Peak heights were used in the calculation.

Standards were prepared by weighing 20.0 mg of riboflavin into a 200 ml volumetric flask, then adding three drops of glacial acetic acid and 50 ml of water. The flask was warmed to 80°C in a water bath until the riboflavin dissolved. The flask was allowed to cool, and the solution made to volume with water. Working standards were prepared similarly to those for thiamine.

Niacin

Standards

A 100 μg/ml stock solution of niacin was prepared by dissolving 20 mg in 25% ethanol/water and making to 200 ml with same. The solution was stored at 4°C. Working standards were prepared from aliquots of the stock solution and subjected to the same extraction procedure as the samples.

Sample treatment

An official AOAC (1980*b*) method was used. Essentially, 1 g of sample was weighed accurately into a 50 ml centrifuge tube containing 0.75 g of calcium hydroxide. Then 20 ml of water were added, the tubes were capped and shaken thoroughly. The caps were then loosened and the tubes were autoclaved for 2 h at 121°C and 104 kPa above atmospheric pressure. The digest was mixed thoroughly while still hot, then cooled and diluted to 50 ml with water. After centrifuging at 2500 rpm for 15 min at 0°C, 15 ml of the supernatant was pipetted into a 30 ml plastic tube containing 6 g of ammonium sulphate and 1.5 ml of a pH 8 phosphate buffer. The resulting solution was shaken and warmed to 55–60°C for 10 min, then refrigerated for 15 min. After centrifuging at 2000 rpm for 15 min at 0°C, the solution was filtered using a 0.8 μm cellulose acetate disc filter. A 5.0 ml aliquot of this solution was treated with aqueous 10% cyanogen bromide followed by aqueous 55% sulphanilic acid, and absorbance of the final solution was measured with a Perkin Elmer 55B UV/visible spectrophotometer set at 470 nm, with a 1 cm flowcell.

Ascorbic acid

Sample treatment

Approximately 5 g of the powder was weighed accurately and dissolved in 200 ml of water. An aliquot was diluted five-fold with 0.2% dithiothreitol solution and analysed by capillary electrophoresis (Thompson & Trenerry, 1995) with erythorbic acid as internal standard, or by reverse phase HLPC (Maeda *et al.*, 1988).

Standards were prepared in dithiothreitol solution according to the method described by Thompson and Trenerry (1995).

Cholesterol

Sample treatment

Approximately 5 g of sample were weighed accurately into a 250 ml conical flask, and 60 ml of 9% potassium hydroxide in 90% ethanol/water was added. After brief swirling and covering with a watchglass, the flasks were placed in a 70–80°C water bath for half-an-hour, with further brief swirling. After cooling to room temperature, the flask contents were transferred to a 500 ml separating funnel containing 60 ml of 10% brine. The flask was rinsed with 50 ml of petroleum ether (40–60°) and the solvent transferred to the funnel, which was then stoppered and shaken for 30 s. After allowing layers to separate, the lower layer was run into the original flask, and the top layer was collected in a 250 ml separating funnel. The lower layer was then returned to the 500 ml funnel and extracted twice more with fresh solvent in a similar fashion, pooling all the solvent extracts. This extract was washed with water (5×50 ml), discarding the washings each time. The

petroleum ether was then transferred to a 250 ml round-bottomed flask, and evaporated to low volume under vacuum. This extract was transferred to a 10 ml volumetric flask. The round-bottomed flask was then rinsed twice with a few ml of petroleum ether, the rinsings being transferred to the 10 ml flask each time. After making to volume with petroleum ether, the flask contents were mixed thoroughly.

For the determination, 1.0 ml of cholestane solution (0.5 mg/ml in hexane) and 2.0 ml of sample extract were pipetted into vials and blown to dryness with warming and a stream of nitrogen. Then 1 ml of redistilled pyridine was added and then the vials were capped and sealed. After adding 0.3 ml of acetic anhydride, the vials were heated at 80°C for 20 min with gentle inversion, then allowed to cool before analysis.

GLC

Analyses were performed on either model 3400 or 3700 gas chromatographs fitted with autosamplers and flame ionization detectors (Varian Associates Inc., Walnut Creek, California, USA) using a fused silica 12QC2/BP1–0.10 capillary column (SGE Pty Ltd, Ringwood, Victoria, Australia). The carrier gas was hydrogen (5 psi pressure) and the injector was operated in the split mode, the split ratio being approximately 50:1. Injector and detector temperatures were 290°C and 300°C respectively. The oven temperature was programmed at 5°C/min from 200 to 260°C, then held at this temperature for 3 min. Data were captured and analysed with a HP 3350A laboratory data system (Hewlett–Packard Co., Palo Alto, California, USA).

Working standards of cholesterol (1 mg/ml and 0.2 mg/ml) were prepared by dissolving 100 mg of cholesterol in 100 ml of hexane and diluting appropriately. Then 1.0 ml aliquots of these solutions were derivatized in the same way as the sample extracts.

Fatty acid profiles

Sample treatment

The lipids from the wheatgerm and canned ham were extracted and purified using a variation of the method described by Bligh and Dyer (1959), while the fish oils were used without further purification. Approximately 10 g of the ham or wheatgerm was blended with 2:1 methanol/chloroform mixture followed by addition of chloroform and further blending. After adding water, the mixture was blended again, then filtered under vacuum. The filtrate was transferred to a separating funnel and left overnight to allow the layers to separate. The lower (chloroform) layer was evaporated to recover the extracted lipid, which was stored at 4°C until required.

For the transesterification, 120–130 mg of lipid were weighed into a 20×150 mm glass culture tube with a teflon-lined screw cap. Then 1.5 ml of 0.5 M sodium methoxide in methanol was added, and the tubes were capped and heated to 70–80°C for 5 min, with periodic shaking. After cooling, 1.5 ml of 1.8 M sulphuric acid in methanol was added. The tubes were heated again (as just described) then cooled to room temperature. Then 20 ml of 1 M sodium bicarbonate/16% potassium chloride solution was added slowly to neutralize excess acidity, followed by 5 ml of hexane. The tubes were capped and shaken for 1 min, the layers allowed to separate, and the hexane transferred to a 25 ml volumetric flask containing anhydrous sodium sulphate. After making to volume and mixing, the extracts were stored in a freezer until required.

GLC

A model 3400 gas chromatograph equipped with autosampler and flame ionization detector (Varian Associates Inc., Walnut Creek, California, USA) and a

Table 1. Initial homogeneity data (represented as %CV) and levels of all analytes, based on seven replicate analyses

Commodity	Analyte	Statistics (units: mg/100 g)		
		Mean	SD	%CV
Wheat germ	Thiamine	2.0	0.042	2.1
Wheat germ	Riboflavin	0.22	0.036	16.3
Wheat germ	Niacin	7.4	0.35	4.8
Canned ham	Cholesterol	54	1.3	2.4
Vitamin C supplement	Ascorbic acid	757	15	2.0
			(units: % total fatty acids)	
Wheatgerm	C18.0	0.9	0.012	12.8
	C18:1	17.1	0.034	0.2
	C18:2	57.1	0.11	0.2
Canned ham	C18:0	13.0	0.19	1.5
	C18:1	46.4	0.23	0.5
	C18:3	0.4	0.082	20.4
Cod liver oil	C18:0	2.5	0	0
	C20:5	13.6	0.068	0.5
	C22:6	5.7	0.063	1.1
ω3 Fish oil	C18:0	3.8	0.049	1.3
	C20:5	20.9	0.063	0.3
	C22:6	13.7	0.069	0.5

25QC2/BPX70–0.25 fused silica column (SGE Pty Ltd, Ringwood, Victoria, Australia) was used. The carrier gas was hydrogen (10 psi pressure) and the injector was operated in the split mode, the split ratio being approximately 50:1. Injector and detector temperatures were 240 and 250°C, respectively. The oven temperature was held at 70° for 1 min then programmed at 30°C/min to 170°C, followed by further programming at 30°C/min to 200°C, and held at this temperature for 8 min. Data was captured and analysed with a HP 3350A laboratory data system (Hewlett–Packard Co., Palo Alto, California, USA).

Standards were prepared by dissolving the fatty acid methyl esters (FAMES) in HPLC grade hexane and transferring to a 10 ml volumetric flask, resulting in concentrations of 0.3–0.5 mg/ml. Standards were stored at −18°C.

RESULTS AND DISCUSSION

Each food type was initially analysed seven times to check the sample homogeneity before the extended time study commenced. The results (Table 1) indicate that the sample preparation and the levels of analytes were satisfactory. The levels of cholesterol were also similar between cans in the same batch (mean value 57 mg/100 g, %CV 3.4), allowing for a separate can to be used for each determination during the study.

After verifying that the samples were homogeneous, each analyte was determined in triplicate at regular intervals over a 5 month period, except for cholesterol, which was evaluated over 4 months. For cholesterol and the fatty acid profiles, the levels used to determine the homogeneity of the sample were not used in the study, as different batches of canned ham and wheatgerm were used for these two experiments and the data were obtained much earlier than the stability study. There is very little difference between the %CV data for the determinations done seven times and the %CV for the triplicate analyses performed during the 5 month study. After the 5 month study, the accumulated data were plotted (Figs 1–9). The figures show the initial level of the analyte in the commodity and the levels of the analytes determined over the 5 month study. Overall analyte mean values for the 5 month study are presented in Table 2.

For cholesterol, vitamin C, thiamine, niacin and most fatty acids, the slope of the line of best fit could not be

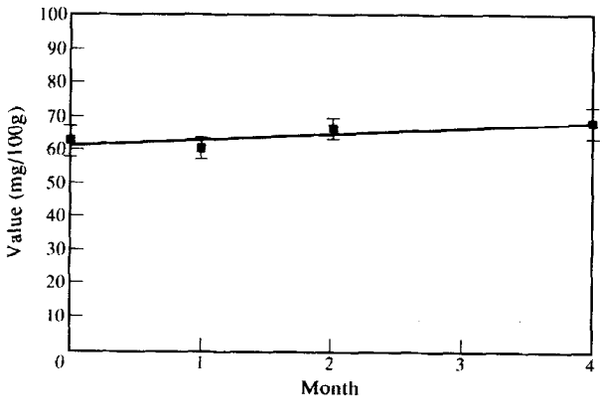

Fig. 1. Chart showing the levels of cholesterol in canned ham determined at regular intervals over a 4 month period, error bars (±2 SD) for each set of values, and the line of best fit.

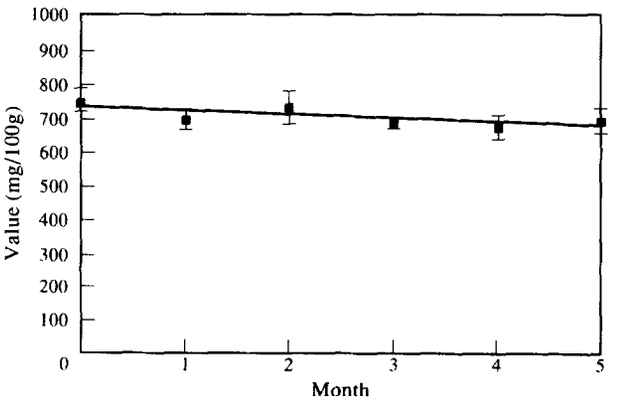

Fig. 2. Chart showing the levels of ascorbic acid in powdered orange drink vitamin C supplement determined at regular intervals over a 5 month period, error bars (±2 SD) for each set of values, and the line of best fit.

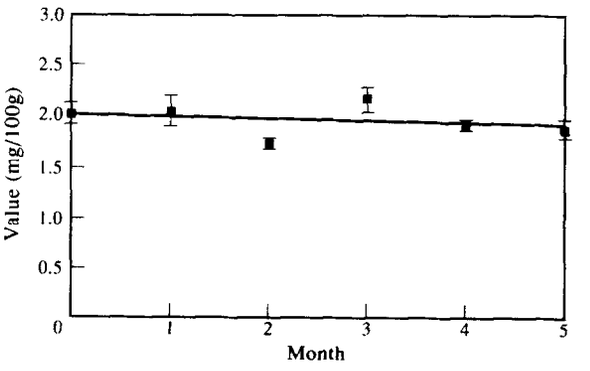

Fig. 3. Chart showing the levels of thiamine in wheatgerm determined at regular intervals over a 5 month period, error bars (±2 SD) for each set of values, and the line of best fit.

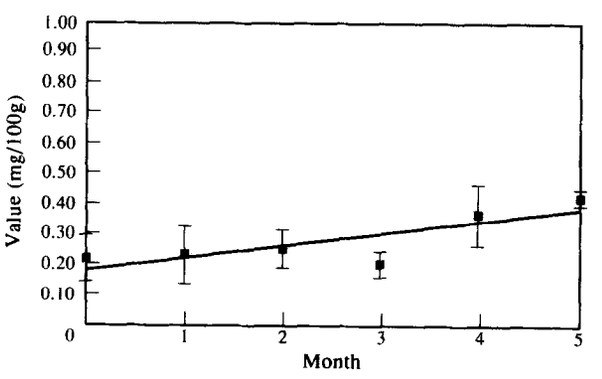

Fig. 4. Chart showing the level of riboflavin in wheatgerm determined at regular intervals over a 5 month period, error bars (±2 SD) for each set of values, and the line of best fit.

distinguished from zero, indicating acceptable stability. The data for riboflavin and some of the fatty acids were not as consistent. The levels of riboflavin showed a slight upward trend over the 5 month period (Fig. 4), while a downward trend in the levels of C18:1 and C18:2 in wheatgerm (Fig. 6) and C18:3 in canned ham (Fig. 7) was demonstrated. The levels of C18:1 and C18:2 in canned ham were more uniform, as were the levels of the unsaturated fatty acids C20:5 and C20:6 determined in the fish oils, even though a greater variation from the initial level was noticed.

The levels of the majority of analytes, with the possible exception of wheatgerm for riboflavin, C18:1 and C18:2 fatty acids, and C18:3 fatty acid in canned ham, were reasonably uniform over the 5 month study, suggesting that the commodities would be suitable reference materials. Variations in the actual levels of the analytes determined over the 5 month period could be attributed to the many factors which contribute to analytical error, for example, different operator, operator efficiency and the use of different analytical instruments.

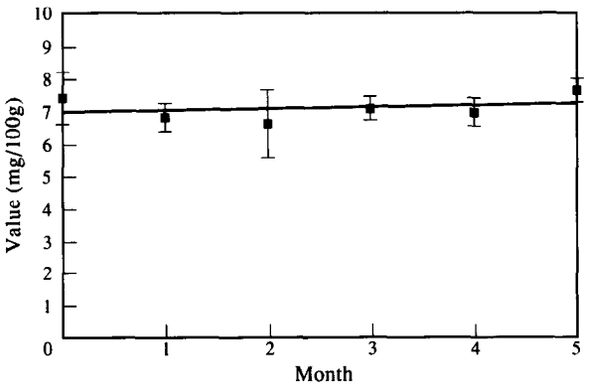

Fig. 5. Chart showing the levels of niacin in wheatgerm determined at regular intervals over a 5 month period, error bars (±2 SD) for each set of values, and the line of best fit.

CONCLUSION

The suitability of a number of 'off-the-shelf' food items as reference materials for incorporation into routine work batches as an additional quality assurance measure has been evaluated over a 5 month period. Canned ham, wheat germ, two fish oils, and powdered orange drink vitamin C supplement were suitable as control samples for the determination of cholesterol, fatty acid profile, thiamine, riboflavin, niacin, and ascorbic acid.

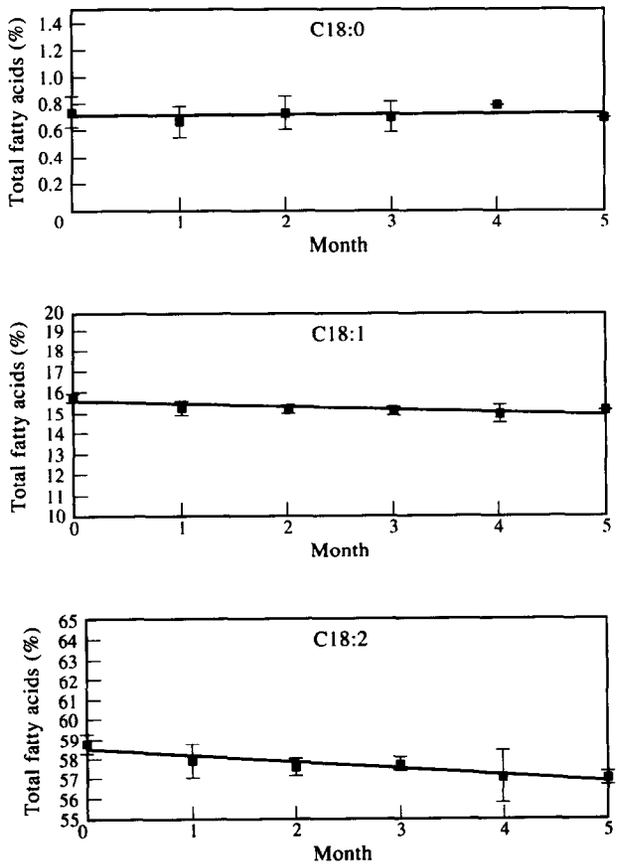

Fig. 6. Chart showing the % total fatty acids for C18:0, C18:1, and C18:2 in wheatgerm, determined at regular intervals over a 5 month period, error bars (±2 SD) for each set of values, and the line of best fit.

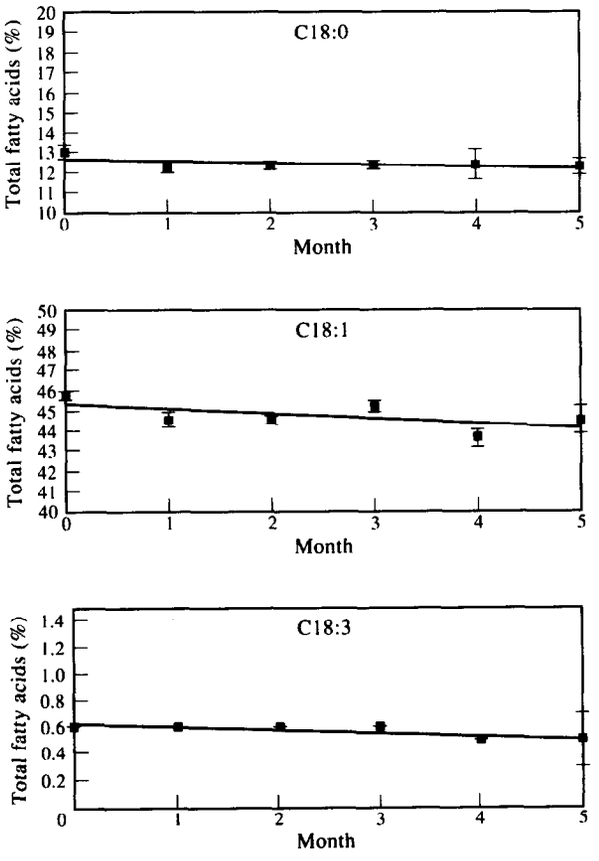

Fig. 7. Chart showing the % total fatty acids for C18:0, C18:1, and C18:3 in canned ham determined at regular intervals over a 5 month period, error bars (±2 SD) for each set of values, and the line of best fit.

Except for riboflavin, the intra-batch and overall precision was excellent for all analytes. Analyte stability during the evaluation was satisfactory, indicating that the samples are suitable for use as reference materials over an extended period. The materials are now in use as references in our laboratory.

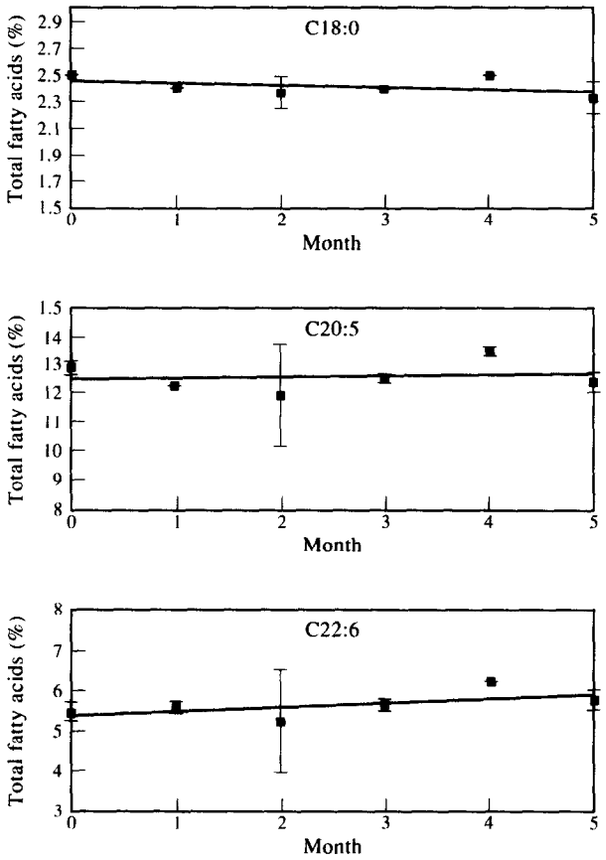

Fig. 8. Chart showing the % total fatty acids for C18:0, C20:5, and C22:6 in cod liver oil determined at regular intervals over a 5 month period, error bars (±2 SD) for each set of values, and the line of best fit.

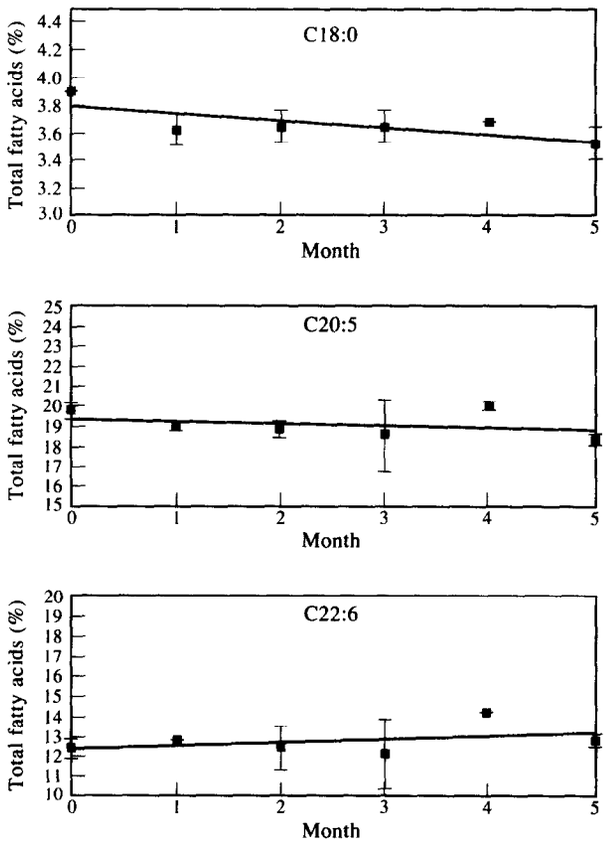

Fig. 9. Chart showing the % total fatty acids for C18:0, C20:5, and C22:6 in ω fish oil capsules determined at regular intervals over a 5 month period, error bars (±2 SD) for each set of values, and the line of best fit.

Table 2. Overall mean levels and %CV data for all analytes from regular triplicate analyses over a 5 month period

Commodity	Analyte	Statistics (units: mg/100 g)		
		Mean	SD	%CV
Wheat germ	Thiamine	1.97	0.13	6.8
Wheat germ	Riboflavin	0.27	0.087	32.2
Wheat germ	Niacin	7.1	0.45	6.4
Canned ham	Cholesterol	65	3.6	5.6
Vitamin C supplement	Ascorbic acid	718	35	4.9
			(units: % total fatty acids)	
Wheat germ	C18:0	0.72	0.055	7.6
	C18:1	15.2	0.26	1.7
	C18:2	57.7	0.68	1.2
Canned ham	C18:0	12.5	0.30	2.4
	C18:1	44.7	0.69	1.6
	C18:3	0.57	0.059	10.5
Cod liver oil	C18:0	2.4	0.071	2.9
	C20:5	12.6	0.58	4.6
	C22:6	5.6	0.38	6.8
ω Fish oil	C18:0	3.7	0.11	3.1
	C20:5	19.2	0.66	3.4
	C22:6	12.8	0.81	6.3

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