J.Assoc.Publ.Analysts, 1993, 29,1-32

Validation of European Community Methods for Microbiological and Chemical Analysis of Raw and Heat-treated Milk.

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A collaborative trial to assess methods of analysis for raw and heat-treated milks was carried out by twenty UK laboratories. The E.C. methods for colony count at 30°C and 21°C, coliform count, somatic cell count and the determination of phosphatase activity were assessed as were a LPS test for the determination of bacterial lipopolysaccharide and an alternative phosphatase test.

The trial has shown that the precision characteristics of the colony count at 30°C, coliform count and somatic cell count were acceptable but that the colony count at 21°C, LPS test and EC phosphatase test all gave poor results for both repeatability and reproducibility.

Many participants were unfamiliar with the LPS test and the E.C. phosphatase test; this may have contributed to the poor precision values determined for these two methods.

Introduction

The European Commission has recently published Decision 91/180/EEC (Anon. 1991a) laying down methods of analysis for raw and heat-treated milk in support of Directive 85/397/EEC (Anon. 1985) on health and animal health problems affecting intra-Community trade in heat-treated milk.

In Directive 85/397/EEC standards are prescribed for raw and heat-treated milk as follows:

A. **Raw milk standards**

	Step 1	Step 2
Plate Count 30°C (per ml)	$\leq 300,000$ [1]	$\leq 100,000$ [1]
Cell Count (per ml)	$\leq 500,000$ [2]	$\leq 400,000$ [2]
Freezing Point (°C)	≤ -0.520	≤ -0.520
Antibiotics (per ml)		
-penicillin	< 0.004 µg	< 0.004 µg
-other	Undetectable	Undetectable

[1] Average recorded over a period of two months, with at least two samples a month.

[2] Average recorded over a period of three months, with at least one sample a month.

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B. **Pasteurised milk standards**

	Step 1	Step2
Pathogens	None	None
Coliform (per ml)	< 5	< 1
Plate count 30°C (per ml)	≤ 50,000	≤ 30,000
Plate count 21°C (per ml) After incubation for 5 d at 6°C	≤ 250,000	≤ 100,000
Phosphatase	-	-
Peroxidase	+	+
Antibiotics (per ml)	undetectable	undetectable
Freezing point (°C)	≤ −0.520	≤ −0.520

C. **Sterilised and UHT milk standards**

	Step 1	Step 2
After incubation for 15 d at 30 °C:		
Plate count 30°C (per 0.1 ml)	≤ 10	≤ 10
Organoleptic check	normal	normal
Antibiotics (per ml)	undetectable	undetectable

For most of the methods prescribed in Decision 91/180/EEC, precision characteristics e.g. repeatability and reproducibility, are not available. Such precision parameters are required to demonstrate the normal variation which can be expected from a method: the Directive intends such precision values to be determined.

A collaborative trial has been carried out to assess the methods of analysis and ascertain their precision values for various methods used in the analysis of milk. The following methods were assessed:

- Enumeration of micro-organisms - colony count at 30°C (Method IV of 91/180/EEC)
- Enumeration of micro-organisms - colony count at 21°C (Method V of 91/180/EEC)
- Enumeration of coliforms - Colony count at 30°C (Method VI of 91/180/EEC)
- Enumeration of somatic cells (Method VII of 91/180/EEC)
- Determination of bacterial lipopolysaccharide (LPS) content (previously considered but not yet adopted by E.C.)
- Determination of phosphatase activity (non E.C. test but as prescribed in Milk (Special Designation) Regulations 1989) (Anon. 1989)

COLLABORATIVE TRIAL ORGANISATION

Methods

The methods for colony count at 30°C and 21°C, coliform count, somatic cell count and one of the tests for phosphatase activity, investigated during this collaborative trial, are fully described in Commission Decision 91/180/EEC (Anon. 1991a) and are outlined in Appendices I - V. The LPS test evaluated in this trial was developed by Sudi and Heeschen (1984) and Schulz (1991b) and is fully described in Appendix VII. The alternative phosphatase test is as prescribed in the Milk (Special Designation) Regulations 1989 and is outlined in Appendix VI.

Participants

Twenty UK laboratories participated in the trial comprising three Public Health laboratories, eleven Public Analyst laboratories, two Government laboratories and four food industry laboratories.

It will be noted that participants were permitted to select which of the 7 methods they wished to assess and thus the numbers of participants for each method is different.

Samples

All samples were prepared by the Milk Marketing Board (MMB) Microbiology Laboratories, Thames Ditton, Surrey as described below and transported by either taxi, to arrive within 6 h of preparation or by overnight carrier (for frozen samples e.g. for LPS and phosphatase tests).

Prior to the trial, a pilot study was undertaken by the MMB to ensure that spiked samples prepared as described for the somatic cell count, coliform count, LPS test and phosphatase test would give acceptable ranges of test results, and to determine the effect of freezing samples for the LPS and phosphatase tests.

Colony counts at 30°C and 21°C.

Five batches of pasteurised milk from the holding tanks and filler heads at two local dairies were collected on the day of the trial and transported at 4°C to the MMB. Each batch of milk was portioned aseptically to produce forty replicates of 20 ml aliquots and 34 replicates of 200 ml aliquots.

Twenty participants received duplicate 20ml samples from each batch of milk, giving a total of 10 samples, numbered randomly, to be used for the colony count at 30°C. Seventeen participants received duplicate 200ml samples from each batch of milk, numbered randomly, to be used for the colony count at 21°C.

Coliform count

Pasteurised milk from the holding tank of a local dairy and raw milk, known to contain coliforms, from a local producer were collected and transported to MMB. The pasteurised milk was portioned aseptically into five sub-samples and each sub-sample spiked with a varying amount of

the raw milk. Forty replicates were prepared from each sub-sample, and twenty participants sent duplicates of each, randomly numbered, to give a total of 10 samples for examination. Samples were dispatched to participants within 6 h of sample preparation and kept at a holding temperature of ≤ 5°C.

Determination of somatic cells

Raw milk was collected from two local producers known to have either high or low cell counts. Five batches of raw milk were prepared by mixing the high and low cell count milks in varying proportions. Two replicates from each batch were preserved by the addition of formalin. These samples were transported by courier the day after preparation to the Genus Veterinary Laboratory for testing using an automated coulter counter method. Additionally, 4 replicates (2 preserved, 2 unpreserved) of each batch were analysed by the MMB Central Testing Laboratory for enumeration of somatic cells using a fluoro-opto-electronic cell count method. The remaining samples were preserved using *ortho*boric acid. Nine participants received duplicate samples of each batch, randomly numbered, giving a total of ten samples for analysis. Samples were transported to participating laboratories the day after preparation.

Determination of bacterial lipopolysaccharide (LPS)

Five batches of UHT milk were prepared from raw milk of known LPS content. Each batch was portioned aseptically into fourteen replicates and deep frozen immediately. The seven participants received duplicates of each batch of UHT milk randomly numbered, and thus analysed ten samples. Participants were instructed to store samples deep frozen for one week prior to analysis in order to spread workload.

Determination of phosphatase activity (both methods)

Raw milk collected from a local dairy was laboratory-pasteurised at the MMB. This was portioned into six batches and raw milk added to each pasteurised sample to give varying levels of phenol (0, 4, 6, 8, 10 and 12 µg/ml). Thirty-eight replicates were prepared from each batch and deep-frozen immediately. Nineteen participants received duplicate samples from each batch of milk, randomly numbered, to give a total of 12 samples for analysis. Participants were instructed to keep samples deep frozen for one week prior to analysis.

Results

Results obtained for all methods are given in Tables I to XXX.

Statistical Analysis of the Results

Results were first converted to a $\log_{10}$ basis, examined for evidence of individual systematic error using Cochran's and Grubb's tests at $P<0.5$ and then statistically analysed by procedures described by the International Dairy Federation (1988) which is based on the

IUPAC/ISO/AOAC Protocol for the Design, Conduct and Interpretation of Collaborative Studies (Horwitz, 1988)

Calculations for repeatability (r) and reproducibility (R), as defined by the procedures given by the International Dairy Federation, were carried out on those results remaining after removal of outliers. The results for each method investigated and accompanying statistical data are given in Tables I - XXX and summarised in Table XXXI.

Discussion

Few documented data on the performance of many microbiological methods mean that an assessment of the precision characteristics for microbiological methods obtained by collaborative trials is to some extent subjective. Notwithstanding this, the following conclusions have been drawn regarding the performance of the methods investigated.

Colony count at 30°C - EC Method IV

Mean colony counts obtained for this method ranged from $\log_{10}$ 2.48 - 4.43. Values for both repeatability and reproducibility of this test were good although the relative standard deviation of reproducibility (RSDR%) (Table XXXI) indicates a general improvement in reproducibility as numbers of cfu/ml increased.

Colony count at 21°C - EC Method V

Mean colony counts obtained for this method were in the range $\log_{10}$ 3.57 - 6.26. Repeatability values for this test were poor for samples contaminated with $<\log_{10}$ 4.5 cfu/ml. Reproducibility of this test was also poor particularly at the lowest level of contamination ($\log_{10}$ 3.57 R=2.83). The RSDR% indicated some increase in reproducibility as cfu/ml increased. Many factors influence the performance of this test. The requirement for pre-incubation of the unopened sample of milk prior to removal of an aliquot for enumeration of psychrotrophic bacteria results in a deterioration of reproducibility values as compared to reproducibility values obtained for the colony count at 30°C where no pre-incubation is required. It has previously been demonstrated that the final count obtained is dependent upon the initial types and numbers of bacteria present in the sample. Samples taken in close proximity from a cartoned milk processing line show considerable sample to sample variation (Wilson & Gilmore, 1990). Accurate control of pre-incubation temperature (6°C $\pm$ 0.2°C) is also critical in this test in order to improve precision but equipment capable of maintaining such strict temperature control is rarely available in routine microbiology laboratories. It is therefore unlikely that the precision of this test can be improved. These results concur with other published data (Hands, 1988).

Coliform count - EC Method VI

Mean contamination levels achieved for this examination were in the range $\log_{10}$ 0.51-1.44. Except for the very lowest level of contamination (where homogeneity in sample preparation is more difficult to achieve),

repeatability of this method was acceptable and improved with increasing numbers of coliforms present in the sample. Reproducibility was generally poorer than expected particularly at the highest level of contamination ($\log_{10}$1.44 R=1.35). It is not clear from the data however, why reproducibility should decrease as numbers of coliforms increase which is in contrast to trends observed in other microbiological tests investigated in this study.

Some problems were encountered by participants with the confirmatory techniques prescribed in the method. The method states that confirmation is required to be carried out only on atypical colonies present on the agar medium. However, in practice, several participants in the trial carried out confirmation on both typical, e.g. in size and colour, and atypical colonies and found some typical colonies which could not be confirmed as coliforms and some atypical colonies which were confirmed as coliforms. In contrast, some laboratories did not carry out any confirmation procedures. The inconsistent approach to confirmation by participants in this study probably resulted in some counting errors of these organisms thus affecting the final results reported. To avoid possible counting errors when carrying out this method and in support of good laboratory practice, a representative number of typical and atypical colonies should always be picked from the agar medium, confirmed and final figures adjusted accordingly. If no confirmation techniques are carried out, results should only be considered as presumptive.

Somatic cell count - EC Method VII

Mean cell counts in the range $\log_{10}$ 5.13 - 6.03 were obtained in this examination. Notwithstanding the fact that most laboratories did not routinely carry out this technique, repeatability and reproducibility were acceptable with a significant increase in reproducibility when cell numbers reached $\log_{10}$ 6.0/ml. In addition to the microscopic method, two automated counting methods, the coulter counter and fluoro-opto electronic methods, were used (Table XXXIII) by expert laboratories on samples identical to those received by participants. The automated methods demonstrated good repeatability and there was also close correlation between cell counts obtained by automated methods and the microscopic method. The use of preservative in the samples did not appear to affect results in any way. Precision data are available for the fluoro-opto-electronic method and are quoted at S_r = 20,000 cells/ml and S_R = 40,000 cells/ml. No statistical analysis of the data for the automated method used in this study could be carried out due to insufficient data.

LPS Test

Although not prescribed in Directive 91/180/EEC, the LPS test for the determination of bacterial lipopolysaccharide in UHT and sterilised milk, as an indicator of raw milk quality prior to processing, is still under consideration by the Commission for possible inclusion in future Directives. Only seven laboratories carried out this test and most were inexperienced with its use. The gelation method for the detection of

bacterial LPS investigated in this trial was in the form of a commercially available microtitre assay (Anon., 1991b). Precision values obtained for this method were possibly affected by operator error and values for both repeatability and reproducibility were poorer than those obtained in a German inter-laboratory study (Feier & Goetsch, 1991) of this method when applied to pasteurised liquid egg products. In the German study precision was quoted as r = $\log_{10}$ 0.25 for samples containing $10^{2\text{-}4.25}$ EU's/ml and R = $\log_{10}$ 0.75 for egg containing $10^{2\text{-}3}$ EU's/ml; R=1 for egg containing $10^{3\text{-}4.25}$ EU's /ml.

Statistical analysis of results was only possible for three of the five batches of milk examined in this study.

Determination of phosphatase activity - EC Method II and alternative phosphatase test

Two methods for the determination of phosphatase activity (to detect pasteurisation of milk) were investigated in this trial. The first method is as prescribed in Decision 91/180/EEC and the second as prescribed in the Milk (Special Designation) Regulations 1989 (Anon., 1989). Statistical evaluation of data was only possible for the EC phosphatase test. As for the LPS test, the relative inexperience of analysts with this spectrophotometric enzyme assay probably affected results and most analysts reported some difficulties with the test. With the exception of one sample, mean levels of μg phenol/ml were consistently underestimated as compared to the levels of phenol spiked into the samples. Two laboratories failed to detect enzyme activity in any of the samples tested. Preliminary precision characteristics have been prescribed in Decision 91/180/EEC for this method, and are quoted as r = 2μg phenol/ml and R = 3μg phenol/ml. Values obtained in this trial were generally worse than those prescribed and there was no apparent relationship between precision of the method and levels of phenol present in the sample.

Conclusions

Of the six methods examined in this collaborative trial, data suggest that the methods for colony count at 30°C, coliform count and somatic cell count demonstrate acceptable precision. Precision for the colony count at 21°C (the pre-incubation test) is poor and is unlikely to be able to be improved so long as the pre-incubation step is required.

The precision values observed for the LPS test and phosphatase tests should be interpreted with caution as fewer laboratories participated in these tests and there was some degree of unfamiliarity with the techniques.

EC Methods for Raw and Heat Treated Milk: Collaborative Trial Results

TABLE I

AEROBIC PLATE COUNT 30°C

SAMPLE 1

LABORATORY	Log_{10} CFU/ml a	b
1	4.18	4.15
2	2.90	2.70
3	3.60	3.53
4	4.43	4.45
5	4.34	4.34
6	4.43	4.43
7	4.52	4.61
8	4.43	4.43
9	4.40	4.38
10	4.51	4.57
11	4.63	4.71
12	3.11	3.66[b]
13	4.45	4.53
14	4.61	4.65
15	4.32	<1.00[a]
16	2.90	2.66
17	4.40	4.40
18	4.42	4.38
19	4.11	4.26
20	1.81	1.86
MEAN	4.06	
r	0.18	
SD_r	0.07	
RSD_r%	1.63	
R	2.22	
SD_R	0.79	
RSD_R%	19.51	

TABLE II

AEROBIC PLATE COUNT 30°

SAMPLE 2

LABORATORY	Log_{10} CFU/ml a	b
1	2.94	3.93[b]
2	2.85	3.00
3	3.08	3.04
4	2.83	2.98
5	3.00	3.08
6	3.04	3.04
7	3.00	3.04
8	3.08	3.04
9	3.04	3.04
10	3.23	3.04
11	3.08	3.20
12	2.66	3.00
13	3.04	3.04
14	3.04	3.11
15	<1.00	<1.00[d]
16	2.84	3.04
17	3.01	3.00
18	3.04	2.97
19	3.04	2.76[e]
20	3.04	3.00
MEAN	3.01	
r	0.28	
SD_r	0.10	
RSD_r%	3.28	
R	0.30	
SD_R	0.11	
RSD_R%	3.57	

For key see Table XXXI

TABLE III

AEROBIC PLATE COUNT 30°C

SAMPLE 3

LABORATORY	Log_{10} CFU/ml a	Log_{10} CFU/ml b
1	3.18	3.89[b]
2	3.11	3.28
3	3.00	3.20
4	2.76	2.93[c]
5	3.00	3.04
6	3.08	3.11
7	3.11	3.11
8	3.08	3.08
9	3.04	3.08
10	3.11	3.11
11	3.18	3.20
12	2.72	2.70[c]
13	3.11	3.08
14	3.11	3.15
15	2.82	<1.00[a]
16	3.11	2.86[c]
17	3.08	3.08
18	3.00	3.08
19	4.04	2.86[b]
20	3.15	3.08
MEAN	3.10	
r	0.16	
SD_r	0.06	
RSD_r%	1.80	
R	0.18	
SD_R	0.06	
RSD_R%	2.03	

TABLE IV

AEROBIC PLATE COUNT 30°C

SAMPLE 4

LABORATORY	Log_{10} CFU/ml a	Log_{10} CFU/ml b
1	2.40	3.48[b]
2	2.70	2.78
3	2.67	2.43
4	2.73	2.71
5	2.52	2.42
6	2.59	2.59
7	2.56	2.57
8	2.70	2.64
9	2.51	2.66
10	2.42	2.42
11	2.63	2.59
12	2.49	2.52
13	2.53	2.66
14	2.61	2.68
15	3.36	3.20[c]
16	2.38	2.20
17	2.56	2.45
18	2.56	2.59
19	2.54	2.45[e]
20	2.49	2.53
MEAN	2.56	
r	0.20	
SD_r	0.07	
RSD_r%	2.79	
R	0.33	
SD_R	0.12	
RSD_R%	4.65	

For key see Table XXXI

TABLE V

AEROBIC PLATE COUNT 30°C

SAMPLE 5

LABORATORY	Log$_{10}$ CFU/ml a		Log$_{10}$ CFU/ml b
1	3.51		3.36[c]
2	2.60		2.70
3	2.58		2.62
4	2.48		2.51
5	2.40		2.38
6	2.51		3.53[b]
7	2.63		2.59
8	2.67		2.57
9	2.42		2.42
10	2.34		2.20
11	2.59		2.60
12	2.34		2.28
13	2.52		2.51
14	2.57		2.56
15	2.45		<1.00[a]
16	2.43		2.34
17	2.43		2.40
18	2.36		2.34
19	2.36		2.48
20	2.52		2.20[b]
MEAN		2.48	
r		0.13	
SD_r		0.05	
RSD_r%		1.91	
R		0.35	
SD_R		0.13	
RSD_R%		5.06	

For key see Table XXXI

TABLE VI

PRE-INCUBATED COUNT 21°C

SAMPLE 1

LABORATORY	Log$_{10}$ CFU/ml a		Log$_{10}$ CFU/ml b
1	5.90		5.96
2	6.48		6.30
3	6.38		6.15
4	5.28		6.20[b]
5	7.04		7.20
7	6.08		5.78
9	6.83		6.98
10	6.34		6.46
11	4.42		4.56
13	6.30		6.11
15	6.52		6.51
17	7.31		7.05
18	5.86		5.62
19	f		f
20	f		f
MEAN		6.26	
r		0.37	
SD_r		0.13	
RSD_r%		2.12	
R		2.04	
SD_R		0.73	
RSD_R%		11.65	

Laboratories 6, 8, 12, 14 & 16 did not carry out the p-inc test

TABLE VII

PRE-INCUBATED COUNT 21°C

SAMPLE 2

LABORATORY	Log_{10} CFU/ml a	Log_{10} CFU/ml b
1	<5.00[d]	<5.00[d]
2	5.30	4.88
3	4.84	5,00
4	4.69	4.97
5	5.69	5.91
7	4.45	4.32
9	5.18	5.26
10	4.49	4.38
11	2.90	2.80
12	<1.00	<1.00[d]
13	4.26	4.90
15	4.89	4.73
16	<1.00	<1.00[d]
17	5.49	5.61
18	4.18	4.51
19	f	f
20	f	f
MEAN	4.73	
r	0.56	
SD_r	0.2	
RSD_r%	4.19	
R	2.13	
SD_R	0.76	
RSD_R%	16.07	

TABLE VIII

PRE-INCUBATED COUNT 21°C

SAMPLE 3

LABORATORY	Log_{10} CFU/ml a	Log_{10} CFU/ml b
1	<5.00	5.30[a]
2	5.75	5.59
3	5.74	5.79
4	5.53	5.57
5	7.00	6.00[b]
7	4.71	4.63
9	6.04	5.89
10	5.26	5.36
11	2.72	3.04[c]
12	<1.00	<1.00[d]
13	5.36	5.40
15	5.30	5.82[b]
16	<1.00	<1.00[d]
17	6.26	6.23
18	4.86	4.76
19	f	f
20	f	f
MEAN	5.48	
r	0.19	
SD_r	0.07	
RSD_r%	1.22	
R	1.44	
SD_R	0.51	
RSD_R%	9.35	

For key see Table XXXI

TABLE IX

PRE-INCUBATED COUNT 21°C

SAMPLE 4

LABORATORY	Log_{10} CFU/ml a	b
1	<5.00[d]	<5.00[d]
2	<5.00[d]	<4.00[d]
3	4.18	3.57
4	3.83	3.81
5	5.15	5.51
7	2.80	3.61
9	4.40	4.69
10	4.42	4.28
11	<2.00	<2.00[d]
12	<1.00	<1.00[d]
13	4.04	<4.00[a]
15	3.18	4.04
16	<1.00	<1.00[d]
17	5.63	4.72
18	2.69	3.15
19	f	f
20	f	f
MEAN	4.24	
r	1.18	
SD_r	0.42	
RSD_r%	9.97	
R	2.26	
SD_R	0.81	
RSD_R%	19.05	

TABLE X

PRE-INCUBATED COUNT 21°C

SAMPLE 5

LABORATORY	Log_{10} CFU/ml a	b
1	<5.00[d]	<5.00[d]
2	<3.00[d]	<3.00[d]
3	3.93	3.42
4	3.92	3.99
5	4.32	5.23
7	2.45	3.15
9	4.48	4.00
10	2.18	2.11
11	<2.00	<2.00[d]
12	<1.00	<1.00[d]
13	<4.00	<4.00[d]
15	3.59	3.76
16	<1.00	<1.00[d]
17	4.20	5.08
18	2.11	2.38
19	f	f
20	f	f
MEAN	3.57	
r	1.08	
SD_r	0.39	
RSD_r%	10.80	
R	2.83	
SD_R	1.01	
RSD_R%	28.33	

For key see Table XXXI

TABLE XI

COLIFORM COUNT

SAMPLE 2

LABORATORY	Log_{10} CFU/ml a	b
1	0.30	0.48
2	0.70	0.48
3	<1.00	<1.00[d]
4	0.70	0.95[e]
5	<1.00	<1.00[d]
6	0.78	0.48
7	0.30	0.48
8	0.30	0.60
9	0.30	0.00
10	0.78	0.78
11	0.48	0.00[e]
12	0.00	0.00
13	0.60	0.30
14	0.70	0.70
15	1.57	0.48
16	<1.00	<1.00[d]
17	1.11	0.60[e]
18	0.30	0.48
19	0.00	0.00
20	0.85	0.70
MEAN	0.51	
r	0.71	
SD_r	0.26	
RSD_r%	50.91	
R	0.99	
SD_R	0.35	
RSD_R%	69.43	

Note : No calculations performed on sample 1 as insufficient data

For key see Table XXXI

TABLE XII

COLIFORM COUNT

SAMPLE 3

LABORATORY	Log_{10} CFU/ml a	b
1	0.85	0.95
2	1.08	1.18
3	0.90	1.04
4	1.36	0.70[e]
5	<1.00	<1.00[d]
6	1.11	1.00
7	1.04	0.95
8	0.95	0.90
9	1.26	1.30
10	1.28	1.40
11	1.00	0.95[e]
12	0.30	0.95
13	1.04	1.00
14	1.30	1.15
15	1.00	1.11
16	<1.00	<1.00[d]
17	1.23	1.36[e]
18	1.11	0.78
19	0.85	1.15
20	1.36	1.48
MEAN	1.07	
r	0.52	
SD_r	0.18	
RSD_r%	17.27	
R	0.64	
SD_R	0.23	
RSD_R%	21.54	

TABLE XIII

COLIFORM COUNT

SAMPLE 4

LABORATORY	a (Log_{10} CFU/ml)	b (Log_{10} CFU/ml)
1	0.78	<0.48[a]
2	1.38	1.34
3	0.48	1.36[b]
4	<1.00	1.76[a]
5	0.00	0.00[c]
6	1.30	1.26
7	1.26	1.36
8	1.18	0.90
9	1.58	1.40
10	1.70	1.53
11	1.26	0.60[b]
12	0.30	0.30[c]
13	0.28	1.20
14	1.53	1.40
15	1.34	1.42
16	<1.00	<1.00[d]
17	1.49	1.60[e]
18	1.15	1.18
19	0.90	1.04
20	1.65	1.49
MEAN	1.34	
r	0.27	
SD_r	0.10	
RSD_r%	7.13	
R	0.59	
SD_R	0.21	
RSD_R%	15.81	

TABLE XIV

COLIFORM COUNT

SAMPLE 5

LABORATORY	a (Log_{10} CFU/ml)	b (Log_{10} CFU/ml)
1	1.23	1.43
2	1.62	1.62
3	1.11	1.43
4	2.01	1.96[e]
5	0.00	0.48
6	1.58	1.65
7	1.54	1.59
8	1.64	1.43
9	1.43	1.51
10	1.81	1.79
11	1.53	1.52[e]
12	0.60	0.70
13	1.58	1.67
14	1.60	1.59
15	1.66	1.64
16	0.30	0.48[e]
17	1.94	1.88[e]
18	1.66	1.70
19	1.45	1.52
20	1.90	1.85
MEAN	1.44	
r	0.31	
SD_r	0.11	
RSD_r%	7.69	
R	1.35	
SD_R	0.48	
RSD_R%	33.50	

For key see Table XXXI

TABLE XV

SOMATIC CELL COUNT

SAMPLE 1

LABORATORY	Log_{10} Cells/ml a	b
9	4.68	4.64
11	4.86	4.86
12	5.55	5.55
13	5.30	5.00
14	5.08	5.04
19	6.17	5.54
20	4.83	4.79
MEAN	5.13	
r	0.53	
SD_r	0.19	
RSD_r%	3.67	
R	1.26	
SD_R	0.45	
RSD_R%	8.74	

TABLE XVI

SOMATIC CELL COUNT

SAMPLE 2

LABORATORY	Log_{10} Cells/ml a	b
9	4.88	5.05
11	5.40	5.61
12	5.0 7	5.59
13	5.60	5.48
14	5.60	5.51
19	5.57	6.07
20	5.35	5.41
MEAN	5.49	
r	0.45	
SD_r	0.16	
RSD_r%	2.94	
R	0.81	
SD_R	0.29	
RSD_R%	5.29	

For key see Table XXXI

TABLE XVII

SOMATIC CELL COUNT

SAMPLE 3

LABORATORY	Log_{10} Cells/ml a	b
9	5.15	4.74
11	5.72	5.74
12	5.85	5.9
13	5.90	5.78
14	5.86	5.91
19	6.23	5.98
20	5.66	5.70
MEAN	5.72	
r	0.37	
SD_r	0.13	
RSD_r%	2.33	
R	1.07	
SD_R	0.38	
RSD_R%	6.68	

TABLE XVIII

SOMATIC CELL COUNT

SAMPLE 4

LABORATORY	Log_{10} Cells/ml a	b
9	5.19	4.95b
11	5.96	5.95
12	6.14	6.02
13	6.00	5.95
14	6.00	6.01
19	6.06	6.14
20	5.85	5.97
MEAN	6.00	
r	0.15	
SD_r	0.06	
RSD_r%	0.92	
R	0.23	
SD_R	0.08	
RSD_R%	1.39	

For key see Table XXXI

TABLE XIX

SOMATIC CELL COUNT

SAMPLE 5

LABORATORY	Log_{10} Cells/ml a	b
9	5.75	5.29[b]
11	5.98	6.08
12	6.07	6.07
13	6.00	6.00
14	6.22	5.99
19	6.18	5.98
20	5.90	5.93
MEAN	6.03	
r	0.26	
SD_r	0.09	
RSD_r%	1.54	
R	0.27	
SD_R	0.09	
RSD_R%	1.58	

TABLE XX

LPS TEST

SAMPLE 1

LABORATORY	Log_{10} EU's/ml a	b
9	2.00	2.00
11	2.50	1.50
12	2.00	1.25
13	2.00	1.75
18	1.50	1.50
19	2.50	2.00
20	2.00	1.75
MEAN	1.88	
r	1.04	
SD_r	0.37	
RSD_r%	19.84	
R	1.04	
SD_R	0.36	
RSD_R%	19.35	

Laboratories 1-8,10, 14-17 did not carry out LPS test.

For key see Table XXXI

TABLE XXI

LPS TEST

SAMPLE 2

LABORATORY	Log$_{10}$ EU's/ml a	b
9	3.00	3.00
11	3.00	3.00
12	2.50	2.25
13	3.00	3.00
18	2.50	2.75
19	2.00	2.50
20	2.75	2.75
MEAN	2.71	
r	0.46	
SD_r	0.16	
RSD_r%	6.03	
R	0.93	
SD_R	0.33	
RSD_R%	12.27	

TABLE XXII

LPS TEST

SAMPLE 3

LABORATORY	Log$_{10}$ EU's/ml a	b
9	3.00	3.50
11	4.00	3.50
12	3.25	3.50
13	3.00	3.00
18	3.25	3.25
19	3.00	4.00
20	4.25	3.75
MEAN	3.45	
r	1.00	
SD_r	0.36	
RSD_r%	10.44	
R	1.18	
SD_R	0.42	
RSD_R%	12.26	

For key see Table XXXI

TABLE XXIII

LPS TEST

SAMPLE 4

LABORATORY	Log_{10} EU's/ml a	b
9	4.00	4.00
12	3.50	1.75[b]
13	3.50	3.75[b]
18	3.50	3.50
19	3.50	3.50
20	4.25	4.25
MEAN		
r		
SD_r		
RSD_r%	INSUFFICIENT	
R	DATA	
SD_R		
RSD_R%		

TABLE XXIV

LPS TEST

SAMPLE 5

LABORATORY	Log_{10} EU's/ml a	b
9	4.00	4.00
11	>4.50	>4.50[d]
12	3.75	3.50
13	>4.50	>4.50[d]
18	4.50	4.50
19	4.00	4.00
20	4.00	2.75[b]
MEAN		
r		
SD_r		
RSD_r%	INSUFFICIENT	
R	DATA	
SD_R		
RSD_R%		

For key see Table XXXI

TABLE XXV

EC PHOSPHATASE TEST

SAMPLE 1

LABORATORY	μg phenol/ml a	b
5	8.16	4.08
7	0.00	0.60[a]
8	5.76	8.40
9	0.00	0.00[d]
10	<1.00	<1.00[d]
11	4.56	4.56
12	0.00	0.00[d]
14	0.00	2.22[a]
16	0.00	0.00[d]
17	2.15	2.85[e]
18	0.00	2.80[a]
20	1.44	1.44

MEAN	4.02
r	3.99
SD_r	1.42
RSD_r%	35.45
R	6.94
SD_R	2.48
RSD_R%	61.72

TABLE XXVI

EC PHOSPHATASE TEST

SAMPLE 2

LABORATORY	μg phenol/ml a	b
5	7.20	12.72
7	2.98	0.50
8	16.08	7.30
9	0.00	0.20[d]
10	<1.20	<1.20[d]
11	2.28	2.64
12	0.00	0.00[d]
14	1.97	2.06
15	2.16	3.36
16	0.00	0.00[d]
17	2.15	2.95[e]
18	0.04	1.19
20	3.36	3.48

MEAN	4.13
r	7.15
SDr	2.55
RSDr%	61.73
R	12.02
SDR	4.29
RSDR%	103.82

Laboratories 1-4,6, 12-13 & 19 did not carry out EC phosphatase test

For key see Table XXXI

TABLE XXVII

EC PHOSPHATASE TEST

SAMPLE 3

LABORATORY	μg phenol/ml a	b
5	151.36	8.40[b]
7	2.88	2.88
8	8.52	5.64
9	2.90	2.64[d]
10	<1.20	<1.20[d]
11	5.04	4.56
12	0.00	0.00[d]
14	3.07	2.76
15	4.56	6.00
16	0.00	0.00[d]
17	5.05	4.55[e]
18	0.00	4.17[a]
20	4.32	4.32

MEAN	4.36
r	2.32
SD_r	0.83
RSD_r%	19.05
R	4.47
SD_R	1.60
RSD_R%	36.66

TABLE XXVIII

EC PHOSPHATASE TEST

SAMPLE 4

LABORATORY	μg phenol/ml a	b
5	151.36	8.40[b]
7	2.88	2.88
8	8.52	5.64
9	2.90	2.64[d]
10	<1.20	<1.20[d]
11	5.04	4.56
12	0.00	0.00[d]
14	3.07	2.76
15	4.56	6.00
16	0.00	0.00[d]
17	5.05	4.55[e]
18	0.00	4.17[a]
20	4.32	4.32

MEAN	4.36
r	2.32
SD_r	0.83
RSD_r%	19.05
R	4.47
SD_R	1.60
RSD_R%	36.66

For key see Table XXXI

TABLE XXIX

EC PHOSPHATASE TEST

SAMPLE 5

LABORATORY	μg phenol/ml a	b
5	13.68	11.52
7	3.29	5.66
8	7.32	10.68
9	0.00	0.96[a]
10	<1.20	<1.20[d]
11	2.64	2.64
14	4.75	5.04[a]
15	5.76	5.76
16	0.00	0.00[d]
17	5.75	5.77[e]
18	3.02	6.31
20	7.92	7.92

MEAN	6.41
r	3.76
SD_r	1.34
RSD_r%	20.95
R	8.78
SD_R	3.14
RSD_R%	48.90

TABLE XXX

EC PHOSPHATASE TEST

SAMPLE 6

LABORATORY	μg phenol/ml a	b
5	22.32	30.24[c]
7	6.07	6.26
8	9.24	9.00
9	6.24	6.96
11	3.12	1.20
14	6.02	7.26
15	7.68	9.84
17	8.35	8.65
18	5.47	3.86
20	10.08	9.36

MEAN	6.93
r	2.44
SD_r	0.87
RSD_r%	12.60
R	7.01
SD_R	2.50
RSD_R%	36.13

For key see Table XXXI

TABLE XXXI

E.C Methods of Analysis for Raw and Heat Treated Milk

Key to Tables I TO XXX	
a	single result reported, not used in calculation of mean, repeatability or reproducibility.
b	outlying result by Cochran's Test at P<0.01 level, not used in calculation of mean, repeatability or reproducibility.
c	outlying result by Grubbs' Test at P<0.01 level, not used in calculation of mean, repeatability or reproducibility.
d	result not included in calculations.
e	laboratory deviated from protocol but insufficient to remove data.
f	laboratory data not used
r	repeatability (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.
SD_r	the standard deviation of the repeatability.
$RSD_r\%$	the relative standard deviation of the repeatability $SD_r \times 100/x$.
R	reproducibility (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.
SD_R	the standard deviation of the reproducibility.
$RSD_R\%$	the relative standard deviation of the reproducibility $S_R \times 100/x$.

TABLE XXXII

Summary of Precision of EC Methods of Analysis for Raw and Heat-treated Milk

Method	No. of data	Mean	r	SD_r	RSD_r%	R	SD_R	RSD_R%
Colony count at 30°C (log_{10} cfu/ml)	16	2.48	0.13	0.05	1.91	0.35	0.13	5.06
	17	2.56	0.20	0.07	2.79	0.33	0.12	4.65
	17	3.01	0.28	0.10	3.28	0.3	0.11	3.57
	14	3.10	0.16	0.06	1.80	0.18	0.06	2.03
	14	4.43	0.12	0.04	0.95	0.45	0.15	3.35
Colony count at 21°C (log_{10} cfu/ml)	9	3.57	1.08	0.39	10.8	2.83	1.01	28.33
	9	4.24	1.18	0.42	9.97	2.26	0.81	19.05
	12	4.73	0.56	0.20	4.19	2.13	0.76	16.07
	9	5.48	0.19	0.07	1.22	1.44	0.51	9.35
	12	6.26	0.37	0.13	2.12	2.04	0.73	11.65
Coliform count * (log_{10} cfu/ml)	14	0.51	0.72	0.26	50.91	0.99	0.35	69.43
	15	1.07	0.52	0.18	17.27	0.64	0.23	21.54
	12	1.34	0.27	0.10	7.13	0.59	0.21	15.81
	20	1.44	0.31	0.11	7.69	1.35	0.48	33.50
Somatic cell count (log_{10} cells/ml)	7	5.13	0.53	0.19	3.67	1.26	0.45	8.74
	7	5.49	0.45	0.16	2.94	0.81	0.29	5.29
	7	5.72	0.37	0.13	2.33	1.07	0.38	6.68
	6	6.00	0.15	0.06	0.92	0.23	0.08	1.39
	6	6.03	0.26	0.09	1.54	0.27	0.09	1.58
LPS# (Log_{10} EU's/ml)	7	1.88	1.04	0.37	19.84	1.04	0.36	19.35
	7	2.71	0.46	0.16	6.03	0.93	0.33	12.27
	7	3.45	1.00	0.36	10.44	1.18	0.42	12.26

TABLE XXXII (continued)

Method	No. of data	Mean	r	SD_r	RSD_r%	R	SD_R	RSD_R%
Phosphatase test (μg phenol / ml)	8	2.56	1.95	0.70	27.20	2.21	0.79	30.81
	5	4.02	3.99	1.42	35.45	6.94	2.48	61.72
	8	4.13	7.15	2.55	61.73	12.02	4.29	103.82
	6	4.36	2.32	0.83	19.05	4.47	1.60	36.66
	7	6.41	3.76	1.34	20.95	8.78	3.14	48.90
	9	6.93	2.44	0.87	12.60	7.01	2.50	36.13
	12	1.34	0.27	0.10	7.13	0.59	0.21	15.81
	20	1.44	0.31	0.11	7.69	1.35	0.48	33.50

Key to Table XXXII

r	repeatability (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.
SD_r	the standard deviation of the repeatability.
RSD_r%	the relative standard deviation of the repeatability $SD_r \times 100 / x$.
R	reproducibility (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.
SD_R%	the standard deviation of the reproducibility.
RSD_R%	the relative standard deviation of the reproducibility $S_R \times 100 / x$
*	no statistical analysis carried out on 5th batch of milk.
#	insufficient data for statistical analysis after removal of outliers for 2 batches examined;

Table XXXIII

Log_{10} results of automated somatic cell counts

	SAMPLES									
	1		2		3		4		5	
METHOD	a	b	a	b	a	b	a	b	a	b
Coulter counter	5.41	5.34	5.51	5.51	5.84	5.84	5.92	5.97	6.05	6.03
Fluoro-opto electronic (preserved)	4.98	4.99	5.5	5.51	5.86	5.84	6	5.99	6.11	6.1
Fluoro-opto electronic (unpreserved)	5.06	5.02	5.49	5.5	5.83	5.84	6	5.99	6.1	6.1

Acknowledgements

The authors wish to thank the following analysts and their staff for participation in this trial:

R Barrell, Public Health Laboratory (PHLS), Withington Hospital, Manchester.

T Humphrey, PHLS, Heavitree, Exeter.

P Hansford, PHLS, W. Dorset, Dorchester.

A Harrison, Public Analyst's Laboratory, Bristol.

D Lord, Public Analyst's Laboratory, Preston.

D Robertson, Government Laboratory, Douglas, Isle of Man.

M Billington, City of Birmingham Analyst's Laboratory, Birmingham.

R Andrew, Public Analyst's Laboratory, Maidstone, Kent.

L. McIntosh, Strathclyde Regional Laboratory, Glasgow, Scotland.

G Hooke, Ruddock and Sherratt Public Analyst's Laboratory, Chester.

E.B Reynolds, Tickle and Reynolds Public Analyst's Laboratory, Exeter.

G. Baker, Melling and Ardern Public Analyst's Laboratory, Salford.

D. Dunn, A.H Allen and Partners Public Analyst's Laboratory, Sheffield.

G.S Meadows, Lincolne, Sutton and Wood Public Analyst's Laboratory, Norwich.

F. Brookes, Northern Foods Group, Nottingham.

H. Evans, St Ivel Foods, Swansea.

S. Johal, Laboratory of the Government Chemist, Teddington, Middx.

A. Hands, Milk Marketing Board, Thames Ditton, Surrey.

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APPENDIX 1

Summary of Colony Count Test at 30°C - E.C.Test IV

A defined volume of the milk sample is mixed with the culture medium (milk plate count agar) in Petri dishes and incubated at 30°C for 72 hours. The colonies are counted and the number of micro-organisms per 1 ml of raw or pasteurised milk or per 0.1 ml of preincubated UHT-treated or sterilised milk is calculated.

APPENDIX 2

Summary of Colony Count Test at 21°C - E.C.Test V

The pasteurised milk is incubated (preferably in an unopened container e.g. in carton) at 6°C for five days. A defined volume of the milk sample is mixed with the culture medium (milk plate count agar) in Petri dishes and incubated at 21°C for 25 hours. The colonies are counted and the number of micro-organisms per 1 ml of pasteurised milk is calculated.

APPENDIX 3

Summary of Coliform Count - E.C. Test VI

A defined volume of the milk sample is mixed with the culture medium (violet red bile lactose agar) in Petri dishes and incubated at 30°C for 24 hours. Characteristic colonies are counted and, if necessary, the identity of any non-characteristic colonies is confirmed by testing for the ability to ferment lactose in the presence of bile salts. The number of coliforms per 1 ml of pasteurised milk is then calculated.

APPENDIX 4

Summary of Somatic Cell Count - E.C.Test VII

0.01 ml of milk is spread over 1 cm^2 of a slide. The film is dried and stained. Counting is carried Counting of stained cells is carried out using a microscope. The number of somatic cells counted in a defined area is multiplied by the calculated working factor to obtain the number of cells/ml.

APPENDIX 5

Summary of Determination of Phosphatase Activity - E.C.Test II

The principle on which the test is based is that although phosphatase enzymes are invariably present in raw milk, they are inactivated by pasteurisation. The phosphatase activity is calculated from the amount of phenol liberated from the disodium phenyl-phosphate added to the sample. The phenol liberated reacts with dibromoquinonechlorimide producing dibromoindophenol (bluish in colour) which is measured colorimetrically at 610 nm. A comparison is made with a sample where the phosphatase enzyme has been destroyed.

APPENDIX 6

Summary of Determination of Phospahatase Activity Non E.C.Test

The test prescribed in The Milk (Special Designation) Regulations (Anon. 1989) involves incubation of the milk with di-sodium-*p*-nitrophenol phosphate under alkaline conditions. If the milk contains phosphatase a yellow colour is produced due to the formation of *p*-nitrophenol. The degree of destruction of phosphatase in the milk during pasteurisation is then assessed by comparing the colour produced with the standard colours on an APTW comparator disc. Milk is deemed to be satisfactory using this test if it gives a reading of 10 μg or less of *p*-nitrophenol/ml milk.

APPENDIX 7

Determination of Bacterial Lipopolysaccharide Content *Limulus* Amoebocyte Lysate Test - Non E.C.Test

1 Definition

For the purposes of this method, the following definition applies:

1.1 *Limulus* amoebocyte lysate test (LAL): A semi-quantitative assay in the form of microtitre plates containing lyophilised amoebocytes from the horseshoe crab (*Limulus polyphemus*) which react to form a gel in the presence of lipopolysaccharide (endotoxin/pyrogen) from the cell walls of viable and non-viable Gram-negative bacteria.

2 Principle

In general the determination of the concentration of endotoxin present in the test sample is carried out in 3 successive stages:

2.1 Preparation of dilutions in microtitre wells of initial test sample .

2.2 Incubation of microtitre plates at 37°C for 1 h.

2.3 Visualising gel clots and calculation of endotoxin titres.

3 Diluent and reagents

3.1 Pyrogen free water (PFW, Baxter Healthcare, Thetford)

3.2 Lyophilised reference standard E.C 5 (Labortechnik Peter Schulz, Munich)

3.3 Toluidine blue dye

3.3.1 Composition

Toluidine blue dye	0.2 g
Tween 20	1.0 g
Water	100 ml

Preparation: Dissolve the constituents in water and mix thoroughly.

4 Apparatus and glassware

Usual microbiological laboratory equipment and in particular:

4.1 All glassware coming into contact with the sample must be pyrogen free. This may be achieved by heating at 180°C for 4 h. Sterile plasticware may be used in preference to glass and is normally pyrogen free.

4.4 Non-circulating water bath capable of maintaining a temperature of 37 ± 0.5°C

4.5 LAL test microtitre plates (Labortechnik Peter Schulz)

Note: The microtitre plates must be handled with care as any form of vibration will disturb the adsorbed lysate and may lead to false negative results.

4.7 Automatic pipettes capable of dispensing 64.8 µl and 30 µl volumes

4.8 Parafilm

4.9 Suction pump (water or vacuum)

4.10 Vortex mixer

5 Procedure

5.1 Prepare microtitre plates in the following manner:-

5.1.1 Using the microtitre plates provided, add 64.8 µl PFW to each dilution well. Remove 30 µl of test sample of milk and add to first dilution well containing 64.8 µl PFW. Using a fresh pipette tip, mix the well contents thoroughly by filling and emptying pipette tip taking care not to introduce air bubbles into the microtitre well.

5.1.2 Transfer 30 µl from the first dilution well to the first test well and 30 µl from the first dilution well to the second dilution well. Using a clean pipette tip, mix thoroughly as above.

5.1.3 Transfer 30 µl from the second dilution well to the second test well and 30 µl to the third dilution well. Using a clean pipette tip mix thoroughly.

5.1.4 Continue with dilution of the test material in the microtitre plate as above until all vertical test wells have been filled for each sample.

5.2 In addition to the samples, positive and negative controls must also be incorporated onto the plate:

5.2.1 Positive control: Resuspend the lyophilised reference standard (**5.2**) in 10 ml PFW to prepare a 50 Endotoxin units/ml solution. Vortex for at least 2 min. Test as for sample assay described above (**5.1**).

5.2.2 Negative control: Add 30 µl PFW (**4.1**) to two spare test wells in the microtitre plate.

5.3 Incubation of the LAL test

Immediately after preparation, cover the microtitre plate in parafilm and float on a water bath at 37°C ± 0.5°C for 1 h.

5.4 Visualisation of gel clots

After the incubation period, remove the plate from the water bath and dry carefully. Remove the parafilm and add 1 drop of toluidine blue dye to each test well. To test for gel formation, aspirate (suck out) each test well with the aid of a pasteur pipette fitted to a gentle suction pump by placing the tip of the pasteur into the well. **Do not move tip in the well**. If a gel has formed the contents of the test well will not be completely removed but a hole marked where the pasteur pipette was placed. If no gel has formed the liquid contents will be completely removed. Record a positive reaction where a gel has formed in the well. Record a negative reaction when the contents of the well are completely aspirated i.e. well is empty. Record a partial reaction when there is incomplete gel formation. Calculate the titre of the last positive well in the sequence of dilutions as shown in the examples:

Example 1 :

Well No	Gel	Dilution
1	+	$10^{-0.5}$
2	+	10^{-1}
3	+	$10^{-1.5}$
4	-	$10^{-2.0}$
Titre	=1.5	

Example 2 :

Well No	Gel	Dilution
1	+	$10^{-0.5}$
2	+	10^{-1}
3	+/-	$10^{-1.5}$
4	-	$10^{-2.0}$
5	-	$10^{-2.5}$
Titre	=1.25	

Calculate the number of endotoxin units per g sample using the following equation:

EU's /ml = $10^{(titre)}$ × sensitivity of the lysate (given by manufacturer).

Note: If the pyrogen free water does not show a negative reaction the whole test must be repeated using a new unopened bottle of pyrogen free water.

If the result for the positive control does not correspond to approximately the true value e.g. in this case 50 EU/ml (Titre 1.5 ± 0.25) this must be recorded in the final report.

6 Test Report

The test report should include:

a) Date sample received/analysed

b) The temperature/condition of the sample upon arrival.

c) A statement of

i) the sensitivity of the amoebocyte lysate used (given by manufacturer)

ii) the endotoxin titre determined and

iii) the calculated EU/ml in the sample.

d) Any operations/occurrences during the course of the test which may have influenced the results.

g) Results of positive and negative controls

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MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

No. V 25

SOYA PROTEIN IN MEAT PRODUCTS

Correspondence on this method may be sent to R. Wood, Statutory Methods Ministry of Agriculture, Fisheries and Food, Food ScienceLaboratory, Food Safety Directorate, Norwich Research Park, Colney, Norwich NR4 7UQ

1. Scope and Field of Application

The method allows the determination of the level of soya protein in a raw or pasteurised meat product of entirely unknown composition containing an unknown soya ingredient (whether grits, flour, isolate, concentrate or texturate).

2. Definition

Soya protein content: the total content of all soya proteins as determined by the method specified.

3. Principle

The Biokits Soya Protein Assay is an indirect competitive enzyme immunoassay. The samples of meat product are homogenised and then extracted (solubilised) in a urea-dithiothreitol buffer at 100°C followed by rapid renaturation in a cystine-containing diluent. The assay is performed in plastic microwells which have been pre-coated with a purified preparation of soya protein. In the initial competition reaction, a fixed amount of the diluted extract of the meat sample is added into the soya protein-coated microwell along with a fixed volume of specific rabbit anti-soya protein antiserum. With increased concentrations of soya protein in the diluted extract, the amount of rabbit anti-soya protein antibody binding to the soya protein attached to the well, will decrease. After allowing this reaction to proceed, the unbound material is removed by aspiration and washing.

The amount of rabbit anti-soya protein antibody remaining bound to the soya protein-coated well is determined by reacting a fixed amount of peroxidase-conjugated swine anti-rabbit globulin antibody. After incubation, the excess conjugate is removed by aspiration and washing, and the bound peroxidase activity determined by adding a fixed amount of substrate *(2:2'-azino-di-(3-ethylbenzthiazoline) sulphonic acid*, "ABTS"), which develops a green coloration in the presence of peroxidase. The colour development is an inverse measure of the original soya protein concentration in the original extract. The concentration of soya protein in the meat product can be determined by reading off a calibration curve derived from standards of known soya protein concentration.

0004-5780/93 +16 $20.00

4. Kit Components

The following components are provided in each kit. The reagents (**4.1**-**9**) and the soya protein-sensitised microwell module (**4.10**) should be stored at 2-6°C. The shelf lives of the kit and its components are indicated by the expiry data on the respective labels. Once the kit reagents have been opened they should be used within two weeks; some contain thiomersal as preservative.

4.1 Soya protein standards

Five vials, each containing 3 ml of one of the following pre-diluted soya protein standards: 3.5; 7; 15; 35; 70 µg of soya protein per ml of phosphate buffered saline (*p*H 7.2), with 0.1% *m/V* bovine serum albumin and 0.01% *m/V* thiomersal.

4.2 Soya protein control

One vial containing 0.5 g of soya protein isolate powder with no other additives. The powder has been calibrated for soya protein content by Kjeldahl nitrogen analysis using 6.25 as the conversion factor; the soya protein content is stated on the vial label.

4.3 Anti-soya protein antiserum

One vial containing 6 ml of pre-diluted rabbit anti-soya protein antiserum in phosphate buffered saline (*p*H 7.2) with 0.1% *m/V* bovine serum albumin and 0.01% *m/V* thiomersal.

4.4 Peroxidase conjugate

One vial containing 11.5 ml of peroxidase conjugated swine anti-rabbit globulin antibody in phosphate-buffered saline (*p*H 7.2) with 0.1% *m/V* bovine serum albumin, 0.01% *m/V* thiomersal and stabiliser.

4.5 ABTS concentrate

One vial containing 0.55 ml of a solution of 15 mg of ABTS per ml of distilled water.

ABTS is an abbreviation for 2:2'-azino-di-(3-ethylbenzthiazoline) sulphonic acid, and is a peroxidase substrate.

4.6 Peroxide-citrate buffer (*p*H 4.0)

One vial containing 12.0 ml of a solution of 2.3% *m/V* citric acid monohydrate and 0.015% *m/V* hydrogen peroxide.

4.7 Wash solution concentrate

One bottle containing 100 ml of a ten-fold concentrate of tris-buffered saline with 5% *m/V* Tween 80 and 0.01% *m/V* thiomersal. The *p*H of this concentrate is approximately 7.9; "tris" is an abbreviation for tris(hydroxymethyl)methylamine.

4.8 Diluent concentrate

One vial containing 20 ml of a five-fold concentrate of phosphate-buffered saline with 0.5% *m/V* bovine serum albumin and 0.05% thiomersal. The *p*H of this concentrate is approximately 6.9.

4.9 Stop solution

One vial containing 6 ml of 1.5% *m/V* sodium fluoride in distilled water. *Caution: sodium fluoride is toxic; avoid ingestion or contact with skin or eyes.*

4.10 Soya protein-sensitised microwell module

Soya protein-sensitised microwell module, comprising six double strips of microwells held in a plastic frame and packed in a foil laminate pouch with desiccant bag. Each strip has two columns of eight microwells; there are 96 assay wells in all. The interior of each microwell has been coated with a pre-determined amount of soya protein and dried.

4.11 Stationery

Three sheets of pre-labelled graph paper, an example work sheet (Fig. 1) and an assay layout guide (Fig. 2).

5. Reagents and Apparatus not Provided in Kit

5.1 Reagents

All reagents should be of recognised analytical grade unless specified otherwise.

5.1.1 Urea

5.1.2 Dithiothreitol, DTT, Cleland's reagent.

5.1.3 Tris(hydroxymethyl)methylamine, commonly abbreviated to "tris".

5.1.4 *L*-cystine

5.1.5 Sodium chloride

5.1.6 Sodium hydroxide solution, 1.0 mol/l.

5.1.7 Hydrochloric acid, 1.0 mol/l.

5.2 Equipment for sample preparation

5.2.1 Waring blender, with 37-110 ml capacity bowl (e.g. as available from Gallenkamp, Belton Road West, Loughborough, Leicestershire LE11 0TR), or equivalent.

5.2.2 Ultra Turrax homegeniser, model TP18/10, with 18N shaft, speed control and stand (e.g. as available from Scientific Supplies Co. Ltd, Scientific House, Vine Hill, London EC1 5EB), or equivalent.

5.2.3 Borosilicate conical flasks, 50 ml, with 19/26 neck size, and 19/26 stoppers.

5.2.4 Whatman filter papers, 18.5 cm diameter, grade no. 1., or equivalent.

5.2.5 Water or steam bath, at 100°C.

5.2.6 Water bath, at 50°C.

5.2.7 Miscellaneous glassware, including measuring cylinders, volumetric flasks and pipettes.

5.3 Equipment for enzyme immunoassay

5.3.1 Precision micropipette, capable of delivering 50 µl (e.g. Gilson Pipetman P200, as available from Anachem, Anachem House, 20 Charles Street, Luton, Bedfordshire LU2 0EB), or equivalent.

5.3.2 Precision repeating dispenser, capable of delivering 50 and 100 µl aliquots (e.g. Labsystems Finnpipette Stepette 10-1000 µl; with steptip 2 syringe (800 µl) and steptip 3 syringe (3200 µl), as available from Jencons (Scientific) Ltd, Cherrycourt Way Industrial Estate, Leighton Buzzard, Bedfordshire LU7 8UA), or equivalent.

5.3.3 Orbital plate shaker, with mixing speed of 1200-1400 rpm (e.g. Flow Titertek Plate Shaker, fixed speed, as available from Flow Laboratories Ltd, Woodcock Hill, Harefield Road, Rickmansworth, Hertfordshire WD3 1PQ), or equivalent.

5.3.4 Microwell washer, (e.g. NUNC Immuno Wash 8, as available from Gibco Ltd, PO Box 35, Washington Road, Abbotsinch Industrial Estate, Paisley PA3 4EP, Scotland), or equivalent.

5.3.5 Microwell plate reader, fitted with a 414 nm interference filter (e.g. Uniskan I or Uniskan II, as available from Labsystems (UK) Ltd, 12 Redford Way, Uxbridge, Middlesex), or equivalent.

6. Procedure

6.1 Preparation of extraction reagents

The extraction reagents should be prepared on the day of use and not stored for longer than the working day. The given quantities are sufficient for 32 assay wells.

6.1.1 Stock buffer concentrate, 0.25 mol/l tris-HCl, *p*H 8.6 (200 ml).

Weigh out exactly 6.06 g of tris (**5.1.3**) into a 250 ml beaker. Add approximately 150 ml of distilled water and mix until all the tris is in solution. Adjust the *p*H to 8.6 by adding 1.0 mol/l HCl (**5.1.7**) (approximately 11-13 ml). Quantitatively transfer to 200 ml volumetric flask and make up to exactly 200 ml with distilled water.

6.1.2 Buffer, 0.05 mol/l tris-HCl, *p*H 8.6 (600 ml)

Dilute the stock buffer to one-fifth concentration by adding 120 ml of the concentrate (**6.1.1**) to 480 ml of distilled water.

6.1.3 Urea-DTT extraction buffer, 100 ml.

Weigh out 80.0 g of urea (**5.1.1**) into a 250 ml conical flask. Add 20 ml of stock buffer concentrate (**6.1.1**) and 20 ml of distilled water. Heat gently over a Bunsen flame with constant mixing by swirling until all the urea has dissolved. Add 0.29 g of DTT (**5.1.2**) to the hot urea solution and mix by swirling until dissolved. Transfer the flask to a water bath at 100°C (**5.2.5**), and maintain at this temperature.

6.1.4 Renaturation solution, 1000 ml

Weigh out 1.8 g of *L*-cystine (**5.1.4**) in a 50 ml beaker. Add 20.0 ml of 1.0 mol/l NaOH solution (**5.1.6**) using a volumetric pipette. Allow the *L*-cystine to dissolve completely by gentle mixing. Weigh out 3.5

g of NaCl (**5.1.5**) and dissolve in 900 ml of distilled water in a 1000 ml beaker. With constant mixing, slowly add the 20 ml of *L*-cystine solution to the NaCl solution; complete the transfer by rinsing out the residual *L*-cystine solution with a little distilled water. Continue mixing and slowly add 8 ml of 1.0 mol/l HCl (**5.1.7**) to the *L*-cystine/NaCl solution while monitoring the pH; finally adjust to *p*H 9.0 by adding a further 1.5-3.0 ml of 1.0 mol/l HCl. Make up the total volume to 1.0 litre with distilled water. Pre-warm to 50°C before use.

6.2 Preparation of meat slurry

The given quantities are appropriate for soya protein contents between 1% and 10%.

6.2.1 Weigh accurately about 12.0 g of the sample of meat product into the blender container (**5.2.1**). Record the weight as W_1 on the work sheet (**4.11**).

6.2.2 Weigh accurately about 48.0 g of 0.05 mol/l tris-HCl buffer *p*H 8.6 (**6.1.2**) into the blender container (**6.2.1**). Record the weight as W_2 on the work sheet (**4.11**).

6.2.3 Blend the meat sample until a fairly smooth homogeneous mixture is obtained.

6.2.4 Transfer as much as possible of the slurry to a 100 ml beaker, taking care not to leave any significantly large pieces of tissue in the blender bowl.

6.2.5 Using the Ultra Turrax tissue homogeniser (**5.2.2**), complete the sample homogenisation. The final mixture should be a smooth homogenate, easily pipettable using Pasteur pipettes with tip diameter 1 mm.

6.2.6 Weigh accurately about 2.5 g of each meat homogenate into separate 50 ml conical flasks (**5.2.3**). This may be done by placing the flask directly on the balance pan and transferring the homogenate using a Pasteur pipette. Before pipetting, ensure that the homogenate is thoroughly mixed and has not settled out. Record the weight as W_3 on the work sheet (**4.11**).

6.2.7 Place each flask in a water bath at 50°C (**5.2.6**); this pre-warms the flask and meat homogenate before addition of the urea-DTT extraction buffer (**6.1.3**).

6.3 Preparation of soya protein control

6.3.1 Weigh accurately about 40 mg of the soya protein control (**4.2**) into a 50 ml stoppered flask (**5.2.3**). Record the weight as W_4 on the work sheet (**4.11**).

6.3.2 Add 2.5 ml of 0.05 mol/l tris-HCl buffer *p*H 8.6 (**6.1.2**) to the flask; gently mix the powder into suspension by swirling.

6.3.3 Place the flask in a water bath at 50°C (**5.2.6**) to pre-warm, as for the meat samples (**6.2.7**).

6.4 Extraction of meat slurry and soya protein control

6.4.1 Using a graduated pipette, add 7.5 ml of the urea-DTT extraction buffer at 100°C (**6.1.3**) to each of the meat sample flasks (**6.2.7**) and to the soya protein control flask (**6.3.3**) in the water bath at 50°C. Leave the pipette standing in the flask of urea-DTT extraction buffer to prevent cooling and crystallisation of urea in the pipette tip.

6.4.2 Stopper each flask and mix by gentle swirling to achieve a uniform suspension. Immediately transfer all the flasks to a water bath at 100°C (**5.2.5**).

6.4.3 Incubate the flasks at 100°C for 60 min. with occasional mixing.

6.4.4 Remove all the flasks from the water bath at 100°C and place them in the water bath at 50°C (**5.2.6**) to prevent the urea crystallising out of the buffer.

6.5 Renaturation of extracted samples and control

6.5.1 With constant mixing, slowly add 20 ml of renaturation solution (**6.1.4**), pre-warmed to 50°C, to each flask in the water bath at 50°C (**6.3.3** and **6.4.4**). Mix the contents of each flask thoroughly by swirling, and replace the flasks in the water bath at 50°C.

6.5.2 Remove each flask in turn from the water bath at 50°C and quantitatively transfer its contents to a 100 ml volumetric flask, using three 10 ml volumes of renaturation solution (**6.1.4**) at 50°C. Mix thoroughly by swirling and allow to cool to room temperature. Make up the volume to exactly 100.0 ml with renaturation solution at room temperature.

6.5.3 Mix thoroughly by repeated inversion and immediately filter the contents of each flask through a pleated or folded 18.5 cm diameter Whatman no. 1 filter paper (**5.2.4**). Collect only the first 10 ml of filtrate. The filtrates (sample and control extracts) may be stored for up to 48 hr. at 2-6°C prior to assay.

6.6 Preparation of reagents from kit materials

6.6.1 Soya protein standards (**4.1**) are supplied pre-diluted in buffer. No preparation is necessary other than mixing the contents of the vial thoroughly by repeated inversion. Do not shake.

6.6.2 Soya protein control (**4.2**) is supplied as a powder which requires to be extracted in a similar fashion to the meat samples (**6.3-6.5; 6.8.2**)

6.6.3 Anti-soya protein antiserum (**4.3**) is supplied pre-diluted in buffer. No preparation is necessary other than mixing the contents of the vial thoroughly by repeated inversion. Do not shake.

6.6.4 Peroxidase conjugate (4.4) is supplied pre-diluted in buffer. No preparation is necessary other than mixing the contents of the vial thoroughly by repeated inversion. Do not shake.

6.6.5 ABTS substrate solution

ABTS is supplied as a 25-fold concentrate (**4.5**) and requires dilution in peroxide-citrate buffer (**4.6**) to prepare working ABTS solution.

Mix the contents of each vial by inversion; dilute one volume of the ABTS concentrate (**4.5**) with twenty-four volumes of the peroxide-citrate buffer (**4.6**).

Either of the following procedures may be used.

(i) For 96 assay wells: add 0.5 ml of ABTS concentrate (**4.5**) to the 12.0 ml of buffer contained in the peroxide-citrate buffer vial (**4.6**), stopper the vial and mix well by repeated inversion.

(ii) For 32 assay wells: pipette 3.6 ml of peroxide-citrate buffer (**4.6**) into a clean container and add to this 0.15 ml of ABTS concentrate (**4.5**); mix well.

Take care not to cross-contaminate the ABTS concentrate and the peroxide-citrate buffer in their respective vials. Dilutions of ABTS concentrate should be freshly prepared and used within 4 hr. of preparation.

6.6.6 Wash solution

A ten-fold concentrate (**4.7**) is supplied and requires dilution to prepare the working wash solution.

Dilute one volume of wash solution concentrate (**4.7**) with nine volumes of distilled or deionised water: for instance, either of the following procedures may be used.

(i) For 96 assay wells: use the total contents (100 ml) of the wash solution concentrate vial (**4.7**), by rinsing out and making up to 1.0 litre in a volumetric flask with distilled or deionised water.

(ii) For 32 assay wells: add 30 ml of wash solution concentrate (**4.7**) to 270 ml of distilled or deionised water.

The *p*H of the working wash solution should be in the range 7.7-7.9.

6.6.7 Diluent solution

A five-fold concentrate (**4.8**) is supplied and requires dilution to prepare the working assay diluent solution; this diluted reagent is used for the final tenfold dilution of the sample extract and the soya protein control extract (**6.8.2**).

Dilute one volume of diluent concentrate (**4.8**) with four volumes of distilled or deionised water: for instance, either of the following procedures may be used.

(i) For 96 assay wells: use the total contents (20.0 ml) of the diluent concentrate vial (**4.8**) by rinsing out and making up to 100 ml in a volumetric flask with distilled or deionised water.

(ii) For 32 assay wells: add 6.5 ml of diluent concentrate (**4.8**) to 26 ml of distilled or deionised water.

The *p*H of the working assay diluent solution should be in the range 7.1-7.3.

6.6.8 Stop solution

No preparation is necessary other than mixing the contents of the vial (**4.9**) by repeated inversion. Stop solution contains 1.5% *m/m* sodium

fluoride, which is toxic. If contact with the skin is made, wash the affected area immediately with copious amounts of water.

6.6.9 Soya protein-sensitised microwell module

Open the foil laminate pouch with label side uppermost, by cutting along the inside margin of the crimp seal with a sharp pair of scissors. Remove the module, keeping the open ends of the wells uppermost. If only a small number of assays are to be run (e.g. 32 assay wells), then remove the strips of wells required (e.g. 2) and fit into a spare frame; replace the remaining frame and strips in the pouch, taking care that the desiccant bag lies underneath the module. Reseal the pouch with adhesive tape.

6.7 Enzyme immunoassay: introduction

The Biokits soya protein assay kit can be divided into three 32 assay well groups (2-strip assay); two 48 assay well groups (3-strip assay) or the entire plate of 96 wells (6-strip assay) may be used. Example assay layouts are supplied (**4.11**).

6.7.1 Familiarisation

It is recommended when first familiarising oneself with the kit that a 2-strip assay is selected (32 assay wells). All reaction wells, except the substrate blank (B) and maximum binding (M) wells, should be run in duplicate and the mean absorbance value of each pair of wells calculated. The results may be recorded on the example work sheet provided (4.11). Of the 32 assay wells, ten (five duplicates) are used for the standards, two (one duplicate) are used for the control (C) and one well each is used to monitor substrate blank (B) and maximum binding (M). This leaves 18 wells (nine duplicates) for test samples.

6.7.2 Identification of wells

With a pencil, number the columns on the lower frosted edge of the strips (1-4 from left to right for a 2-strip assay); this preserves the identity of the strips should they become detached from the frame. The rows of wells are designated by letters A to H from the top, but need not be marked.

6.7.3 When an assay has been started, all steps should be completed without interruption.

6.8 Enzyme immunoassay: procedure

6.8.1 Allow all reagents and the soya protein-sensitised microwell module (**6.6**) to reach room temperature before starting the assay.

6.8.2 Prepare the meat product samples (**6.5**), the soya protein control (**6.5**) and the necessary kit materials (**6.6**). Immediately prior to assay, dilute the sample and control filtrates tenfold by adding 0.90 ml of working assay diluent solution (**6.6.7**) to 0.10 ml of filtrate (**6.5.3**).

6.8.3 Using the precision micropipette (**5.3.1**), place 50 µl of each soya protein standard (**6.6.1**), the prepared soya protein control (**6.8.2**) and each of the prepared samples (**6.8.2**), into duplicate microwells (**6.6.9**).

Use a separate disposable tip for each pipetting step to avoid cross contamination.

Ensure that the addition of standards, control and samples follows the layout on the template guide provided (**4.11**).

6.8.4 Using the precision micropipette (**5.3.1**), place 50 µl of working assay diluent solution (**6.6.7**) into the maximum binding microwell (M). The substrate blank microwell (B) should be left empty.

6.8.5 Using the precision dispenser with the steptip 2 syringe (**5.3.2**), add 50 µl of the anti-soya protein antiserum (**6.6.3**) to each microwell, except the substrate blank microwell (B).

The repeating dispenser unit with steptip 2 syringe in place should be used to dispense aliquots to one column of eight microwells, before refilling the syringe. Do not allow the tip of the pipette to contact the material already in the microwells. Work in an orderly sequence, starting at position B1 (microwell M in column 1), and completing the addition to column 1 before refilling the dispenser and adding aliquots to the eight microwells in column 2, starting at position A2. Repeat this procedure with the other columns. Once this step (**6.8.5**) has been started, it must be completed at a steady pace without interruption.

6.8.6 Place the microwell module on the orbital shaker (**5.3.3**), and incubate with mixing for 10 min.

6.8.7 At the end of the incubation period, aspirate the material from all the microwells of column 1 using the microwell washer (**5.3.4**). Then fill all the microwells of column 1 with working wash solution (**6.6.6**). Complete this sequence for each successive column of microwells; all the microwells are now filled with wash solution. Return the microwell washer to column 1 and repeat the whole aspiration/fill sequence a further four times across all the columns; each microwell will now have received a total of five aspirations and five fills. Finally, use the microwell washer to empty all the microwells in each column by aspiration, and then tap the plate while upside down on several layers of absorbent tissue to remove any residual droplets of wash solution.

When inverting the plate, be sure to squeeze the plastic frame at the centre of the long edges to prevent the strips from falling out of the frame.

6.8.8 Using the repeating dispenser with the steptip 3 syringe (**5.3.2**), add 100 µl of peroxidase conjugate to each microwell, except the substrate blank (B).

The repeating dispenser unit with steptip 3 syringe in place should be used to dispense aliquots to two columns of eight microwells each, before refilling the syringe. Use the same orderly sequence as in **6.8.5** above. Once this step (**6.8.8**) has been started, it should be completed at a steady pace without interruption.

6.8.9 Place the microwell module on the orbital shaker (**5.3.3**) and incubate with mixing for 10 min.

6.8.10 At the end of the incubation period, repeat the washing sequence described in **6.8.7** above.

6.8.11 Using the repeating dispenser with a clean steptip 3 syringe (**5.3.2**), add 100 µl of the working ABTS solution to each microwell.

Refill the syringe after dispensing aliquots to two columns of eight microwells each. Use the same orderly sequence and steady pace described in **6.8.8** above.

6.8.12 Place the microwell module on the orbital shaker (**5.3.3**) and incubate with mixing for 10 min.

6.8.13 At the end of the incubation period, add 50 µl of stop solution (**6.6.8**) to each microwell, using the repeating dispenser with a clean steptip 2 syringe (**5.3.2**).

Add the aliquots of stop solution to each column of eight microwells in the same orderly sequence and at the same steady pace as that used at **6.8.11** to add the working ABTS solution.

6.8.14 Mix for 10 sec. on the orbital shaker (**5.3.3**) to distribute the stop solution uniformly.

6.8.15 Using a microplate reader fitted with a 414 nm filter (**5.3.5**), set the reader to zero on the substrate blank microwell (at co-ordinates A1). Then measure the absorbance of each of the assay microwells, starting at microwell M in column 1; complete the reading of column 1 before moving to the top of column 2. Repeat this process until all the microwells have been measured. All readings should be completed within 90 min. of adding the stop solution (**6.8.13**). Record the results on the worksheet provided (**4.11**) and calculate the mean absorbance values.

7. COSHH

Analysts are reminded that appropriate hazard and risk assessments required by the Control of Substances Hazardous to Health Regulations, 1988 (See "Control of Substances Hazardous to Health - Approved Code of Practice, Control of Substances Hazardous to Health Regulations, 1988") must be made before using this method.

8. Expression of Results

The unknown values for soya protein concentration in the samples of meat product are determined from a calibration curve. To construct the calibration curve, use the pre-labelled graph paper provided (4.11). Plot the mean absorbance value for each of the five soya protein standards and draw straight lines to join each pair of neighbouring points. To determine the soya protein levels of the samples and of the soya protein control, take the mean absorbance value from each duplicate and interpolate the corresponding soya protein concentration from the calibration curve.

Fig. 1 illustrates the data and results of a typical Biokits soya protein assay. Fig. 3 shows the calibration curve and the interpolation of soya

protein concentrations from the mean absorbance values, for the soya protein control and for a sample of meat product containing soya protein.

9. Reference

9.1 CC Hall, CHS Hitchcock and R Wood, J. Assoc. Publ. Analysts, 1987, **25**, 1-27.

9.2 Ministry of Agriculture, Fisheries and Food, Food Safety Directorate, MAFF Validated Methods for the Analysis of Food, Introduction, General Considerations and Analytical Quality Control, J. Assoc. Publ. Analysts, 1992, **28**, 11-16.

APPENDIX 1

Analytical Quality Control

General principles of analytical quality control are outlined in protocol V.0 of the series[2].

A1 Repeatability

The absolute difference between two test results obtained under repeatability conditions should not be greater than the repeatability, r, deduced from the collaborative trial data summarised below (Table 1). There is a slight indication that the presence of texturate has an adverse effect on precision (r=1.14); otherwise, the value of r ranged from 0.49 to 1.03, displaying little correlation with type of meat product or type of soya ingredient. Overall at the levels tested, r may be taken to be about 0.7 g of soya protein per 100 g of sample; this corresponds to a relative standard deviation of repeatability (coefficient of variance of repeatability), RSD_r, of approximately 16%.

A2 Reproducibility

The absolute difference between two test results obtained under reproducibility conditions should not be greater than the reproducibility, R, deduced from the collaborative trial data summarised below (Table 1). R may be taken to be about 1.1 g of soya protein per 100 g of sample. This corresponds to a relative standard deviation of reproducibility (coefficient of variance of reproducibility), RSD_R, of approximately 25%.

A3 Trueness (Bias)

The mean observed level of soya protein after 104 separate determinations of the pre-trial sample was 1.61 g per 100 g, corresponding to 92.5% recovery. This underestimation was consistently repeated during the trial itself (Table 1). However, the mean observed level depends on the response of the standard soya protein relative to the soya protein actually present in the sample; since the recoveries varied from 102% to 83% over a range of soya ingredients and meat product

types, the standard chosen minimises the chance of overestimation rather than maximises accuracy. Such a systematic bias is therefore generally acceptable.

A4 Limit of Detection

The collaborative trial data suggests an accuracy which, if maintained, corresponds to an extrapolated lower limit of roughly 0.7 g of soya protein per 100 g of sample for a single determination. This is indeed the limit claimed by the kit manufacturer, who also states that it can be halved by doubling the weight of sample taken. Samples containing no soya protein always give a satisfactory blank corresponding to the maximum observed absorbance.

A5 Statistical Data Derived from the Results of Interlaboratory Tests

Participants in the collaborative trial each analysed sixteen samples of meat product once (eight samples in blind duplicate). In addition, one pre-trial sample was analysed eight times, and two blanks were included in the trial. A selection of recipe combinations was tested: these included different meat levels (52-80%), soya protein levels (0-4.9%), casein levels (0, 1%), product types (sausage, beefburger, uncooked pate, cooked pate), soya ingredient types (grits, concentrate, isolate, texturate).

Sample A: beefburger containing soya grits;

Sample B: beefburger containing soya concentrate;

Sample C: beefburger containing soya texturate;

Sample D: sausage containing soya concentrate;

Sample E: sausage containing soya isolate;

Sample F: uncooked pate containing soya isolate;

Sample G: cooked pate containing soya isolate (sample F after cooking);

Sample H: cooked pate containing both isolate and concentrate.

Table 1 summarises the statistical data; the levels of soya protein are expressed as a percentage by mass of the sample. No corrections were made for any response variations of the soya components used relative to the standard.

A6 Key to Table 1

Symbol	Definition
$\bar{x}$	Overall mean value
S_r	The standard deviation of repeatability
RSD_r	The relative standard deviation of repeatability, expressed as a percentage of the mean (coefficient of variance of repeatability CV_r)
r	Repeatability
S_R	The standard deviation of reproducibility
RSD_R	The relative standard deviation of reproducibility, expressed as a percentage of the mean (coefficient of variance of reproducibility CV_R)
R	Reproducibility

TABLE I

Statistical Analysis of % Soya Protein in Meat Products

Sample	A	B	C	D	E	F	G	H
Number of Laboratories retained after eliminating outliers	14	14	14	13	14	13	12	13
Number of Laboratories eliminated as outliers	0	0	0	1	0	1	2	1
Number of results accepted after eliminating outliers	28	28	28	26	28	26	24	26
LEVEL OF ANALYTE								
Mean observed value $\bar{x}$	4.08	1.18	1.69	1.20	1.61	1.45	1.52	2.50
Actual (Target) value	4.90	1.30	1.98	1.18	1.74	1.74	1.74	2.92
Recovery (%)	83	91	85	102	93	83	87	86
REPEATABILITY								
Standard Deviation S_r	0.37	0.28	0.41	0.20	0.36	0.21	0.18	0.35
Relative Standard Deviation RSD_r(%)	9	24	24	17	22	14	12	14
Repeatability r [2.8 × S_r]	1.03	0.78	1.14	0.57	0.67	0.58	0.49	0.97
REPRODUCIBILITY								
Standard Deviation S_R	0.81	0.29	0.48	0.21	0.28	0.31	0.29	0.45
Relative Standard Deviation RSD_R(%)	20	25	29	18	17	22	19	18
Reproducibility R [2.8 × S_R]	2.28	0.81	1.35	0.60	0.77	0.88	0.81	1.27

BioKits

Soya Protein Assay

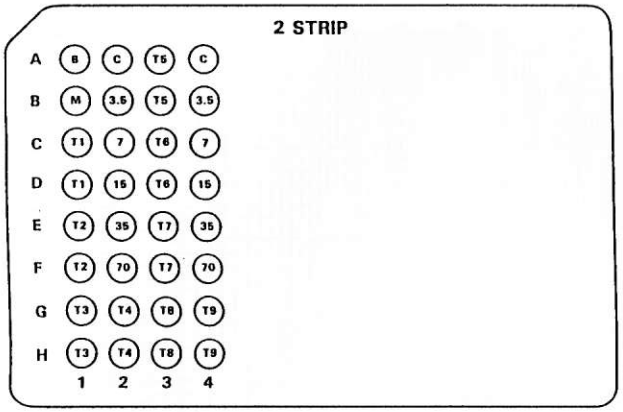
QUANTITATION OF SOYA PROTEIN IN MEAT PRODUCTS

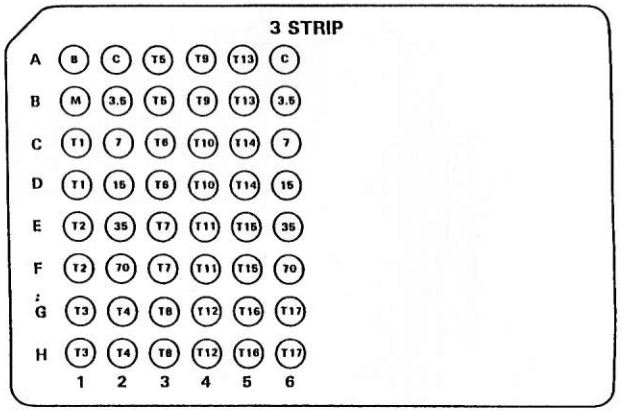
Date: 11-7-85 Operator:

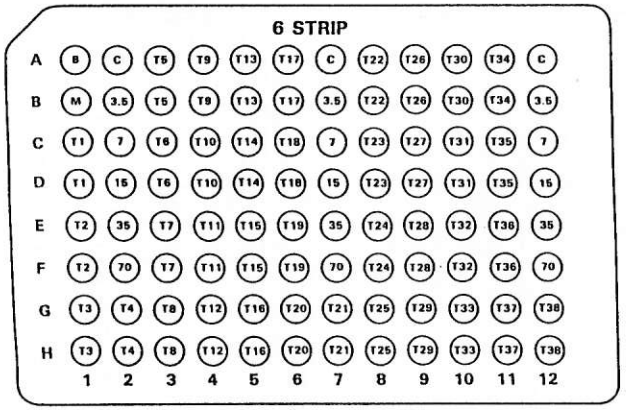
SOLUTION TESTED		Absorbance (414nm) 1	2	Mean	Soya Protein Concentration Read from Calibration Curve (µg/ml)	Weight of Meat Sample (grams)	Weight of .05M Tris-HCl Buffer (grams)	Weight of Homogenate Added to Extraction Flask (grams)	Weight of Soya Protein Control Added to Extraction Flask (mg)	Weight of Meat Extracted (mg) $\frac{W1}{W1 \div W2} \times W3 \times 1000$	SAMPLE $\frac{(S.P.)}{W5} \times 100$	CONTROL $\frac{(S.P.)}{W4 \times \text{PROTEIN VALUE}} \times 100$
3.5µg/ml SOYA PROTEIN STANDARD		1·139	1·184	1·162								
7.0µg/ml SOYA PROTEIN STANDARD		·864	·809	·837								
15µg/ml SOYA PROTEIN STANDARD		·646	·580	·563								
35µg/ml SOYA PROTEIN STANDARD		·335	·329	·332								
70µg/ml SOYA PROTEIN STANDARD		·206	·192	·199	(S.P.)	W1	W2	W3	W4	W5	% SOYA PROTEIN IN MEAT SAMPLE	% RECOVERY OF SOYA PROTEIN CONTROL
C	SOYA PROTEIN CONTROL PROTEIN VALUE: ·83	·349	·368	·359	21·5				40·6			93·5
M	MAXIMUM BINDING	1·963										
1	ECONOMY SAUSAGE	·744	·694	·719	9·6	12·47	47·96	2·47		509·7	1·9	
2												
3												
4												
5												
6												
7												
8												
9												

0020100BM

Fig 1: Example Work Sheet with Data and Results





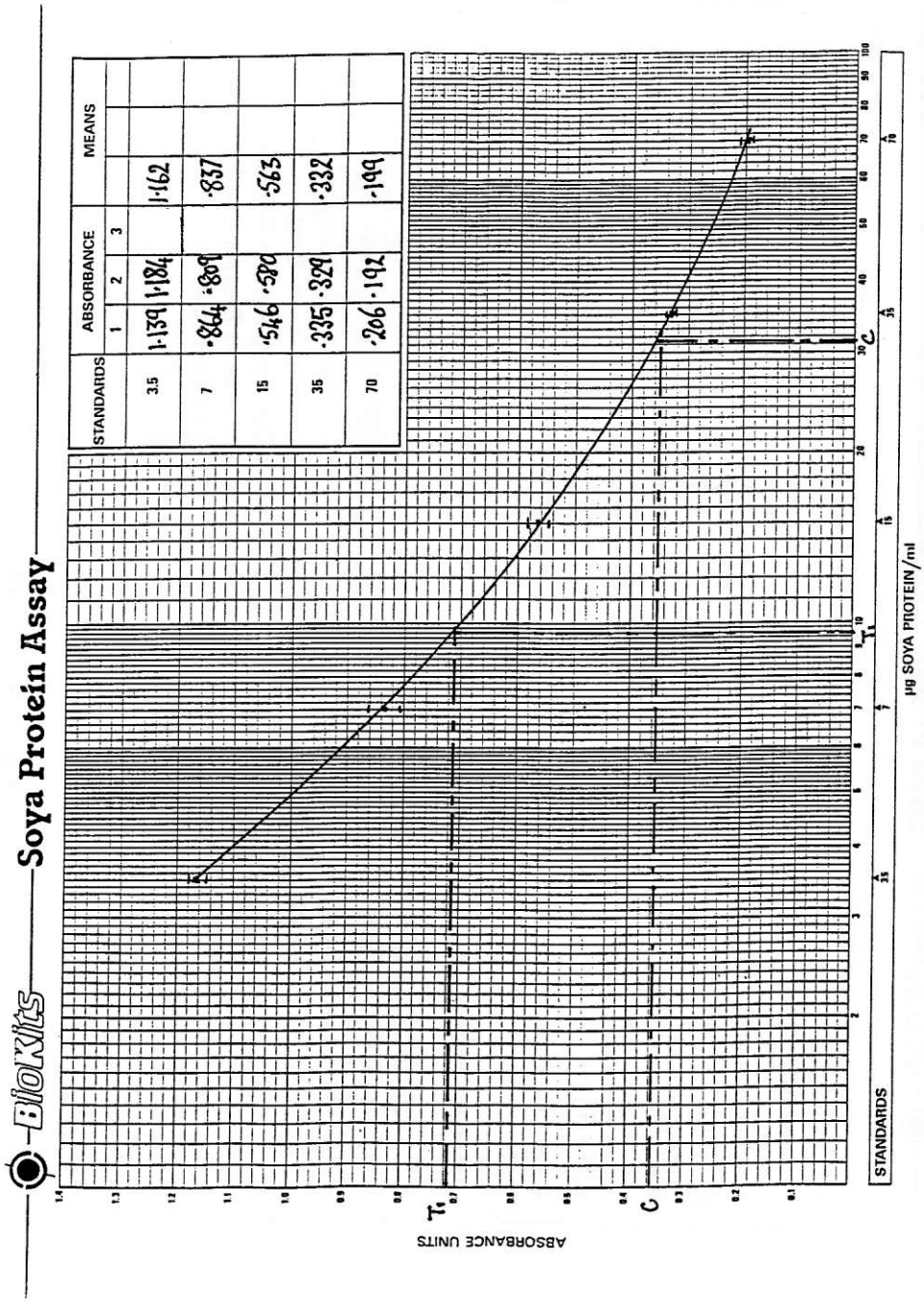


EXAMPLE ASSAY LAYOUTS

B	Substrate Blank
M	Maximum Binding
3.5-70	Soya Protein Standards
C	Soya Protein Control
T1-T38	Test Sample

Fig 2: Example Assay Layouts (Template)

Fig 3: Example Calibration Curve



J. Assoc. Publ. Analysts, 29, 49-55

MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

No. V 26

CRUDE FIBRE IN FLOURS

Correspondence on this method may be sent to R. Wood, Statutory Methods, Ministry of Agriculture, Fisheries and Food, Food ScienceLaboratory, Food Safety Directorate, Norwich Research Park, Colney, Norwich NR4 7UQ

1. Scope and Field of Application

The method allows the determination of crude fibre in bread and flour. It is applicable to samples that contain less than 2% of crude fibre, but higher contents can be accommodated.

2. Definition

Crude fibre content: the content of crude fibre as determined by the method specified.

3. Principle

The sample undergoes oxidative digestion, followed by oxidation of the residue in solution by potassium dichromate and determination of the excess dichromate by means of titration with thiosulphate.

4. Reagents

All reagents should be of recognised analytical grade unless specified otherwise. Wherever the use of water is required, distilled or water of equivalent purity is to be used.

4.1 Diethyl ether

4.2 Sulphuric acid, 96% *m/m*.

4.3 Scharrer-Kurschner reagent

Dissolve 25.0 g of trichloroacetic acid (**4.3.1**) and 62 ml of nitric acid (**4.3.2**) in acetic acid (**4.3.3**) and make up with the acetic acid (**4.3.3**) to 1 l.

4.3.1 Trichloroacetic acid

4.3.2 Nitric acid, 65% *m/m* (13.1 gram equivalent per kg).

4.3.3 Acetic acid, 70% *m/m* (11.7 gram equivalent per kg).

4.4 Potassium dichromate in sulphuric acid, approximately 0.033 mol/l. Dissolve 2.45 ± 0.05 g of potassium dichromate in 160 ± 5 ml of water and add 120 ± 5 ml of concentrated sulphuric acid (**4.2**).

4.4.1 Potassium dichromate

4.5 Potassium iodide solution, 1 mol/l. Dissolve 16.6 ± 0.5 g of potassium iodide in 100 ± 5 ml of water.

4.5.1 Potassium iodide

0004-5780/93 +7 $20.00

4.6 Sodium thiosulphate solution, approximately 0.1 mol/l, standardised with a precision of 0.5%.

4.6.1 Sodium thiosulphate

4.7 Starch indicator

Add a suspension of 5 g of soluble starch in 30 ml of water to 1 litre of boiling water, and continue boiling for 3 min; cool and add, if desired, 10 mg of mercury(II) iodide as a preservative.

4.7.1 Soluble starch

4.7.2 Mercury(II) iodide

5. Apparatus

5.1 Digestion assembly, illustrated in Fig. 1 and consisting of the following parts.

5.1.1 Pear-shaped acetylation flasks, 150 ml, with ground glass joint NS 24/29 female.

Note that if the sample contains less than 0.2% crude fibre, larger flasks are necessary to accommodate the volume of diethyl ether required for the extraction.

5.1.2 Air condenser, with ground glass joint NS 24/29 male.

5.1.3 Argand burner

5.2 Extraction assembly, illustrated in Fig. 2 and consisting of the following parts.

5.2.1 Erlenmeyer suction flask, 500 ml, with the bottom cut off and ground edge to fit onto a glass plate ground flat; or a Witt filter chamber (e.g. Jencons) or equivalent apparatus.

5.2.2 All-glass Buchner funnel, with sintered glass disc filter of pore diameter 15-40 µm (e.g. 17G3 Schott-Jena), overall diameter 65 mm; to fit suction flask (**5.2.1**).

5.2.3 Erlenmeyer receiving flasks, 100 ml.

5.3 Erlenmeyer suction flasks, 500 ml.

5.4 Erlenmeyer flasks, 1 l, with wide necks and ground glass stoppers.

6. Procedure

6.1 Sample preparation

Grind the sample so that it passes completely through a 1 mm sieve.

6.2 Digestion

Weigh, to the nearest 1 mg, an amount of sample that is expected to contain between 5 and 25 mg of crude fibre. Transfer to an acetylation flask (**5.1.1**). Add Scharrer-Kurschner reagent (**4.3**), 15 ml per g of test sample, but not less than 10 ml. Attach the air condenser (**5.1.2**). Bring the contents of the flask to the boil, and boil gently for 60 ± 1 min, swirling periodically. Cool to room temperature.

6.3 Extraction

Add diethyl ether, 1.5 times the volume of Scharrer-Kurschner reagent used, swirl for a few seconds and filter the top layer only through the Buchner funnel (**5.2.2**) into a suction flask (**5.3**). Repeat the extraction twice with the same volume of diethyl ether, and reject the filtrate. Add 25 ml of cold reagent (**4.3**) to the acetylation flask. Apply suction to the suction flask and pour the entire contents of the acetylation flask directly onto the frit of the Buchner funnel. Transfer and wash the collected residue with cold reagent (**4.3**) until the filtrate no longer shows cloudiness on the addition of water; usually washing 3 times with 10 ml is sufficient. Remove the filtrate from the suction flask. Place the Buchner funnel on it again, wash with hot water until acid-free to litmus; usually 3-5 times is sufficient.

6.4 Dissolution

Pipette 25 ml of potassium dichromate solution (**4.4**) into an Erlenmeyer receiving flask (**5.2.3**) and place it in the modified suction flask (**5.2.1**). Fit the Buchner funnel with the washed residue after extraction to the suction flask (**Fig. 2**). Place 5 ml of sulphuric acid (**4.2**) on the filter and stir until the residue has dissolved completely. Draw off the acid solution into the receiving flask by suction. Wash five more times with 5 ml of sulphuric acid, each portion always being completely transferred before the next portion is added.

6.5 Oxidation

Take the Erlenmeyer receiving flask with the solution of crude fibre in dichromate-sulphuric acid out of the suction flask. Place it in a boiling water bath for 10 min.

6.6 Titration

After cooling, wash the contents of the Erlenmeyer receiving flask with 500 ml of water into an Erlenmeyer flask (**5.4**). Cool again; then pipette, while swirling, 10 ml of potassium iodide solution (**4.5**) into the flask and close it. After standing it for 10 min in the dark, titrate with sodium thiosulphate solution (**4.6**), using starch (**4.7**) as indicator.

6.7 Blank titration

Carry out a blank titration with 25 ml of potassium dichromate, 50 ml of water, 30 ml of sulphuric acid and 10 ml of potassium iodide.

7. COSHH

Analysts are reminded that appropriate hazard and risk assessments required by the Control of Substances Hazardous to Health Regulations, 1988 (See "Control of Substances Hazardous to Health - Approved Code of Practice, Control of Substances Hazardous to Health Regulations, 1988") must be made before using this method.

8. Expression of Results

The content of crude fibre, calculated as a percentage by mass of the prepared sample on a dry basis, is given by:

$$\% \text{ crude fibre} = 0.686 \times \frac{(V_2 - V_1) \times T}{m} \times \frac{100}{100 - H}$$

Where:

V_1 is the volume, in ml, of thiosulphate solution used;

V_2 is the volume, in ml, of thiosulphate solution in the blank titrations;

T is the concentration, in mol/l, of the thiosulphate solution used;

m is the mass, in g, of the test sample;

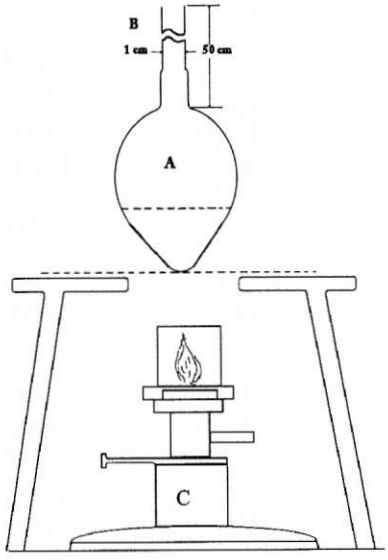
H is the moisture content, in g per 100 g of the sample.

9. References

9.1 RB Player and R Wood, J. Assoc. Publ. Analysts, 1980, 18, 29-40.

9.2 Ministry of Agriculture, Fisheries and Food, Food Safety Directorate, MAFF Validated Methods for the Analysis of Food, Introduction, General Considerations and Analytical Quality Control, J. Assoc. Publ. Analysts, 1992, **28**, 11-16.

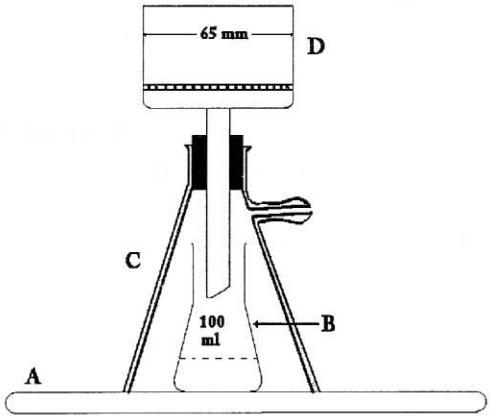
Fig. 1



Digestion assembly:

A, acetylation flask; B, air condenser; C, Argand burner.

Fig. 2



Filter flask arrangement:

A, glass plate; B, receiving flask; C, suction flask; D, Buchner funnel.

APPENDIX 1

Analytical Quality Control

General principles of analytical quality control are outlined in protocol V.0 of the series[2].

A1 Repeatability

The absolute difference between two test results obtained under repeatability conditions should not be greater than the repeatability, r, deduced from the collaborative trial data summarised below (Table 1). At levels lower than 0.5 g of crude fibre per 100 g of dry sample, r may be taken as 0.05 g per 100 g; at higher levels, r would be expected to increase to over 0.2 g per 100 g and may be taken to be 10% of the observed level. This would correspond to a relative standard deviation of repeatability (coefficient of variance of repeatability), RSD_r, of 4% at the higher levels.

A2 Reproducibility

The absolute difference between two test results obtained under reproducibility conditions should not be greater than the reproducibility, R, deduced from the collaborative trial data summarised below (Table 1). At levels lower than 0.7 g of crude fibre per 100 g of dry sample, R may be taken as 0.25 g per 100 g; at higher levels, R would be expected to increase to over 0.6 g per 100 g and may be taken to be 36% of the observed level. This would correspond to a relative standard deviation of reproducibility (coefficient of variance of reproducibility), RSD_R, of 13% at the higher level.

A3 Trueness (Bias)

The collaborative trial established satisfactory precision parameters for the method; a partial check on the precision is possible as Samples C and D were prepared from fixed mixtures of the two single flours represented by Samples A and B. When the fibre contents of the former are calculated from the observed fibre contents of the latter, and expressed in g of crude fibre per 100 g of dry sample, the following comparisons can be made:

Sample C: calculated, 1.69; observed, 1.69

Sample D: calculated, 1.48; observed, 1.41.

The agreement is good, and the difference between the calculated and observed values for sample D is no higher than expected from the observed precision.

The accuracy of the method was not tested by spiking with known amounts of fibre. However, there is no reason to suspect systematic bias.

A4 Limit of Detection

This limit has not been established, but the collaborative trial data suggests an accuracy which, if maintained, corresponds to an extrapolated lower limit of roughly 0.05 g of crude fibre per 100 g of dry sample, for a single determination.

A5 Statistical Data Derived from the Results of Interlaboratory Tests

Participants in the collaborative trial each received four samples on which he was required to carry out duplicate determinations of:

moisture content, by drying at 103 ± 2°C to constant weight;

crude fibre content, by the method above (V26);

crude fibre content, by two other methods, which are not reported here.

The composition of the samples were:

A: 100% wholewheat stone-ground flour;

B: plain white flour;

C: a homogenised mixture of A and B in the ratio of 7:2,

D: a homogenised mixture of A and B in the ratio of 2:1.

Table 1 summarises the statistical data; the levels of analyte are expressed as g of crude fibre per 100 g of sample on a dry weight basis.

TABLE 1

Statistical Analysis of Crude Fibre (g/100g) in Flour

Sample	A	B	C	D
Number of Laboratories retained after eliminating outliers	16	16	16	16
Number of Laboratories eliminated as outliers	2	2	2	2
Number of results accepted after eliminating outliers	30	32	32	32
LEVEL OF ANALYTE				
Mean observed value $\bar{x}$	2.10	0.23	1.69	1.41
REPEATABILITY				
Standard Deviation S_r	0.072	0.016	0.094	0.068
Relative Standard Deviation RSD_r(%)	3.4	7.1	5.6	4.8
Repeatability r [2.8 × S_r]	0.202	0.046	0.264	0.190
REPRODUCIBILITY				
Standard Deviation S_R	0.236	0.082	0.213	0.148
Relative Standard Deviation RSD_R(%)	11	36	13	11
Reproducibility R [2.8 × S_R]	0.662	0.231	0.597	0.415

A6 Key to Table 1

Symbol	Definition
$\bar{x}$	Overall mean value
S_r	The standard deviation of repeatability
RSD_r	The relative standard deviation of repeatability, expressed as a percentage of the mean (coefficient of variance of repeatability CV_r)
r	Repeatability
S_R	The standard deviation of reproducibility
RSD_R	The relative standard deviation of reproducibility, expressed as a percentage of the mean (coefficient of variance of reproducibility CV_R)
R	Reproducibility