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HOMOGENEITY AND STABILITY OF MAJOR NUTRIENTS AND VITAMINS IN FIVE FOOD CANDIDATE REFERENCE MATERIALS

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ABSTRACT

HOMOGENEITY AND STABILITY OF MAJOR NUTRIENTS AND VITAMINS IN FIVE FOOD CANDIDATE REFERENCE MATERIALS

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21 tables, 8 figures, 2 annexes

As part of a Community Bureau of Reference (BCR) certification project on food reference materials for nutrients, homogeneity and stability of 5 candidate reference materials (RMs), whole milk powder, pork muscle, wheat and rye flour and haricots verts beans were studied.

Homogeneity was studied by comparing the within-sachet and between-sachets variation of the nutrients of interest using the F-test. Total protein nitrogen, total fat, available carbohydrates, fructose, lactose, total dietary fibre, ash, retinol, α-tocopherol, vitamin B1 and vitamin B2 proved to be homogeneously distributed in these candidate RMs (F-test, $p < 0.01$). However except for wheat flour, evidence of inhomogeneity of moisture was found. Consequently future certified values will have to be expressed on dry weight.

Major nutrients were monitored at regular intervals for 24 months in samples stored at -18 °, 4 °, 20 °, and 37 °C. In addition vitamins were monitored in samples stored at -18 ° and 4 °C. No evidence of change of the following major nutrients in the candidate RMs was found: total protein nitrogen, fructose, total dietary fibre, and ash. In some of the candidate RMs evidence of degradation only at 37 °C of the following major nutrients was found: total fat, available carbohydrates, and lactose. Stability of the following vitamins in

these candidate RMs proved to be adequate: retinol, α-tocopherol, vitamin B1, vitamin C. No change in the content of vitamin B2 in milk powder (RM 380) was observed, however vitamin B2 in pork muscle (RM 384) showed a tendency of degradation with time at each temperature.

Key words: reference materials, major nutrients, vitamins, foods, food analysis, stability, homogeneity, BCR

CONTENTS

1. INTRODUCTION

Information on the nutrient content of foods is used for many purposes: for research on the relation between diet and health, for counselling, for planning of diets, in education, for legal control, for nutrition labelling, and for the manufacture of food products. The only way to provide this information is by chemical analyses of foods for their nutrient content. However, routine food analyses, even performed by experienced laboratories, may produce widely different values for major nutrients in common foods [1].

Reference materials will be valuable tools to improve the quality of nutrient analyses. With this in view, the Community Bureau of Reference (BCR) of the Commission of the European Communities started a project on the preparation and certification of food reference materials.

Batches of materials intended for use as RMs were produced and packaged at Leatherhead Food RA (UK). Homogeneity and stability of the nutrients in these materials had to be proven. BCR contracted RIKILT to carry out these homogeneity and stablility studies. In this report results of these studies are described.

2. MATERIALS AND METHODS

2.1 Materials

Whole (full-cream) milk powder (RM 380), rye flour (RM 381), wheat flour (RM 382), freeze-dried haricots verts beans (RM 383), and freeze-dried pork muscle (RM 384) were provided by Leatherhead Food RA in heat sealed laminated foil sachets of 100 g (50 g for pork muscle). During packaging, sachets were coded by numbers in ascending order and sachets were taken at regular intervals to study the between-sachets variation as described in section 2.2.

2.2 Homogeneity study

The between-sachets homogeneity was studied on the following nutrients: dry weight, total protein nitrogen, total fat, available carbohydrates, fructose, lactose, ash, retinol, α-tocopherol, vitamin B1, and vitamin B2. The analytical methods used are described in section 2.4. In order to determine the between-sachets variation, ten sachets of each RM, taken at regular intervals during packaging (see section 2.1), were analysed for the nutrients of interest by single analyses. The repeatability of the methods used (within-

sachet variation) was studied by analysing one sachet of each RM for each nutrient ten times. All results were calculated on dry matter.

2.3 Stability study

Directly after receipt of the RMs in the beginning of September 1988, sachets were stored at -40 ± 2 °C, -18 ± 2 °C, +4 ± 2 °C, +20 ± 2 °C, and +37 ± 2 °C for at least 24 months. The freezers (-40 °C and -18 °C) and the refrigerator (+4 °C) were provided with an alarm set at 2 °C above the target temperature. No anomalies were detected during the study. The stove (+20 °C) was checked five times during the study by continueously registrating of the temperature for a period of three weeks. Temperature proved to be 20 ± 0.5 °C. The stove (+37 °C) was checked weekly during the first half of the study. During the second half temperature was registrated contineously. No anomalies occured, temperature was 37 ± 1 °C.

Nutrients were monitored at regular intervals by duplicate analyses using the methods given in section 2.4. Repeatability criteria as determined in the homogeneity study (section 2.2) were applied. Whenever results did not meet these repeatability criteria, analyses were repeated in duplicate. All results were expressed on dry matter. The scheme of analyses of the RMs is given in Table 1.

For major nutrients, the laboratory regularly participated in interlaboratory intercomparisons. No anomalies in the level of the analyses were observed. For vitamins an internal control sample, milk powder stored at -18 °C, was used. In addition to analyses at the respective storage periods, this control sample was analysed monthly to quantify possible degradation of vitamins in this control sample.

Table 1. Scheme of analyses

Temperature	-18 °C						4 °C					20 °C					37 °C			
Period (months)	0	3	6	12	18	24	3	6	12	18	24	3	6	12	18	24	6	12	18	24
Dry matter	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Protein N	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Total fat	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Avail.carb.	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Sugars	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Dietary fibre	+	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Ash	+	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Retinol	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
α-Tocopherol	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Vitamin B1	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Vitamin B2	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-

2.4 Methods of analysis

- Dry weight

Milk Powder: 2 g sample is dried at 103 °C during 4 hours.

Wheat and Rye Flour: 5 g sample is dried at 130 °C during 2 hours.

Pork Muscle and Haricots Verts Beans: 5 g sample is dried at 103 °C during 4 hours.

- Total protein nitrogen

Milk Powder, according to NEN 3198 [2]: 0.5 g sample is digested with 20 ml H_2SO_4 and $CuSO_4$(0.05 g)/K_2SO_4(10 g) in a block digestion apparatus. After alkalisation, the ammonia is transfered by steam distillation into excess of boric acid. The ammonia bound by boric acid is determined by titration with HCl.

Pork Muscle, Wheat and Rye Flour, Haricots Verts Beans, according to NEN 5396 [3]: 1-1.5 g sample is digested with 26 ml H_2SO_4 and CuSO4(1.2 g)/K_2SO_4(10 g) in a block digestion apparatus. After alkalisation, the ammonia is transfered by steam

distillation into excess of boric acid. The ammonia bound by boric acid is determined by titration with HCl.

- Total fat

Pork Muscle, according to Weibull-Stoldt, NEN 3444 [4]: 4 g sample is boiled in 4 M HCl for 1 hour, and filtered on fluted filter paper. The filter is washed, dried for 1 hour at 103 °C, and extracted with petroleum ether for 4 hours in a Soxhlet type extraction apparatus. After evaporation of the solvent, the fat is dried for 1 hour at 103 °C, and weighed.

Wheat and Rye Flour and Haricots Verts, according to Weibull-Stoldt, EC L15/29 (1984) [5]: 2.5 g sample is boiled in 3 M HCl for 1 hour, and filtered on fluted filter paper. The filter is washed, dried for 1.5 hour at 95-98 °C, and extracted with petroleum ether for 6 hours in a Soxhlet type extraction apparatus. After evaporation of the solvent, the fat is dried for 1.5 hour at 75 °C in a vacuum oven, and weighed.

Milk Powder, according to Röse-Gottlieb, NEN 3197 [6]: 1 g sample is dissolved in water at 65 °C, 2 ml ammonia 25% (v/v) is added and the mixture is heated for 15 min at 65 °C. 10 ml ethanol is added, followed by mixing and addition of 25 ml diethyl ether. After shaking, 25 ml petroleum ether is added followed by shaking, centrifuging and decantation of the solvent. 5 ml ethanol is added and a second extraction is carried out with 25 ml diethyl ether and 25 ml petroleum ether. A third extraction is carried out with 15 ml diethyl ether and 15 ml petroleum ether. After evaporation of the combined solvents, the fat is dried for 30 min at 100 °C and weighed.

- Available carbohydrates

Rye and Wheat Flour, Haricots Verts Beans: 30 ml water is added to 1 g sample, followed by solubilisation of starch in an autoclave at 120 °C for 2 hours. Starch is hydrolysed with pancreatin (α-amylase) for 1 hour at 40 °C. Disacharides are hydrolysed to monosaccharides with 1 M HCl at 100 °C for 1.5 hour. Sugars are determined by a reductiometric procedure according to Luff/Schoorl.

- Lactose

Milk Powder, according to NEN 6853 [7]: 1.2 g sample is dissolved in 50 ml water at 50-70 °C. After deproteination of the extract, lactose is split into glucose and galactose with the enzyme β-galactosidase. Glucose is determined by measurement of the absorbance of reduced nicotinamide adenine- dinucleotide phosphate (NADPH) formed by conversion of glucose catalysed by hexokinase in the presence of adenosine triphosphate and nicotinamide adenine- dinucleotide phosphate.

- Total Dietary Fibre

Rye and Wheat Flour, Haricots Verts Beans, according to the AOAC method [8], the enzymatic-gravimetric procedure.

- Ash

All RMs: sample is pre-ashed at a hot plate, followed by ashing at 550 °C, until a white ash remains.

- Retinol

Milk Powder: 10 g sample is saponified in alkaline ethanol and extracted with dichloroethane according to Grimm [9]. Retinol is determined with reversed phase HPLC (C18) and UV-detection at 325 nm.

- α-Tocopherol

Milk Powder: 10 g sample is saponified in alkaline ethanol and extracted with dichloroethane according to Grimm [9]. α-Tocopherol is determined with reversed phase HPLC (C18) and fluorescence detection at 290/326 nm.

- Vitamin B1

Milk Powder, Pork Muscle: 0.5 g sample is extracted by autoclaving for 15 min at 120 °C, followed by enzymatic hydrolysis of the phophorylated thiamin with Takadiastase for 18 hours at 45 °C. Thiamin is separated with ion pair reversed HPLC (C18) and determined with fluorescence detection at 368/420 nm after post column derivatisation of thiamin to thiochrome.

- Vitamin B2

Milk Powder, Pork Muscle: 0.5 g sample is extracted by autoclaving for 15 min at 120 °C, followed by enzymatic hydrolysis of the phophorylated thiamin with Takadiastase for 18 hours at 45 °C. Riboflavin is separated with ion pair reversed HPLC (C18) and determined with fluorescence detection at 468/520 nm.

- Vitamin C

Haricots Verts Beans: 2 g sample is extracted with metaphosphoric acid 1%, followed by enzymatic oxidation of ascorbic acid with ascorbate oxidase at 37 °C for 5 min. A fluorescent quinoxaline is formed by reaction of dehydroascorbic acid with o-phenylenediamine for 30 min at 37 °C. The quinoxaline is separated with reversed HPLC (C18) and determined with fluorescence detection at 355/425 nm.

3 RESULTS AND DISCUSSION

3.1 Homogeneity

For each nutrient and RM the between-sachets standard deviation was calculted and compared with the within-sachet standard deviation (analytical precision). The results are summarised in Table 2, the between-sachet standard deviation is expressed as the coefficient of variation CVhom, the within-sachet standard deviation is given as CVrep. Individual data are presented in Annex I.

The results show that there is no significant difference (F-test, 5% level) between these standard deviations for most of the RMs and nutrients. For available carbohydrates in wheat flour (RM 382), and vitamin B2 in whole milk powder (RM 380) differences between CVhom and CVrep are somewhat higher, however there is no significant difference at 1% level.

One exception has to be made for dry matter. The variation between the samples differs significantly (1% level) from the analytical variation, indicating that moisture is not homogeneously distributed. In consequence, nutrients will have to be expressed on dry weight to account for these differences in moisture content between sachets.

Although fat in milk powder proved to be homogeneous comparing the within-sachet and between-sachets variation, the analytical variation in the homogeneity study was rather high (CVrep = 0.94 %). This poor precision proved to be caused by a faulty analytical balance. However, results of the stability study at -18 °C confirm that fat in milk powder measured by the same method is homogeneously ditributed between sachets (CVrep = 0.22 %, CV between different storage periods = 0.19 %).

The candidate reference materials can thus be regarded as homogeneous, provided results are expressed on dry weight.

Table 2. Homogeneity study, comparing for each material and nutrient the variation between sachets (CVhom) with the analytical precision (CVrep). Mean values are expressed as g/100 g dry weight, except for vitamins which are expressed as mg/100 g dry weight

	Whole Milk Powder (RM 380)	Rye Flour (RM 381)	Wheat Flour (RM 382)	Haricots Verts Beans (RM 383)	Pork Muscle (RM 384)
Dry weight					
mean	97.89	87.78	87.34	96.19	97.37
sample weight (g)	2	5	5	5	5
CVhom	0.22%	0.32%	0.08%	0.24%	0.31%
n hom	10	10	10	10	10
CVrep	0.03%	0.04%	0.07%	0.05%	0.05%
n rep	10	10	10	10	10
Total protein nitrogen					
mean	4.5004	1.2338	2.098	1.0494	13.749
sample weight (g)	0.6	1	1	1	0.25
CVhom	0.13%	0.46%	0.35%	0.47%	0.22%
n hom	10	10	10	10	10
CVrep	0.10%	0.34%	0.25%	0.27%	0.33%
n rep	10	10	10	10	10
Total fat					
mean	27.090	1.150	1.425	2.440	10.993
sample weight (g)	1	2.5	2.5	2.5	2.5
CVhom	0.75%	7.20%	8.69%	5.85%	0.74%
n hom	10	10	10	10	10
CVrep	0.94%	8.40%	5.26%	6.71%	0.84%
n rep	9	10	10	10	10

	Whole Milk Powder (RM 380)	Rye Flour (RM 381)	Wheat Flour (RM 382)	Haricots Verts Beans (RM 383)	Pork Muscle (RM 384)
Available carbohydrates					
mean		80.75	81.739	73.10	
sample weight (g)		1	1	1	
CVhom		0.54%	1.41%	0.98%	
n hom		10	10	10	
CVrep		0.78%	0.79%	0.72%	
n rep		10	10	10	
Fructose					
mean				4.63	
sample weight (g)				1	
CVhom				3.68%	
n hom				10	
CVrep				3.50%	
n rep				10	
Lactose					
mean	36.14				
sample weight (g)	1				
CVhom	0.71%				
n hom	10				
CV rep	1.36%				
n rep	10				

	Whole Milk Powder (RM 380)	Rye Flour (RM 381)	Wheat Flour (RM 382)	Haricots Verts Beans (RM 383)	Pork Muscle (RM 384)
Ash					
mean	6.051	0.7988	0.7949	2.236	4.459
sample weight (g)	1.5	5	5	5	5
CVhom	0.33%	1.26%	1.08%	1.53%	0.58%
n hom	10	10	10	10	10
CVrep	0.45%	0.90%	0.64%	1.45%	0.95%
n rep	10	10	10	10	10
Retinol					
mean	0.3975				
sample weight (g)	10				
CVhom	2.09%				
n hom	9				
CVrep	1.75%				
n rep	10				
α-Tocopherol					
mean	0.739				
sample weight (g)	10				
CVhom	4.58%				
n hom	10				
CVrep	2.81%				
n rep	9				

	Whole Milk Powder (RM 380)	Rye Flour (RM 381)	Wheat Flour (RM 382)	Haricots Verts Beans (RM 383)	Pork Muscle (RM 384)
Vitamin B1					
mean	0.2852				2.5729
sample weight (g)	0.5				0.5
CVhom	3.23%				1.37%
n hom	9				9
CVrep	2.27%				1.63%
n rep	10				9
Vitamin B2					
mean	1.471				0.7644
sample weight (g)	0.5				1.0
CVhom	3.88%				2.54%
n hom	10				10
CVrep	2.10%				1.40%
n rep	10				9

As the vitamin content of the cereals was too low to be of interest, homogeneity of α-tocopherol, vitamin B1 and B2 in an already existing certified reference material CRM 189, a whole rye meal, was studied following the same procedure. Results are summarised in Table 2a, individual data are given in Annex I. For α-tocopherol and vitamin B1 the variation between the bottles (CVhom) differs significantly (1% level) from the analytical variattion indicating that these vitamins are not homogeneously distributed in CRM 189.

Table 2a.Homogeneity study of CRM 189, comparing for α-tocopherol, vitamin B1 and B2 variation between bottles (CVhom) with the method repeatability (CVrep). Values are expressed as mg/100 g

	α-Tocopherol	Vitamin B1	Vitamin B2
mean	1.005	1.034	0.1776
CVhom	22%	5.0%	4.1%
n hom	10	10	10
CVrep	5.2%	1.7%	4.5%
n rep	9	10	10

3.2 Stability

Results of the stability study are presented in Figures 1 - 6 and Tables 1a - 1j of Annex II. In the figures the value "average" given is the mean value of the results at all temperatures for each nutrient and RM. The value "sd" is the standard deviation of the analyses including the repeatability standard deviation s_{rep} and the variation between different series of analyses performed on diferent days within the laboratory, s_{time}. Assuming that all nutrients are stable at -18 °C, s_{time} and s_{rep} were calculated only using the determinations in the sachets stored at -18 °C. Then sd was calculated as follows:

$$sd = (s_{time}^2 + \tfrac{1}{2} s_{rep}^2)^{\frac{1}{2}}$$

For **total protein nitrogen** (Fig.1) no change in protein measured with time or temperature in none of the RMs is noticeable.

Fat (Fig.2) shows evidence of deterioration only at the highest storage temperature, viz. 37 °C, especially for wheat flour (RM 382). As fat content in sachets stored at 20 °C and 37 °C always are lower compared to sachets stored at -18 °C, it is recommended by way of precaution to store samples at a lower temperature, f.i. 4 °C. However, it is emphasized that the variation in fat contents are smaller than 2*sd, the analytical variation determined in sachets stored at -18 °C.

No change in the content of **available carbohydrates** (Fig.3) was measured, except perhaps for wheat flour at 37 °C. At this temperature, always the lowest results were measured.

Lactose in milk powder (Fig. 3) clearly degraded at 37 °C. Fig. 3 also shows doubts

about stability at 20 °C. Therefore, it was decided to extend the stability study for lactose. Lactose content of sachets of milk powder stored at -40 °C, 4 °C, 20 °C and 37 °C for 31 months was determined by single analyses. Results given in Table 3 confirm stability of lactose at 4 °C and 20 °C, and degradation of lactose at 37 °C.

Table 3. Lactose content (g anhydrous lactose/100 g dry weight) of sachets of milk powder (RM 380) stored at different temperatures for 31 months

-40 °C		4 °C		20 °C		37 °C	
Sachet code	lactose	code	lactose	code	lactose	code	lactose
609	36.42	247	36.90	390	36.56	268	35.73
315	36.71	381	35.96	667	36.76	284	35.47
310	36.30	861	36.92				
mean	36.477		36.593		36.66		35.600
(st.dev.)	(0.21)		(0.55)				

Fructose in haricots verts (RM 383) proves to be stable at all temperatures (Fig. 3).

No change in **dietary fibre** contents of cereals (RM 381 and RM 382) and haricots verts beans (RM 383) was observable with time and temperature (Fig. 4).

As could be expected **ash** (Fig. 5) is stable at all temperatures.

Retinol content in milk powder (RM 380) rapidly decreased during the first six months of storage both at -18 °C and 4 °C, but stabilised afterwards (Fig. 6). Retinol content of sachets stored at -18 °C is higher compared to sachets stored at 4 °C, however, these differences seem constant after six months.

No change in **α-tocopherol** content of milk powder (RM 380) stored at -18 °C and 4 °C is noticeable (Fig. 6). Again α-tocopherol content of sachets stored at -18 °C is higher compared to sachets stored at 4 °C. However, α-tocopherol content of sachets stored at -18 °C decreases gradually to the level found in sachets stored at 4 °C. In addition to milk powder a limited study on the stability of α-tocopherol in haricots verts beans (RM 383) was carried out. α-Tocopherol content of sachets of haricots verts beans stored at -40 °C, 4 °C, and 37 °C for 25 months was determined by duplicate analyses. Results given in Table 4 confirm stability of α-tocopherol at 4 °C and even at 37 °C.

Table 4. α-Tocopherol content (mg α-Tocopherol acetate/100 g dry weight) of sachets of haricots verts beans (RM 383) stored at different temperatures for 25 months

-40 °C		4 °C		37 °C	
Sachet code	α-Tocopherol	code	α-Tocopherol	code	α-Tocopher.
465	0.293	898	0.294	57	0.327
556	0.295	250	0.282	647	0.314
877	0.312	414	0.273	926	0.327
mean	0.2998		0.2830		0.3227
(st.dev.)	(0.011)		(0.011)		(0.008)

Vitamin B1 proves to be stable in milk powder (RM 380) and pork muscle (RM 384) at both temperatures (Fig. 6).

No change in **vitamin B2** content of milk powder (RM 380) was observed. However, evidence of degradation of vitamin B2 in pork muscle even at -18 °C was found (Fig. 6).

A limited study on the stability of **vitamin C** was performed. Vitamin C content of sachets of haricots verts beans (RM 383) stored at -40 °C, 4 °C, and 37 °C for 25 months was determined by duplicate analyses. This study was repeated by analysing samples which had been stored for 31 months at -40 °C and 4 °C. Results given in Table 5 demonstrate adequate stability for samples stored at 4 °C, however storage at 37 °C resulted in almost complete destruction of vitamin C.

Table 5. Vitamin C content (mg /100 g dry weight) of sachets of haricots verts beans (RM 383) stored at different temperatures for 25 and 31 months

Storage period									
25 months						31 months			
-40 °C		4 °C		37 °C		-40 °C		4 °C	
Sach code	Vit.C	code	Vit.C	code	Vit.C	code	Vit.C	code	Vit.C
465	18.30	898	17.67	57	0.78	121	16.24	424	16.21
556	18.66	250	17.22	647	1.20	874	17.95	633	18.13
877	18.08	414	16.56	926	0.28	901	17.37	914	17.72
mean	18.347		17.150		0.753		17.187		17.35
s.d.	0.29		0.55				0.87		1.01

Results of the internal control sample are shown in Figures 7a,7b. In these figures "s_m" indicates the standard deviation of the mean values of three replicates (retinol, α-tocopherol) or four replicates (vitamin B1, B2) found in the control sample in the first seven months of monitoring. The average of these mean values is also indicated ("average"). Results of control samples incorporated in the stability study are marked in the figures. For retinol and vitamin B2 a significant decrease in the control sample was measured: 1.5 IE retinol/(100g.month), 10 μg vitamin B2/(100g.month). It is concluded that the analytical level of the laboratory in this stability study was within narrow limits (average $\pm$ 1 s_m) for all vitamin studied. For retinol and vitamin B2 allowance had to be made for a decrease in content of the internal control sample established by frequent monitoring.

Fig. 1. TOTAL PROTEIN NITROGEN (g/100 g dry weight) monitored at regular intervals in RMs stored at different temperatures for a total period of 24 months.

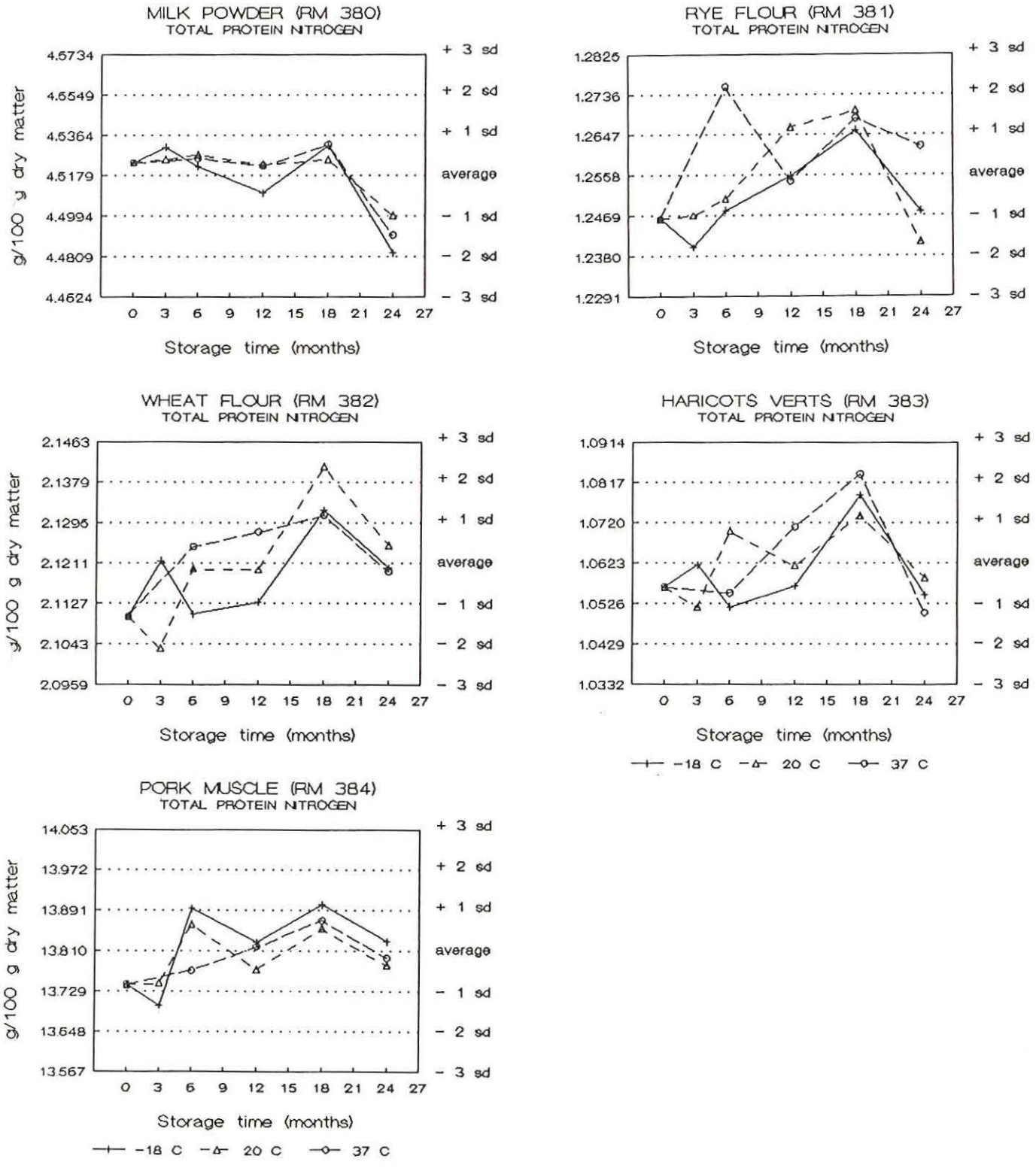


Fig. 2. TOTAL FAT (g/100 g dry weight) monitored at regular intervals in RMs stored at different temperatures for a total period of 24 months.

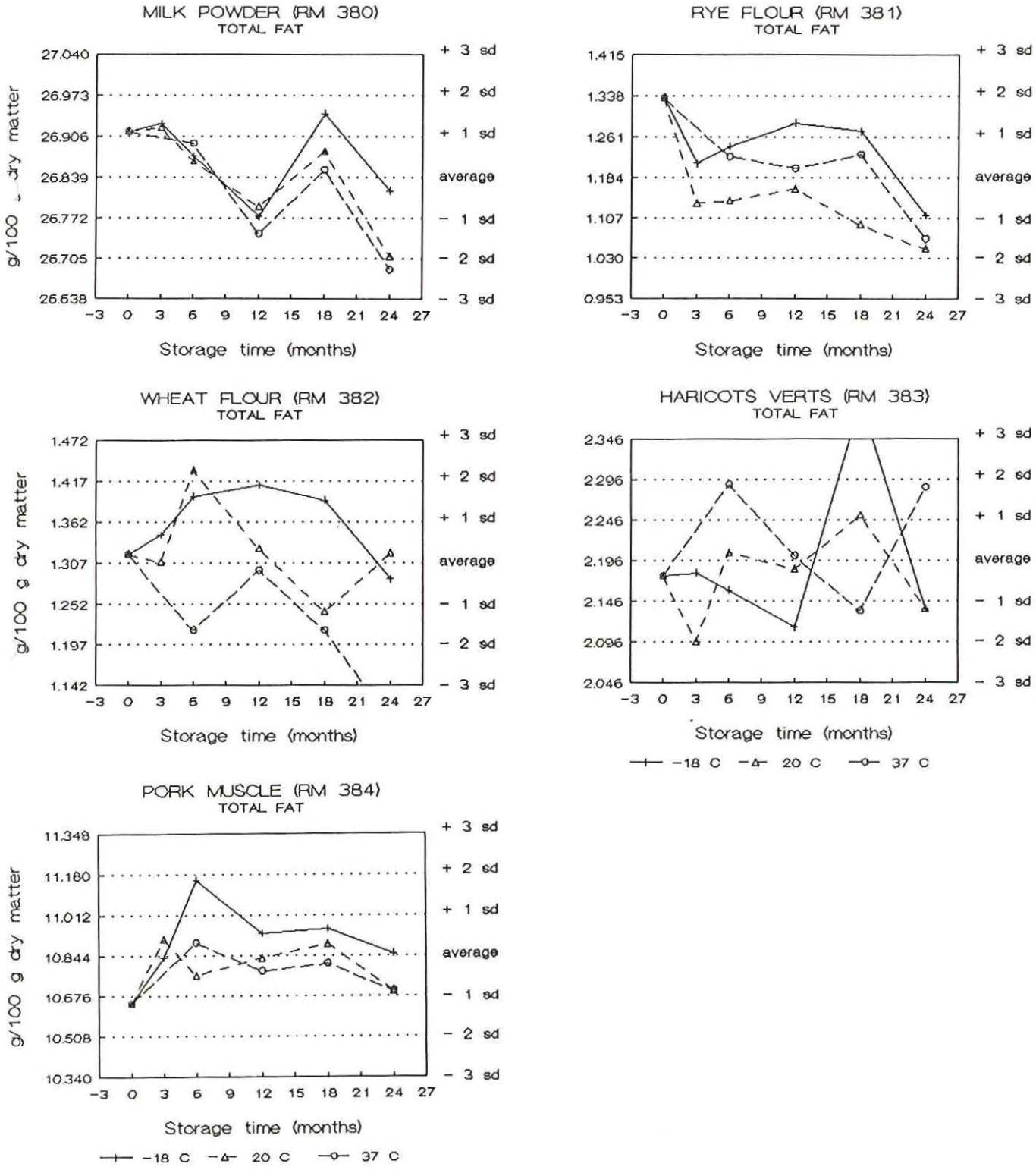


Fig. 3. AVAILABLE CARBOHYDRATES (g monosaccharides/100 g dry weight), LACTOSE (g anhydrous lactose/100 dry weight) and FRUCTOSE (g/100 g dry weight) monitored at regular intervals in RMs stored at different temperatures for a total period of 24 months.

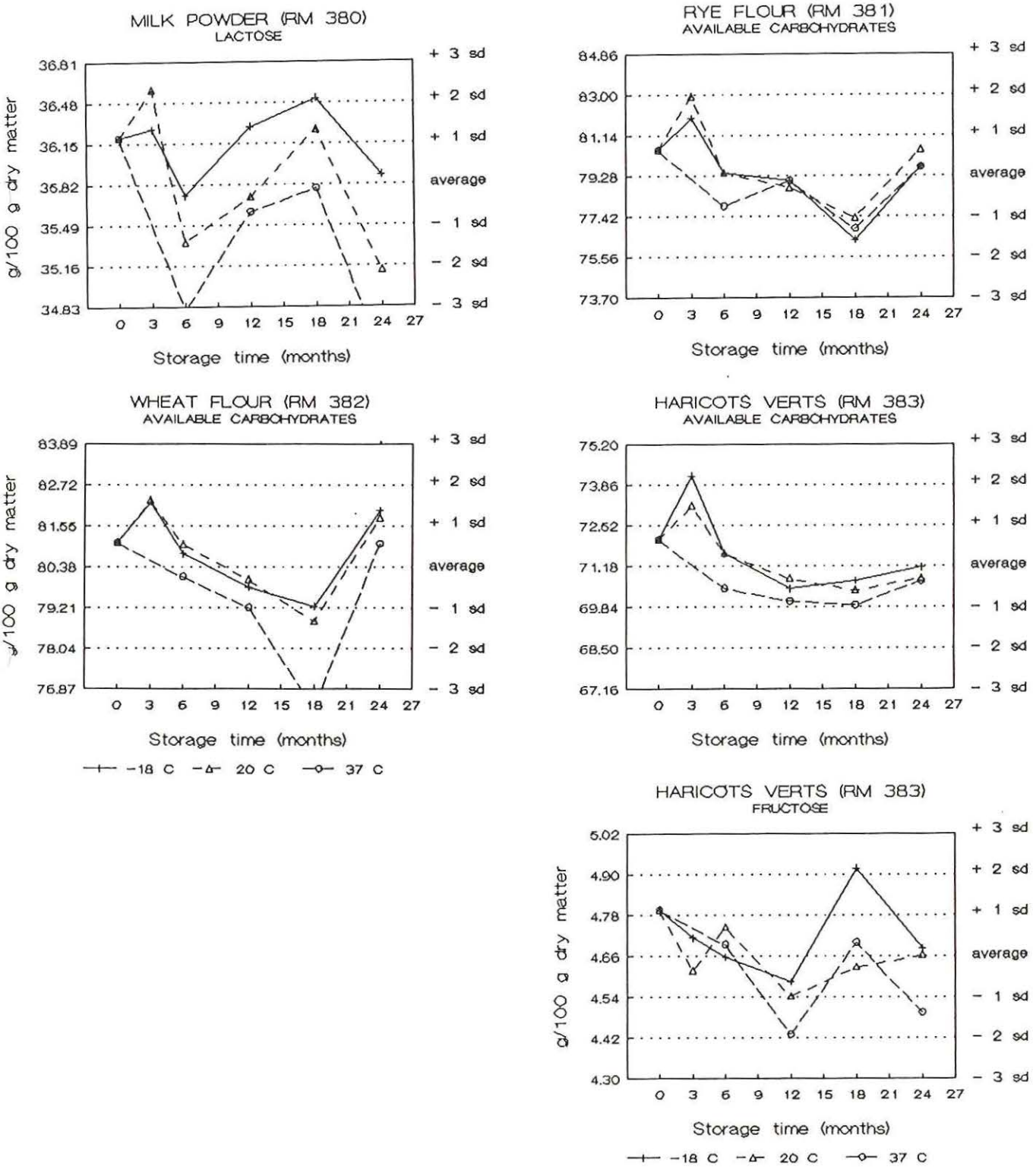


Fig. 4. TOTAL DIETARY FIBRE (g fibre as polymer/100 g dry weight) monitored at regular intervals in RMs stored at different temperatures for a total period of 24 months.

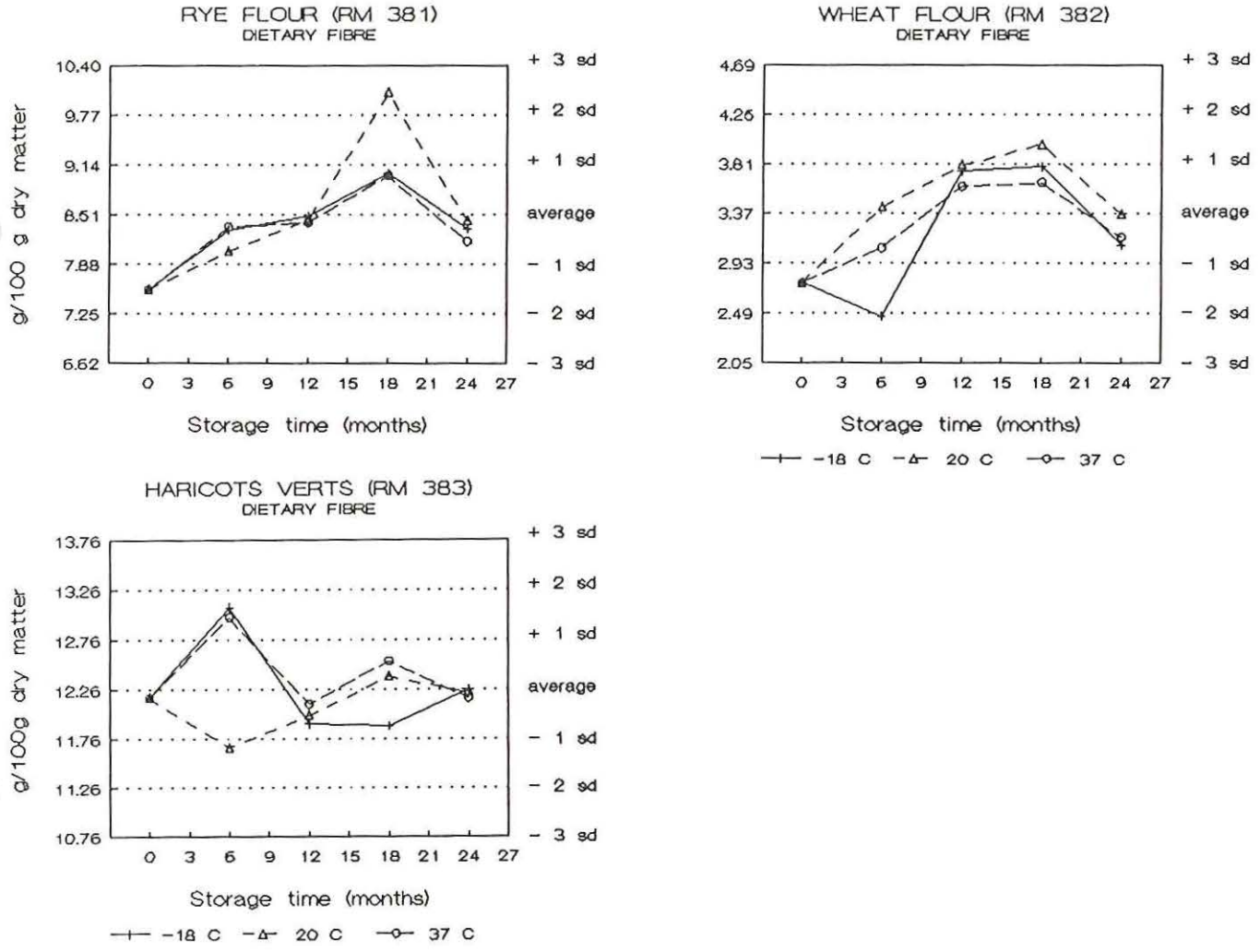


Fig. 5. ASH (g/100 g dry weight) monitored at regular intervals in RMs stored at different temperatures for a total period of 24 months.

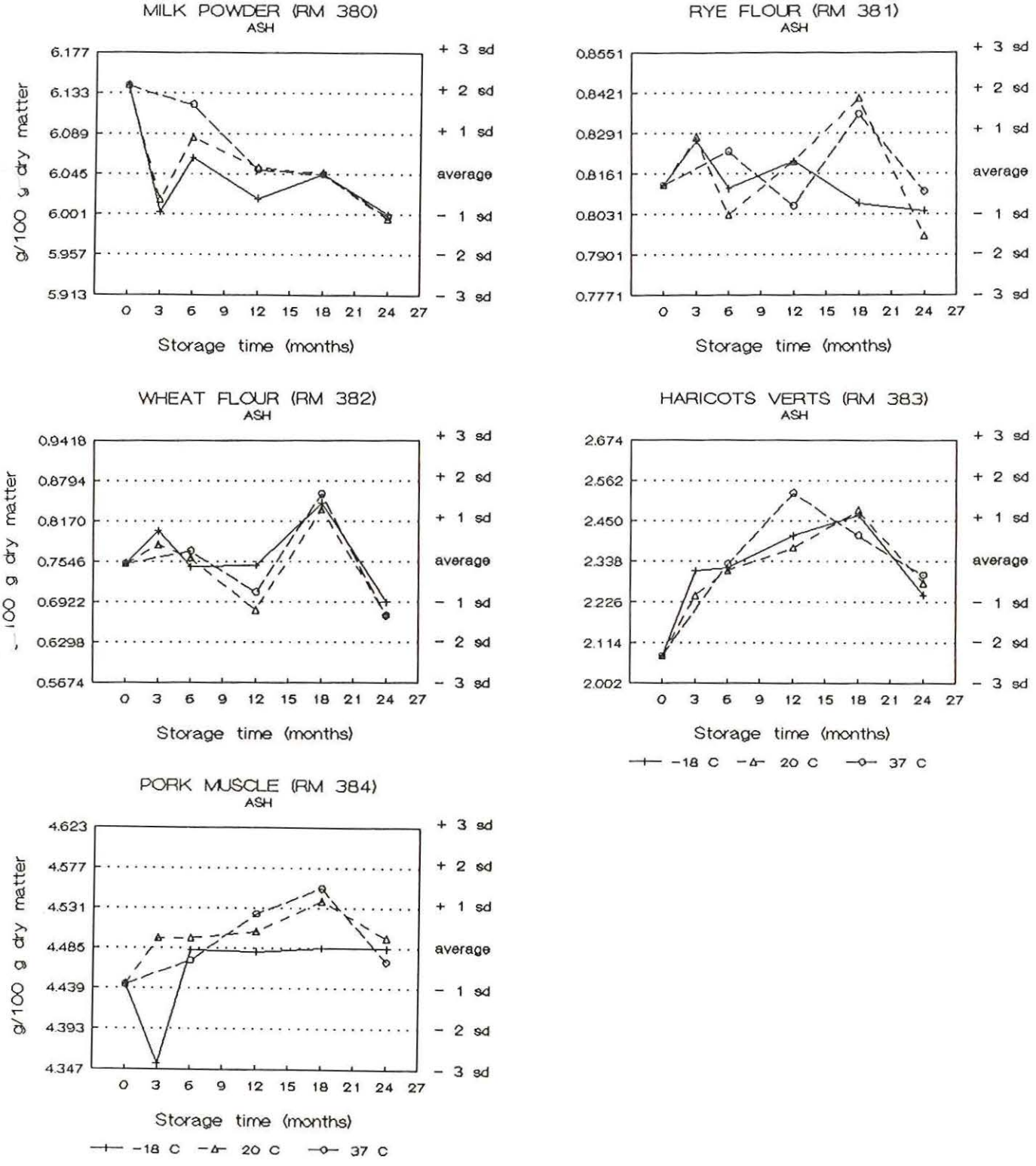


Fig. 6. RETINOL (IE/100 g dry weight), α-TOCOPHEROL, VITAMIN B1 and VITAMIN B2 (all mg/100 g dry weight) monitored at regular intervals in RMs stored at different temperatures for a total period of 24 months.

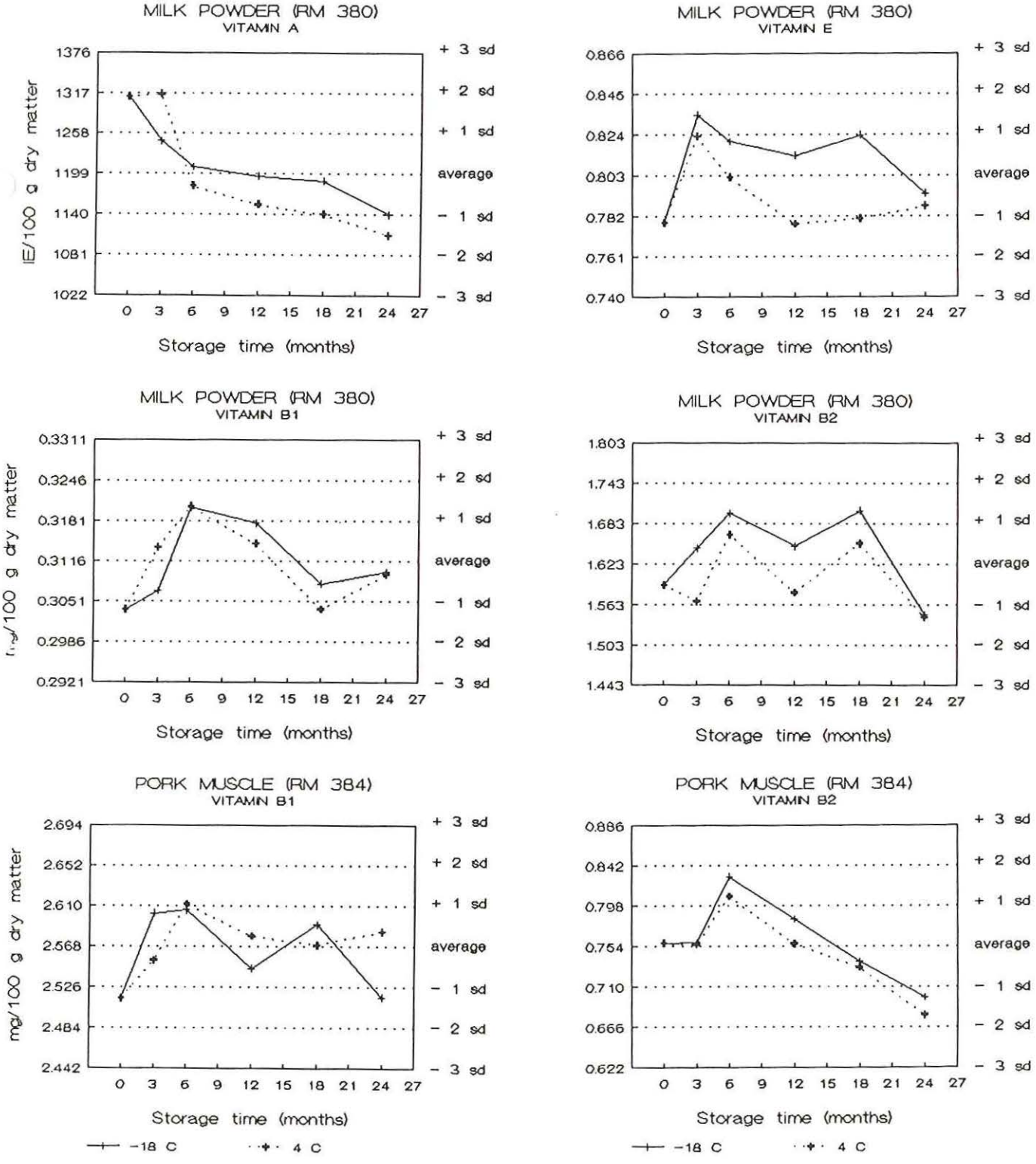
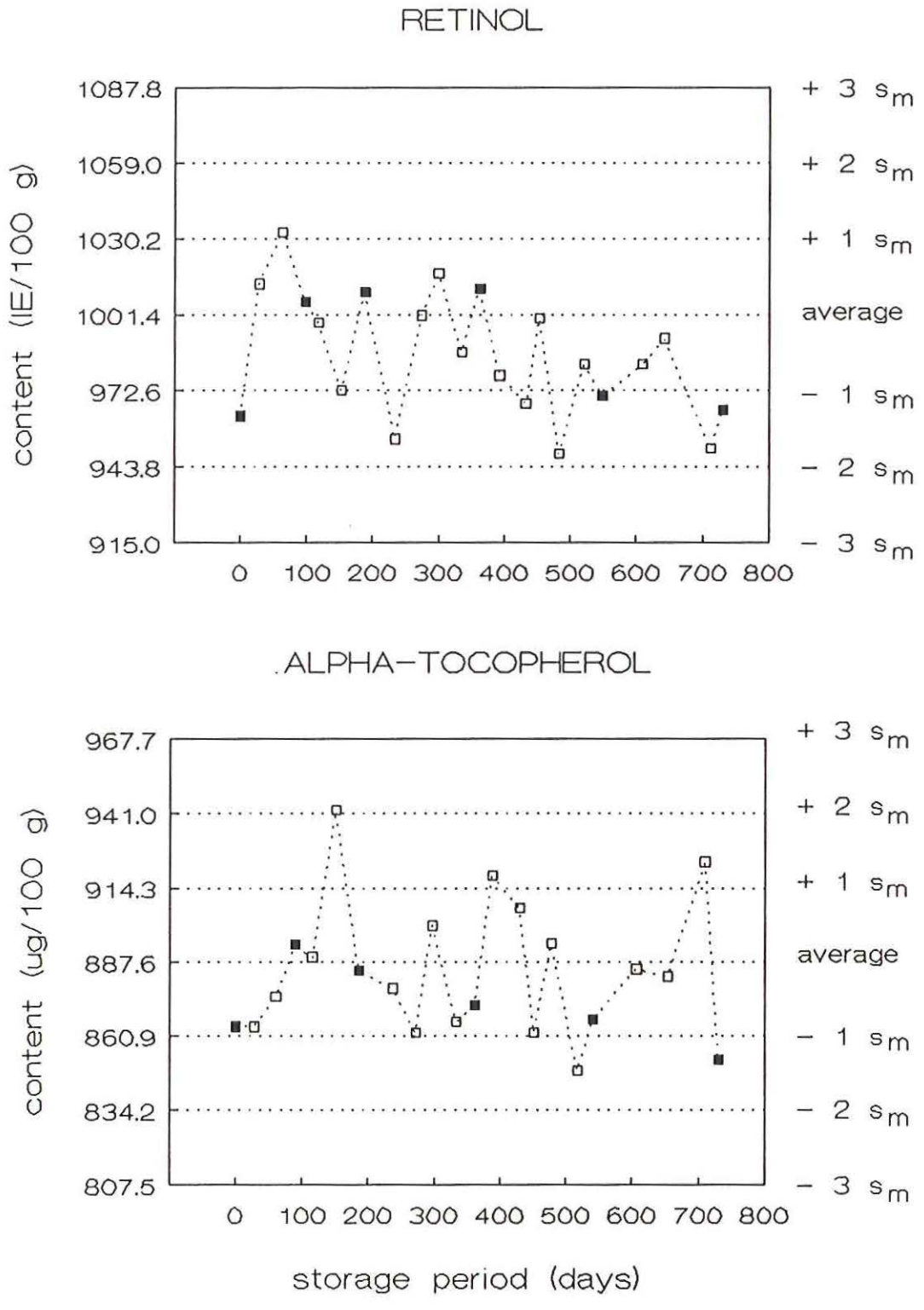


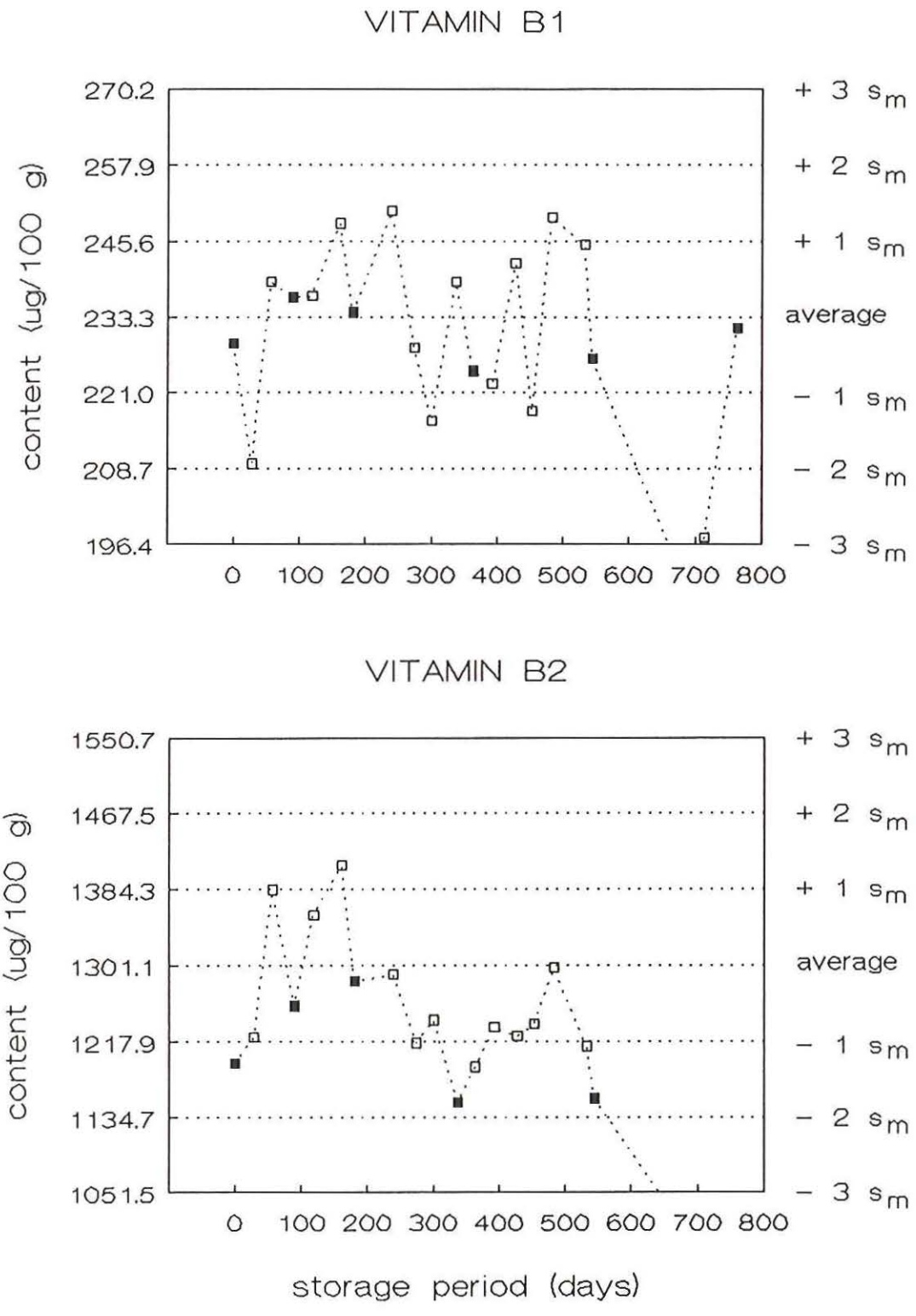
Fig. 7a. Control chart of internal control sample milk powder



mean of 3 replicates

mean of 3 replicates, analysis coincided with stability study

Fig. 7b. Control chart of internal control sample milk powder



4. CONCLUSIONS

The five candidate reference materials studied proved to be homogeneous with respect to total protein nitrogen, total fat, available carbohydrates, fructose, lactose, total dietary fibre, ash, retinol, α-tocopherol, vitamin B1 and vitamin B2, provided results are expressed on dry weight.

A 24 months stability study showed no evidence of change of the following major nutrients in the candidate RMs stored at -18 °C, 20 °C and 37 °C: total protein nitrogen, fructose, total dietary fibre, and ash. In some of the candidate RMs evidence of degradation only at 37 °C of the following major nutrients was found: total fat, available carbohydrates, and lactose. Stability of the following vitamins in these candidate RMs stored at -18 °C and 4 °C proved to be adequate: retinol, α-tocopherol, vitamin B1, vitamin C. No change in the content of vitamin B2 in milk powder (RM 380) was observed, however vitamin B2 in pork muscle (RM 384) showed a tendency of degradation with time at each temperature.

It is recommended to store the reference materials at a temperature not higher than 4 °C.

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ANNEX I

Individual results (major nutrients expressed as g/100 g dry weight, vitamins expressed as mg/100 g dry weight) of the single determinations of each nutrient in ten different sachets of RM 380, RM 381, RM 382, RM 383, RM 384 and of the replicate determinations of each nutrient in one sachet of RM 380, RM 381, RM 382, RM 383, and RM 384 .

MILK POWDER (RM 380)

Sach Code	Dry Weight	Total Prot. Nitro.	Total Fat	Ash	Lactose	Retinol	α-Toco-pherol	Vit. B1	Vit. B2
52	98.022	4.504	27.338	6.035	36.295	1453	0.7417	-	1.525
152	98.095	4.493	27.038	6.078	36.284	1351	0.7931	0.288	1.452
252	98.136	4.508	27.368	6.049	36.532	1270	0.7000	0.288	1.460
352	97.953	4.494	27.137	6.047	36.171	1296	0.7493	0.294	1.447
452	98.045	4.501	27.063	6.068	35.790	1323	0.7558	0.283	1.573
552	97.984	4.509	26.820	6.073	35.729	1322	0.6736	0.289	1.398
652	97.786	4.497	26.991	6.060	36.348	1353	0.7383	0.269	1.489
752	97.585	4.499	26.891	6.032	35.897	1350	0.7727	0.271	1.516
852	97.526	4.496	26.917	6.055	36.147	1330	0.7311	0.295	1.469
952	97.758	4.504	27.356	6.015	36.180	1335	0.7365	0.289	1.385
a	98.115	4.497	26.930	6.025	35.946	1376	0.7563	0.320	1.577
a	98.121	4.491	27.153	6.019	35.839	1301	0.7553	0.326	1.585
a	98.125	4.499	26.866	5.983	36.728	1325	0.7268	0.320	1.537
a	98.060	4.493	-	5.983	36.709	1330	0.7635	0.320	1.602
a	98.128	4.505	26.892	5.999	36.757	1329	0.7798	0.315	1.658
a	98.121	4.497	27.499	5.934	36.082	1306	-	0.332	1.618
a	98.138	4.505	26.814	6.011	36.276	1341	0.7370	0.309	1.585
a	98.064	4.501	27.210	5.962	35.583	1315	0.7370	0.320	1.585
a	98.103	4.501	26.648	5.986	35.637	1352	0.7166	0.326	1.585
a	98.088	4.499	26.879	6.004	36.847	1353	0.7247	0.321	1.610

a =

2	dry matter, total protein N
102	total fat, ash
202	lactose
302	retinol
402	α-tocopherol
502	vitamin B1, vitamin B2

RYE FLOUR (RM 381)

Sachet Code	Dry Weight	Total Protein Nitrogen	Total Fat	Ash	Avail. Carbo-hydrates	Vitamin B1	Vitamin B2
10	87.931	1.2419	1.2339	0.7847	80.291	0.2742	0.0835
110	88.024	1.2258	1.2962	0.8043	80.351	0.2724	0.0821
210	87.911	1.2308	1.0169	0.7940	81.124	0.2714	0.0835
310	88.000	1.2318	1.0943	0.7898	80.591	0.2725	0.0847
410	88.002	1.2295	1.1250	0.7932	81.424	0.2711	0.0834
510	87.868	1.2280	1.2382	0.7978	80.538	0.2701	0.0835
610	87.869	1.2416	1.1130	0.7989	80.378	0.2715	0.0823
710	87.557	1.2323	1.1341	0.8155	80.603	0.2710	0.0818
810	87.253	1.2378	1.1587	0.8149	81.453	0.2720	0.0821
910	87.386	1.2382	1.1009	0.7953	80.749	0.2730	0.0840
b	87.847	1.2401	0.9246	0.8017	82.302	0.2629	0.0781
b	87.777	1.2355	1.0009	0.7880	82.036	0.2586	0.0738
b	87.829	1.2332	0.9952	0.7823	80.814	0.2715	0.0781
b	87.862	1.2344	1.0567	0.7914	82.816	0.2743	0.0781
b	87.776	1.2332	1.0977	0.7868	81.714	-	-
b	87.845	1.2332	1.2048	0.7868	81.282	0.2671	0.0803
b	87.846	1.2275	1.1114	0.7925	81.751	0.2785	0.0803
b	87.760	1.2309	1.2457	0.8028	81.019	0.2743	0.0815
b	87.830	1.2275	1.1159	0.7880	81.159	0.2700	0.0815
b	87.816	1.2264	1.1023	0.8005	82.021	0.2743	0.0790

b =

60	dry matter
160	total protein nitrogen
260	total fat
360	ash
460	available carbohydrates
660	vitamin B1, vitamin B2

WHEAT FLOUR (RM 382)

Sachet Code	Dry Weight	Total Protein Nitrogen	Total Fat	Ash	Avail. Carbo-hydrates
10	87.473	2.0875	1.4805	0.7980	80.139
110	87.365	2.0901	1.3655	0.8001	81.772
210	87.373	2.0945	1.3368	0.8046	81.948
310	87.379	2.0955	1.3024	0.8068	82.869
410	87.315	2.0959	1.2484	0.7891	82.013
510	87.214	2.1052	1.3805	0.7957	81.822
610	87.325	2.1071	1.4200	0.7844	79.725
710	87.320	2.0969	1.5392	0.7902	81.906
810	87.285	2.1092	1.5237	0.7997	81.503
910	87.343	2.1032	1.6544	0.7808	83.693
c	87.35	2.1000	1.5121	0.7984	82.170
c	87.37	2.1115	1.4389	0.7984	81.770
c	87.34	2.1034	1.5384	0.8018	81.702
c	87.48	2.1046	1.3954	0.7892	82.285
c	87.53	2.0954	1.4892	0.7995	80.764
c	87.44	2.1103	1.4995	0.8029	81.930
c	87.38	2.1034	1.5727	0.8075	81.610
c	87.46	2.1000	1.3108	0.7984	80.901
c	87.46	2.0977	1.4835	0.7984	80.512
c	87.47	2.1069	1.4869	0.8064	82.239

c =

93	available carbohydrates
876	dry matter, total protein nitrogen
384	total fat, ash

HARICOTS VERTS BEANS (RM 383)

Sachet code	Dry Weight	Total Protein Nitrogen	Total Fat	Ash	Avail. Carbo-hydrates	Fructose
10	96.199	1.0541	2.2578	2.173	73.882	4.54
110	96.226	1.0403	2.2021	2.248	73.542	4.62
210	96.411	1.0538	2.2290	2.185	71.527	4.52
310	96.534	1.0421	2.4385	2.283	73.324	4.54
410	96.240	1.0547	2.4377	2.251	73.299	4.57
510	96.274	1.0491	2.4399	2.248	73.448	4.54
610	96.285	1.0479	2.3430	2.254	72.602	4.50
710	96.166	1.0503	2.3428	2.257	73.900	4.58
810	95.752	1.0517	2.3707	2.216	72.698	4.91
910	95.861	1.0505	2.2992	2.243	72.820	4.98
d	96.266	1.0490	2.4823	2.166	73.694	4.72
d	96.316	1.0469	2.4708	2.144	72.755	4.68
d	96.237	1.0500	2.4490	2.177	72.669	4.60
d	96.347	1.0448	2.4885	2.163	71.951	4.38
d	96.302	1.0500	2.3950	2.178	72.102	4.66
d	96.320	1.0511	2.4978	2.167	73.276	4.43
d	96.169	1.0500	2.3462	2.142	72.954	4.54
d	96.324	1.0448	2.3940	2.169	73.189	4.18
d	96.285	1.0469	2.0834	2.173	72.608	4.49
d	96.263	1.0428	2.0564	2.143	72.782	4.57

d =

19	total fat, ash
227	dry weight, total protein nitrogen
945	available carbohydrates, fructose

PORK MUSCLE (RM 384)

Sachet Code	Dry Weight	Total Protein Nitrogen	Total Fat	Ash	Vitamin B1	Vitamin B2
10	97.066	13.7237	11.0162	4.4763	2.57248	0.78854
110	97.516	13.7065	11.0279	4.4434	2.58809	0.75393
210	97.238	13.7446	10.9957	4.4468	2.48524	0.77058
310	97.521	13.7529	11.0366	4.4749	2.58796	0.75389
410	97.587	13.7529	11.0896	4.3992	2.59994	0.72243
510	97.535	13.7879	10.9725	4.4661	2.58758	0.75788
610	96.826	13.7721	11.0342	4.4492	2.59269	0.78429
710	97.058	13.7969	11.0006	4.4705	2.58649	0.78242
810	97.636	13.7173	10.7829	4.4901	-	0.76744
910	97.702	13.7397	10.9752	4.4738	2.55573	0.76283
e	97.591	13.8021	11.286	4.4298	-	0.7709
e	97.519	13.8154	11.204	4.4062	2.6924	0.7709
e	97.424	13.7703	11.128	4.4154	2.6516	0.7709
e	97.508	13.8236	11.203	4.4277	2.6788	0.7914
e	97.534	13.8092	11.095	4.4411	2.6788	0.7812
e	97.590	13.7549	11.034	4.4226	2.6652	0.7853
e	97.523	13.7672	11.066	4.4718	2.5564	0.7812
e	97.468	13.8113	11.050	4.4790	2.6516	0.7607
e	97.489	13.6729	11.004	4.5354	2.6924	0.7607
e	97.571	13.8072	11.031	4.5221	2.6788	-

e =

60	dry matter
160	total protein nitrogen
260	total fat
360	ash
460	vitamin B1, vitamin B2

Additional homogeneity study in WHOLE RYE MEAL (CRM 189)

Bottle code	α-Tocopherol	Vitamin B1	Vitamin B2
A	0.7603	1.028	0.170
B	0.7986	1.096	0.170
C	0.6718	0.936	0.168
D	0.8657	1.056	0.180
E	0.7269	1.002	0.182
F	0.7057	1.002	0.170
G	0.9152	1.032	0.186
H	1.1854	1.080	0.180
I	1.2100	1.104	0.184
J	1.0029	1.008	0.186
K	1.024	1.084	0.170
K	0.977	1.084	0.170
K	0.985	1.086	0.168
K	1.009	1.096	0.164
K	1.076	1.096	0.174
K	1.095	1.092	0.174
K	0.975	1.072	0.168
K	0.936	1.072	0.192
K	0.966	1.066	0.178
K	-	1.040	0.166

ANNEX II

Table 1a. Mean of duplicate determinations of TOTAL PROTEIN NITROGEN content (mass fraction, expressed as g/100 g dry weight) in RMs stored at different temperatures and monitored at regular intervals

Period months	Whole Milk Powder (RM 380)	Rye Flour (RM 381)	Wheat Flour (RM 382)	Haricots Verts Beans (RM 383)	Pork Muscle (RM 384)
Temperature -18 °C					
0	4.524	1.246	2.110	1.056	13.74
3	4.531	1.240	2.122	1.062	13.70
6	4.522	1.248	2.110	1.052	13.89
12	4.510	1.256	2.113	1.057	13.83
18	4.531	1.266	2.132	1.079	13.90
24	4.482	1.248	2.120	1.055	13.83
mean	4.5166	1.2505	2.1178	1.0599	13.815
std.dev.	0.0185	0.0089	0.0085	0.0097	0.0809
Temperature 20 °C					
0	4.524	1.246	2.110	1.056	13.74
3	4.525	1.247	2.103	1.052	13.74
6	4.527	1.251	2.120	1.070	13.86
12	4.523	1.266	2.120	1.062	13.77
18	4.525	1.270	2.141	1.074	13.85
24	4.500	1.241	2.125	1.059	13.78
mean	4.5206	1.2534	2.1198	1.0620	13.793
std.dev.	0.0104	0.0118	0.0130	0.0084	0.0534
Temperature 37 °C					
0	4.524	1.246	2.110	1.056	13.74
3					
6	4.526	1.275	2.124	1.055	13.77
12	4.522	1.254	2.128	1.071	13.82
18	4.532	1.268	2.131	1.084	13.87
24	4.491	1.262	2.119	1.050	13.80
mean	4.5188	1.2612	2.1225	1.0633	13.799
std.dev.	0.0161	0.0114	0.0082	0.0137	0.0491

Table 1b. Mean of duplicate determinations of TOTAL FAT content (mass fraction, expressed as g/100 g dry weight) in RMs stored at different temperatures and monitored at regular intervals

Period months	Whole Milk Powder (RM 380)	Rye Flour (RM 381)	Wheat Flour (RM 382)	Haricots Verts Beans (RM 383)	Pork Muscle (RM 384)
temperature -18 °C					
0	26.91	1.33	1.32	2.18	10.64
3	26.93	1.21	1.35	2.18	10.84
6	26.87	1.24	1.40	2.16	11.16
12	26.77	1.29	1.41	2.11	10.94
18	26.94	1.27	1.39	2.39	10.96
24	26.82	1.11	1.29	2.14	10.85
mean	26.874	1.243	1.358	2.193	10.896
std.dev.	0.067	0.077	0.049	0.101	0.168
temperature 20 °C					
0	26.91	1.33	1.32	2.18	10.64
3	26.92	1.14	1.31	2.10	10.91
6	26.87	1.14	1.43	2.21	10.76
12	26.79	1.16	1.33	2.19	10.83
18	26.88	1.09	1.24	2.25	10.89
24	26.71	1.05	1.32	2.14	10.70
mean	26.847	1.153	1.324	2.176	10.790
std.dev.	0.082	0.098	0.061	0.054	0.108
temperature 37 °C					
0	26.91	1.33	1.32	2.18	10.64
3					
6	26.89	1.22	1.22	2.29	10.90
12	26.75	1.20	1.30	2.20	10.78
18	26.85	1.23	1.22	2.13	10.81
24	26.69	1.07	1.10	2.29	10.70
mean	26.818	1.211	1.229	2.218	10.767
std.dev.	0.098	0.095	0.088	0.069	0.099

Table 1c. Mean of duplicate determinations of AVAILABLE CARBOHYDRATES content (mass fraction, expressed as g monosaccharides/100 g dry weight) in RMs stored at different temperatures and monitored at regular intervals

Period months	Rye Flour (RM 381)	Wheat Flour (RM 382)	Haricots Verts Beans (RM 383)
temperature -18 °C			
0	80.5	81.1	72.0
3	81.9	82.2	74.1
6	79.4	80.8	71.6
12	79.0	79.8	70.4
18	76.3	79.2	70.7
24	79.7	82.0	71.1
mean	79.47	80.85	71.67
std.dev.	1.86	1.17	1.34
temperature 20 °C			
0	80.5	81.1	72.0
3	82.9	82.3	73.2
6	79.4	81.0	71.6
12	78.7	80.0	70.8
18	77.4	78.8	70.4
24	80.5	81.8	70.8
mean	79.90	80.83	71.45
std.dev.	1.89	1.25	1.03
temperature 37 °C			
0	80.5	81.1	72.0
3			
6	77.9	80.1	70.4
12	79.1	79.2	70.0
18	76.8	76.3	69.9
24	79.7	81.0	70.7
mean	78.79	79.55	70.62
std.dev.	1.44	1.97	0.86

Table 1d. Mean of duplicate determinations of LACTOSE content (mass fraction, expressed as g anhydrous lactose/100 g dry weight) in RM 380 and FRUCTOSE content (mass fraction, expressed as g/100 g dry weight) in RM 383 stored at different temperatures and monitored at regular intervals

period (months)	temperature -18 °C	20 °C	37 °C
Lactose			
0	36.19	36.19	36.19
3	36.26	36.58	
6	35.73	35.35	34.81
12	36.28	35.72	35.60
18	36.51	36.26	35.79
24	35.89	35.12	34.42
mean	36.145	35.870	35.361
std.dev.	0.285	0.568	0.728
Fructose			
0	4.79	4.79	4.79
3	4.71	4.62	
6	4.66	4.75	4.69
12	4.59	4.54	4.43
18	4.92	4.63	4.70
24	4.68	4.66	4.50
mean	4.725	4.665	4.622
std.dev.	0.117	0.092	0.153

Table 1e. Mean of duplicate determinations of TOTAL DIETARY FIBRE content(mass fraction, expressed as g fibre as polymer/100 g dry weight) in RMs stored at different temperatures and monitored at regular intervals

Period months	Rye Flour (RM 381)	Wheat Flour (RM 382)	Haricots Verts Beans (RM 383)
temperature -18 °C			
0	7.6	2.8	12.2
3			
6	8.3	2.5	13.1
12	8.5	3.7	11.9
18	9.0	3.8	11.9
24	8.3	3.1	12.3
mean	8.34	3.16	12.26
std.dev.	0.53	0.59	0.49
temperature 20 °C			
0	7.6	2.8	12.2
3			
6	8.0	3.4	11.7
12	8.4	3.8	12.0
18	10.1	4.0	12.4
24	8.4	3.4	12.2
mean	8.51	3.46	12.09
std.dev.	0.94	0.47	0.28
temperature 37 °C			
0	7.6	2.8	12.2
6	8.4	3.1	13.0
3			
12	8.4	3.6	12.1
18	9.0	3.6	12.5
24	8.2	3.2	12.2
mean	8.30	3.24	12.40
std.dev.	0.52	0.38	0.37

Table 1f. Mean of duplicate determinations of ASH content (mass fraction, expressed as g/100 g dry weight) in RMs stored at different temperatures and monitored at regular intervals

Period months	Whole Milk Powder (RM 380)	Rye Flour (RM 381)	Wheat Flour (RM 382)	Haricots Verts Beans (RM 383)	Pork Muscle (RM 384)
temperature -18 °C					
0	6.141	0.812	0.752	2.076	4.443
3	6.003	0.827	0.801	2.310	4.353
6	6.062	0.811	0.746	2.318	4.483
12	6.018	0.820	0.749	2.408	4.480
18	6.045	0.807	0.845	2.465	4.485
24	6.001	0.804	0.692	2.245	4.485
mean	6.0449	0.8134	0.7641	2.3038	4.4548
std.dev.	0.0529	0.0081	0.0527	0.1362	0.0522
temperature 20 °C					
0	6.141	0.812	0.752	2.076	4.443
3	6.017	0.828	0.781	2.243	4.496
6	6.085	0.803	0.761	2.312	4.496
12	6.052	0.820	0.680	2.375	4.504
18	6.046	0.840	0.835	2.479	4.539
24	5.996	0.796	0.673	2.278	4.497
mean	6.0561	0.8165	0.7468	2.2940	4.4958
std.dev.	0.0517	0.0163	0.0619	0.1351	0.0306
temperature 37 °C					
0	6.141	0.812	0.752	2.076	4.443
3					
6	6.120	0.823	0.771	2.330	4.471
12	6.050	0.806	0.707	2.527	4.524
18	6.045	0.835	0.859	2.410	4.553
24	5.999	0.810	0.671	2.300	4.469
mean	6.0710	0.8173	0.7519	2.3287	4.4920
std.dev.	0.0585	0.0118	0.0714	0.1662	0.0451

Table 1g. Mean of duplicate determinations of RETINOL and α-TOCOPHEROL content (mass fraction, expressed as mg/100 g dry weight) in RM 380 stored at different temperatures and monitored at regular intervals

period	temperature	
months	-18 °C	4 °C
Retinol		
0	0.394	0.394
3	0.374	0.395
6	0.363	0.354
12	0.358	0.346
18	0.356	0.342
24	0.342	0.333
mean	0.3644	0.3606
std.dev.	0.0176	0.0268
α-Tocopherol		
0	0.779	0.779
3	0.834	0.823
6	0.820	0.802
12	0.813	0.778
18	0.824	0.781
24	0.793	0.787
mean	0.811	0.792
std.dev.	0.0206	0.0177

Table 1h. Mean of duplicate determinations of VITAMIN B1 content (mass fraction, expressed as mg/100 g dry weight) in RM 380 and RM 384 stored at different temperatures and monitored at regular intervals

Period months	Whole Milk Powder (RM 380)	Pork Muscle (RM 384)
Vitamin B1		
temperature -18 °C		
0	0.304	2.51
3	0.307	2.60
6	0.320	2.61
12	0.318	2.55
18	0.308	2.59
24	0.310	2.52
mean	0.3110	2.562
std.dev.	0.0065	0.0424
temperature 4 °C		
0	0.304	2.51
3	0.314	2.55
6	0.320	2.61
12	0.314	2.58
18	0.304	2.57
24	0.309	2.58
mean	0.3109	2.569
std.dev.		

Table 1i. Mean of duplicate determinations of VITAMIN B2 content (mass fraction, expressed as mg/100 g dry weight) in RM 380 and RM 384 stored at different temperatures and monitored at regular intervals

Period months	Whole Milk Powder (RM 380)	Pork Muscle (RM 384)
Vitamin B2		
temperature -18 °C		
0	1.59	0.757
3	1.65	0.758
6	1.70	0.829
12	1.65	0.783
18	1.70	0.737
24	1.55	0.698
mean	1.639	0.7604
std.dev.	0.0601	0.0441
temperature 4 °C		
0	1.59	0.757
3	1.57	0.756
6	1.67	0.808
12	1.58	0.757
18	1.65	0.731
24	1.54	0.679
mean	1.601	0.7480

Table 1j. Mean of duplicate determinations of α-TOCOPHEROL and VITAMIN C content (mass fraction, expressed as mg/100 g dry weight) in sachets of RM 383 stored at different temperatures for a period of 24 months

	temperature		
	-40°C	4°C	37°C
α-Tocopherol			
	0.267	0.268	0.298
	0.269	0.257	0.286
	0.285	0.249	0.298
mean	0.2736	0.2578	0.2940
std.dev.	0.0100	0.0096	0.0068
Vitamin C			
	16.2	16.2	0.6
	18.0	18.1	1.2
	17.4	17.7	0.2
mean	17.19	17.35	0.66
std.dev.	0.870	1.010	0.50