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Methods of Examination for Eggs and Egg Products: Collaborative Trial

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Five British Standard methods as prescribed in the Egg Products Regulations 1993 were subjected to collaborative trial in order to assess their performance with egg products and where possible to determine precision characteristics. The methods investigated were:-

BS 5763 Part 1, 1991. Enumeration of micro-organisms - colony count at 30°C (pour plate technique)

BS 5763 Part 5, 1981. Enumeration of micro-organisms - colony count at 30°C (surface plate method)

BS 5763 Part 4, 1990. Detection of Salmonella.

BS 5763 Part 7, 1983. Enumeration of Staphylococcus aureus *by colony count technique*

BS 5763 Part 10, 1986. Enumeration of Enterobacteriaceae (colony count method)

One other BS method for the detection of very low numbers of Staphylococcus aureus *was also included in the trial:*

BS 4285 Part 3, Microbiological examination for dairy purposes, Sub-Section 3.10.2 Detection of Staphylococcus aureus.

For the enumeration of colony count at 30°C, the surface plate technique was generally more precise than the pour plate technique. However, for both colony count techniques, precision was greatest with lower count samples (ca $\log_{10}$ 3.0) than with higher count samples (ca $\log_{10}$ 6.0 - 7.0). Repeatability and reproducibility were at best $\log_{10}$ 0.24 and 0.42 for the surface plate count and $\log_{10}$ 0.38 and 0.66 for the pour plate count respectively.

For the qualitative tests, 16% false positive and 3% false negative results were reported for the method for the detection of Salmonella. *A total of 9% false positive and 16% false negative results were reported for the colony count method for the detection of* Staphylococcus aureus *and 22% total false positive and 11% false negative results were reported for the liquid enrichment method. The high level of false positive and false negative results for this organism is a cause for concern and requires further investigation.*

The organisation and results from the methods assessed in this collaborative trial are reported.

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Introduction

On the 14th July 1993 The Egg Products Regulations 1993[1] came into force. These Regulations revoke The Liquid Egg (Pasteurisation) Regulations 1963[2] and the Liquid Egg (Pasteurisation) (Scotland) Regulations 1963[3] and implement, in part, Council Directive 89/437/EEC[4] as amended by Council Directive 89/662/EEC[5] and Council Directive 91/684/EEC [6].

The Regulations make provision in the UK for the preparation and manufacture of egg products used in food intended for sale for human consumption, including the process of pasteurisation and prohibit the manufacture of egg products other than in an approved establishment.

The Regulations require egg products sold or used in the preparation of foods to comply with specified requirements as to heat treatment, sampling, storage and transport. Several microbiological criteria are prescribed in Schedule 4 of the Regulations; these state that "for each batch the sample of egg products which is tested shall comply with the following microbiological criteria:-

a) Salmonellae: absence in 25 g or 25 ml of egg products;

b) mesophilic aerobic bacteria: M = 10^5 in 1 g or 1 ml;

c) Enterobacteriaceae: M = 10^2 in 1 g or 1 ml;

d) *Staphylococcus aureus*: absence in 1 g of egg products.

Where M = maximum value for the number of bacteria; the result is considered unsatisfactory if the number of bacteria in one or more sample units is M or more."

Methods of examination are also prescribed in the Regulations which enforcement laboratories are required to use in order to assess compliance with these microbiological criteria. British Standard (BS) methods are prescribed in Schedule 4 Parts II to V. However, although BS methods are considered as reference methods, no performance characteristics are available for any of the methods prescribed in the Regulations.

The MAFF Food Science Laboratory, as part of its on-going methods validation programme, organised a collaborative trial of the 5 BS methods prescribed in the Regulations in order to assess their performance and determine precision characteristics for the quantitative methods. Additionally, one other method for the detection of low numbers of *Staphylococcus aureus* in foods was also included in the trial. The organisation and results of this trial are reported.

Collaborative Trial Organisation

Participants

Twenty laboratories participated in the trial comprising 9 Public Analyst laboratories, 9 Public Health Laboratories, the MAFF Food Science Laboratory and 1 consultant laboratory.

Sample preparation

Production of the egg reference materials used in the trial was carried out by the British Food Manufacturing Industries Research Association, Leatherhead, Surrey.

Preliminary studies

Cartons of frozen pasteurised liquid whole egg were supplied by a commercial manufacturer. Prior to use, individual cartons were thawed at 5°C for 18-24 h. The microbiological content of both freshly thawed and aged egg was investigated. Three freshly thawed cartons were tested individually to determine the total aerobic colony count at 25°C and 37°C and, additionally, for the total staphylococci count, *Staphylococcus aureus* count and detection, total Salmonellae count and detection and total Enterobacteriaceae count; these results are shown in Table 1. One of these cartons was then aged at 12°C for 72 h and examined again for the tests described above; the results obtained are shown in Table 2.

Table 1

Typical analysis of freshly thawed egg (cfu/g)

Test procedure	Sample 1	Sample 2	Sample 1 (Detection)	Sample 2 (Detection)
MAC*/ 25°C	3.2×10^3	4.3×10^3		
MAC / 37°C	3.6×10^2	2.3×10^2		
Total *Staph.* count	$<5.0 \times 10^1$	$<5.0 \times 10^1$		
Staph. aureus count	$<5.0 \times 10^1$	$<5.0 \times 10^1$		
Staph. aureus detection			negative	negative
Salmonella detection			negative	negative
Total Enterobacteriaceae count	$<5.0 \times 10^0$	$<5.0 \times 10^0$		

* MAC - mesophilic aerobic count

Table 2
Typical analysis for aged (72 h at 5°C) egg

Test procedure	Colony count cfu/g	Detection
MAC */ 25°C	$< 3.0 \times 10^5$	
MAC / 37°C	3.8×10^7	
Total *Staph.* count	1.0×10^2	
Staph. aureus count	$< 5.0 \times 10^1$	
Staph. aureus detection		negative
Salmonella detection		negative
Total Enterobacteriaceae count	1.6×10^6	

* MAC - mesophilic aerobic count

Inoculum stability

The organisms used in this trial for sample preparation are shown in Appendix I. Individual samples (50 g) of freshly thawed egg were inoculated with *Salmonella enteritidis* P167807, *Staphylococcus aureus, Staph. epidermidis and Staph. saprophyticus* at a level of *ca* 10^4 cfu/g. The samples were stored at 5°C and enumerated after 0, 3, 6 and 10 days on appropriate selective agars. Uninoculated samples were also enumerated to detect any growth of naturally occurring *Staph. aureus* and *Salmonella* spp. The results obtained are shown in Table 3 below.

Table 3
Inoculum stability (cfu/g) at 5°C

Sampling day	*S. enteritidis*[1]	*Staph. aureus*[2]	*Staph.*spp. (non-*Staph. aureus*)[3]
0	1.1×10^4	1.0×10^4	1.1×10^4
3	6.7×10^3	1.3×10^4	2.6×10^4
6	6.5×10^3	1.3×10^4	1.3×10^4
10	4.8×10^3	1.3×10^3	5.0×10^3

1. Enumerated on xylose lysine desoxycholate agar;
2. Enumerated on Baird Parker agar;
3. Enumerated on Kranep agar.

Note: In the uninoculated samples, no Staph. aureus or Salmonella spp. were detected.

Stability of the test organisms at 1°C

Samples (50 g) of freshly thawed egg were inoculated with dilutions of an Enterobacteriaceae cocktail, Salmonellae cocktail, *Staph. aureus* and *Staphylococcus* spp. (non *Staph. aureus*) at a level of 10^4 cfu/g. An uninoculated egg sample was used to investigate the stability of the total aerobic count. The samples were stored at 1°C and using appropriate selective media, enumeration of the samples was carried out after 0, 2 and 5 days. The uninoculated samples were also used to test for naturally occurring Enterobacteriaceae, Salmonellae and *Staph. aureus*. The results obtained are shown in Table 4 below.

Table 4
Inoculum stability at 1°C

Sample day	MAC/25°C	MAC/37°C	*Staphylo-coccus* spp.	*Staph. aureus*	Enterob. cocktail**	*Salmonella* cocktail
0	1.6×10^3	4.0×10^2	1.7×10^4	6.9×10^3	8.5×10^3	1.4×10^4
2	4.2×10^3	1.9×10^3	5.0×10^3	2.8×10^3	4.3×10^3	5.8×10^3
5	nd	1.2×10^3	4.8×10^3	7.1×10^3	2.0×10^3	3.8×10^3

* mesophilic aerobic count;

** Enterobacteriaceae;

nd - not determined

Temperature stability for dispatch of samples

To monitor the temperature range expected during the dispatch of samples to participants, disposable plastic universals (Bibby Sterilin Ltd.) were filled with water and packed as applicable to the trial samples. Water blanks were stored at 1°C for *ca* 18 h and then packed to simulate trial samples. Samples were packed in moulded polystyrene cool boxes (WK. Thomas & Co., Surrey) holding four frozen ice packs. Vermiculite (Fisons Scientific Equip., Leics.) was added to provide extra insulation. The temperature of the water blanks was measured again after a further 18 h at ambient temperature (20 - 23°C)

Pre-trial samples

Volumes of egg (*ca* 5 g) were dispensed into plastic universal bottles prior to inoculation with the respective test organism(s). Each test sample was prepared in duplicate. Once inoculated, a further 5 g of egg was added to each universal to produce the following samples:-

2×10 g Enterobacteriaceae negative
2×10 g Enterobacteriaceae, 10^2 cfu/g
2×10 g Enterobacteriaceae, 10^3 cfu/g

2×25 g *Salmonella* negative
2×25 g *Salmonella*, 10^1 cfu/g

2×10 g *Staph. aureus* negative for plate count procedure
2×10 g *Staph. aureus,* 10^1 cfu/g for plate count procedure
2×10 g *Staphylococcus* spp. (non *Staph. aureus*), 10^1 cfu/g for plate count procedure
2×10 g *Staph. aureus* negative for detection procedure
2×10 g *Staphylococcus spp.*(including *Staph. aureus*) 10^1 cfu/g for detection procedure
2×10 g *Staphylococcus spp.* (non-*Staph aureus*)10^1cfu/g for detection procedure

Freshly thawed egg was satisfactory for providing the low level of contamination for the mesophilic aerobic count (MAC) samples (10^3 cfu/g). Adjustment for the higher level of contamination (10^7 cfu/g) was achieved by ageing a carton of thawed egg at 12°C for 72 h. A dilution of this sample (1 in 100) with freshly thawed egg produced the material for the intermediate level MAC sample (10^5 cfu/g). As 2 methods for the enumeration of MAC were being collaboratively tested, the final number of MAC samples were as follows:-

2×15 g MAC low (10^3 cfu/g) : pour plate technique
2×15 g MAC intermediate (10^5 cfu/g) : pour plate technique
2×15 g MAC high (10^7 cfu/g) : pour plate technique
2×15 g MAC low (10^3 cfu/g) : surface plate technique
2×15 g MAC intermediate (10^5 cfu/g) : surface plate technique
2×15 g MAC high (10^7 cfu/g) : surface plate technique

All samples were stored at 1°C for 18 - 24 h before packing, together with 2×10 ml volumes of water for temperature measurement by participants on arrival of the samples.

Trial samples

Trial samples as above were prepared 72 h in advance of the trial and stored at 1°C until packing and distribution. The MAC samples were prepared, as previously described, immediately prior to packing. A single 10 ml water sample was provided for temperature measurement.

Table 5
Pre-trial results

Test	Desired inoculum level	Colony count (cfu/g)	Detection result
Enterobacteriaceae	uninoculated a	$<0.5 \times 10^0$	
	uninoculated b	$<0.5 \times 10^0$	
	10^2 a	2.1×10^1	
	10^2 b	2.1×10^1	
	10^3 a	1.7×10^3	
	10^3 b	1.3×10^3	
MAC 30°C (surface count)	10^3 a	3.4×10^4	
	10^3 b	3.6×10^4	
	10^5 a	7.8×10^6	
	10^5 b	7.3×10^6	
	10^7 a	3.0×10^8	
	10^7 b	3.0×10^8	
MAC 30°C (pour plate count)	10^3 a	1.9×10^4	
	10^3 b	2.2×10^4	
	10^5 a	4.9×10^6	
	10^5 b	3.9×10^6	
	10^7 a	2.8×10^8	
	10^7 b	2.6×10^8	
Staph. spp. count	uninoculated a	$<1.0 \times 10^1$	
	uninoculated b	$<1.0 \times 10^1$	
Staph. spp. detection	uninoculated a		negative
	uninoculated b		negative
Staph. spp. incl. *Staph. aureus* count	10^1 a	5.0×10^0	
	10^1 b	2.0×10^0	
Staph. spp. incl. *Staph. aureus* detection	10^1 a		positive
	10^1 b		positive*
Staph. spp. (non *Staph. aureus*) count	10^1 a	$<5.0 \times 10^0$	
	10^1 b	$<5.0 \times 10^0$	
Staph. spp. (non *Staph. aureus*) detection	10^1 a		negative
	10^1 b		negative**
Salmonella detection	uninoculated a		negative
	uninoculated b		negative
	10^1 a		positive
	10^1 b		positive

* The detection procedure for *Staphylococcus* recovered both *Staph. aureus* and *Staph.* spp.
** The detection of *Staph. aureus* was negative although other *Staph.* spp. were isolated.
Note: The temperature of the 2 water samples was recorded as 3.4 and 3.9°C.

Methods of Examination being Collaboratively Tested

Laboratory protocols

The protocols participants were required to use were dispatched in advance of the trial to allow time for familiarisation with the methods. A total of six methods were collaboratively tested. Five of the methods were as prescribed in the Regulations and one other BS method for the detection of low numbers of *Staphylococcus aureus* was also included. The methods assessed were as follows:-

BS 5763 Part 1, 1991. Methods for the microbiological examination of food and animal feeding stuffs. Enumeration of micro-organisms - colony count at 30°C (pour plate technique).[7]

BS 5763 Part 5, 1981. Methods for the microbiological examination of food and animal feeding stuffs. Enumeration of micro-organisms - colony count at 30°C (surface plate method).[8]

BS 5763 Part 4, 1990. Methods for the microbiological examination of food and animal feeding stuffs. Detection of Salmonella.[9]

BS 5763 Part 7, 1983. Methods for the microbiological examination of food and animal feeding stuffs. Enumeration of *Staphylococcus aureus* by colony count technique.[10]

BS 4285 Part 3, Microbiological examination for dairy purposes. Methods for Detection and/or enumeration of specific groups of micro-organisms. Section 3.10 *Staphylococcus aureus.* Sub-section 3.10.2 Detection.[11]

BS 5763 Part 10, 1986. Methods for the microbiological examination of food and animal feeding stuffs. Enumeration of Enterobacteriaceae (colony count method).[12]

Results and Discussion

Data was returned by all but 2 of the participating laboratories (Labs. 9 & 11). All quantitative data as returned by participants were converted to a $\log_{10}$ basis so that normal statistical procedures could be applied.

The converted results are given in Tables 6, 7 and 11.

The results for the qualitative tests for *Salmonella* and *Staph. aureus* are given in Tables 8 - 10.

The results from the quantitative tests were examined for individual systematic error using Cochran's and Grubb's tests (at $P<0.05$) progressively by procedures described by Horwitz (1988).[13] Calculations for repeatability (r) and reproducibility (R) were carried out on those results remaining after the removal of outliers. A summary of the data from the quantitative and qualitative tests is given in Tables 12 and 13 respectively.

Table 6
Log_{10} Colony count at 30°C
(pour plate technique)

Laboratory code	Sample Code 1		2		3	
1	7.32	7.32	3.57	3.45	5.20	4.70
2	6.26	5.79	3.61	3.48	4.58	4.28
3	8.45	7.82	3.38	3.46	5.78	5.82
4	6.38	7.64	3.41	3.40	6.04	5.38
5	7.04	7.49	3.46	3.38	5.83	5.86
6	7.98	7.88	3.45	3.48	6.20	6.04
7	8.08	7.57	3.76	3.61	6.08	5.66
8	8.48	7.85	3.86	4.15	7.18	6.86
10	8.08	8.04	3.80	3.81	6.43	6.32
12	8.59	8.51	3.79	3.57	6.43	7.26
13	7.96	8.11	3.97	3.88	6.63	6.60
14	7.34	7.38	4.66	3.73[(a)]	5.96	5.49
15	7.41	7.93	4.11	3.75	5.76	5.97
16	7.30	7.11	3.70	3.78	5.80	5.62
17	7.79	7.65	3.52	3.46	5.83	5.48
18	7.40	7.23	3.54	4.08	6.04	6.00
19	6.52	6.93	3.28	3.32	5.20	5.23
20	7.00	7.36	3.45	3.46	4.86	5.00
21	6.26	6.20	2.60	2.53[(a)]	4.45	4.43
BFMIRA[#]	7.48	7.65	3.71	3.62	5.43	6.06
mean	7.46		3.62		5.74	
SD_r	0.31		0.14		0.24	
RSD_r%	4.22		3.79		4.21	
r	0.88		0.38		0.68	
SD_R	0.70		0.24		0.74	
RSD_R%	9.34		6.52		12.84	
R	1.95		0.66		2.07	

(a) denotes outlier result not used in statistical calculations
Note: Laboratories 9 and 11 did not return any results for the trial.
[#] BFMIRA results obtained on day of trial (not used in statistical calculations)

Table 7
Log_{10} Colony count at 30°C
(surface plate technique)

Laboratory code	Sample Code 1		2		3	
1	7.28	7.41	3.53	3.56	5.63	5.60
2	7.04	7.86	3.58	3.52	5.93	5.56
3	7.85	8.00	3.59	3.57	6.23	6.32
4	7.90	7.77	3.57	3.56	5.89	6.41
5	7.49	7.85	3.74	3.92	6.20	6.34
6	11.51	11.60[(a)]	3.65	3.85	11.80	11.61[(a)]
7	6.91	6.79	3.71	3.64	5.41	5.75
8	8.61	8.08	3.78	3.87	7.52	7.59[(a)]
10	8.15	8.15	4.79	3.93[(a)]	6.43	6.46
12	8.76	>9.48[(b)]	5.41	5.46[(a)]	>9.48	8.11[(b)]
13	7.62	8.00	3.71	3.65	6.38	6.36
14	8.36	8.51	3.87	3.65	6.49	6.81
15	7.90	7.83	3.60	3.73	6.28	5.40
16	7.26	7.30	3.81	3.58	6.15	6.15
17	7.75	7.82	3.67	3.70	6.25	6.34
18	7.68	7.80	3.66	3.57	6.36	6.38
19	7.81	7.46	3.62	3.56	6.18	6.20
20	7.48	7.38	3.32	3.18	6.00	5.88
21	nr[(c)]	7.36	nr	3.57	6.18	nr
BFMIRA[#]	7.64	7.57	3.61	3.67	6.03	6.31
mean	7.72		3.64		6.13	
SD_r	0.21		0.09		0.22	
RSD_r%	2.78		2.40		3.60	
r	0.60		0.24		0.62	
SD_R	0.43		0.15		0.35	
RSD_R%	5.58		4.16		5.74	
R	1.21		0.42		0.98	

(a) denotes outlying result not used in statistical calculations
(b) denotes single usable result not used in statistical calculations
nr no result reported
[#] BFMIRA results obtained on day of trial (not used in statistical calculations)

Table 8
Detection of *Salmonella*

Laboratory code	Sample Code*	
	1	2
1	+/+	-/-
2	+/+	+/-
3	+/+	-/-
4	+/+	+/-
5	+/+	+/+
6	+/+	-/-
7	+/+	-/-
8	+/+	-/-
10	+/+	-/-
12	I/+	+/I[(d)]
13	+/+	-/-
14	+/+	-/-
15	+/+	-/+
16	+/+	-/-
17	+/+	-/-
18	+/+	-/-
19	+/+	-/-
20	+/+	-/-
21	+/+	-/-
BFMIRA[#]	+/+	-/-

*Sample 1 was positive for *Salmonella*; Sample 2 was negative for *Salmonella*
+ denotes S*almonella* present; - denotes S*almonella* not detected
(d) I denotes S*almonella* could not be confirmed therefore result inconclusive
[#] BFMIRA results obtained on day of trial (not used in statistical calculations)

Table 9
Detection of *Staphylococcus aureus*
(colony count technique)

Laboratory code	Sample Code* 1	2	3
1	-/-	+/+	-/-
2	-/-	+/+	-/-
3	-/-	+/+	-/-
4	-/+	+/+	-/+
5	-/-	+/-	+/-
6	-/-	+/+	-/+
7	-/-	-/+	-/-
8	+/-	+/+	+/-
10	-/-	+/+	-/-
12	-/-	-/-	-/-
13	-/-	+/+	-/-
14	-/-	-/+	-/-
15	-/-	-/+	-/-
16	-/-	+/+	-/-
17	-/-	+/+	-/-
18	-/+	+/+	-/-
19	-/-	+/+	-/-
20	-/-	+/+	-/-
21	-/-	+/+	-/-
BFMIRA[#]	-/-	+/+	-/-

* Sample 1 was negative for *Staphylococcus aureus*; Sample 2 was positive for *Staphylococcus aureus*; Sample 3 contained *Staphylococcus* spp. (non *Staphylococcus aureus*) i.e. negative

[#] BFMIRA results obtained on day of trial (not used in statistical calculations)

Table 10
Detection of *Staphylococcus aureus* (Liquid enrichment technique)

Laboratory code	Sample Code* 1	2	3
1	-/-	+/+	-/-
2	-/-	+/+	-/-
3	-/-	-/+	-/-
4	+/+	+/+	+/+
5	-/-	+/+	+/-
6	-/-	+/+	-/-
7	-/+	+/+	+/+
8	-/-	+/+	+/+
10	-/-	+/+	-/-
12	-/nr	-/+	-/nr
13	-/-	+/+	-/-
14	-/-	-/+	-/-
15	-/nr	+/+	-/-
16	-/-	+/+	-/-
17	-/-	+/+	-/-
18	-/+	+/+	+/-
19	-/-	+/+	-/+
20	+/-	-/+	+/+
21	-/-	+/+	-/-
BFMIRA[#]	-/-	+/+	-/-

* Sample 1 was negative for *Staphylococcus aureus*; Sample 2 was positive for *Staphylococcus aureus*; Sample 3 contained *Staphylococcus* spp. (non *Staphylococcus aureus* i.e. negative ; nr no result returned

[#] BFMIRA results obtained on day of trial (not used in statistical calculations)

Table 11

Log_{10} Colony count of Enterobacteriaceae

Laboratory code	Sample Code 1		2		3	
1	nd	nd	1.60	1.48	2.53	2.45
2	1.95	1.48	nr	1.74	2.42	2.64
3	1.60	2.23	nr	1.54	2.18	2.20
4	2.65	2.48	2.08	1.98	2.65	2.60
5	nd	2.00	1.65	1.70	2.75	2.81
6	3.08	2.60	2.18	2.11	2.60	2.68
7	1.30	1.40	1.70	1.85	2.68	2.60
8	nd	nd	1.34	1.60	2.65	2.45
10	1.88	1.98	1.76	1.63	2.48	2.43
12	(e)					
13	1.90	3.08	1.93	2.00	2.66	2.68
14	(e)					
15	nr	2.58	2.11	2.41	2.65	2.64
16	nd	1.30	2.08	1.60	1.93	2.45
17	nd	1.85	1.40	1.85	2.38	2.45
18	1.88	1.30	1.18	1.48	2.20	1.93
19	nd	nd	1.60	1.60	2.71	2.76
20	nd	1.00	1.48	1.54	2.30	2.34
21	nr	1.74	1.74	1.54	1.98	2.40
BFMIRA[#]	1.60	1.00	-		-	

***Note** : Sample 1 was uninoculated with the Enterobacteriaceae cocktail but was subsequently found to be contaminated with naturally-occurring Enterobacteriaceae; (See discussion of results).*

nr denotes no result returned; nd not detected;

(e) laboratories 12 & 14 deviated significantly from the protocol and therefore data not recorded.

[#] BFMIRA results obtained on day of trial (not used in statistical calculations)

Table 12
Summary of precision characteristics for quantitative BS methods (Log_{10})

Method	Type	Mean	S_r	RSD_r %	r	S_R	RSD_R %	R
BS 5763 Part1	Colony count/30°C (pour plate)							
		3.62	0.14	3.79	0.38	0.24	6.52	0.66
		5.74	0.24	4.21	0.68	0.74	12.84	2.07
		7.46	0.31	4.22	0.88	0.70	9.34	1.95
BS5763 Part 5	Colony count/30°C (surface plate)							
		3.64	0.09	2.40	0.24	0.15	4.16	0.42
		6.13	0.22	3.60	0.62	0.35	5.74	0.98
		7.72	0.21	2.78	0.60	0.43	5.58	1.21
BS5763 Part 10	Enumeration of Enterobacteriaceae							
		nc*	nc	nc	nc	nc	nc	nc

* nc - not calculable

Table 13
Summary of performance of qualitative methods

Method	Negative samples* No. tests/+ve (%)	Negative samples# No. tests/+ve (%)	Positive samples No. tests/+ve (%)
Detection of *Salmonella*	38 / 6 (15.8)	na	38 / 37 (97.4)
Detection of *S. aureus* (1)	38 / 3 (7.9)	38 / 4 (10.5)	38 / 32 (84.2)
Detection of *S. aureus* (2)	36 / 5 (13.9)	37 / 11(29.7)	38 / 34 (89.5)

* - Sample negative for test organism;

\# - Sample negative for S. *aureus* but other *Staphylococcus* spp. present

na - not applicable

(1) colony count method

(2) liquid enrichment method

Table 14
Key to Tables 6,7 and 11

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 materia under the same conditions may be expected to lie within a 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 laboratory variation). The value below which the absolute difference between two single test results obtained with the same method on identical test material under different conditions may be expected to lie within a 95% probability
SD_R	The relative standard deviation of the reproducibility
$RSD_R\%$	The relative standard deviation of the reproducibility $SD_R \times 100/x$

BS5763 Part 1 1991
Colony count technique at 30°C (pour plate technique)

This method requires the preparation of 2 poured plates using a non-selective agar (plate count agar) and a specified volume of the test sample (1 ml). Dilutions of the test sample are also examined as necessary. Plates are incubated aerobically at 30°C for 72 h. The number of micro-organisms per ml/g of the samples is calculated from the number of colonies obtained on selected plates.

For the trial, 3 samples containing varying levels of bacteria were examined (mean cfu/g $\log_{10}$ 3.62; 5.74 and 7.46, Table 6). In practice, no laboratories reported any major difficulties with this test except that the undiluted egg was difficult to mix fully with the molten medium. The greatest precision was obtained with the lowest count sample i.e. $\log_{10}$ 3.62, where r = $\log_{10}$ 0.38 (S_r 0.14) and R = $\log_{10}$ 0.66 (S_R 0.24). The repeatability of the method deteriorated as the colony count increased towards $\log_{10}$ 7.5 (Table 12) but there was little observed difference in reproducibility between the two higher count samples. This deterioration in precision was probably due predominantly to increased error associated with the preparation of greater numbers of dilutions and less so with counting errors associated with crowded plates[16]. The precision of the pour plate technique was poorer than for the surface count method and the r and R values were worse than those obtained with heat-treated milk where for samples with a mean of *ca* $\log_{10}$ 3.10, r = $\log_{10}$ 0.16 and R = $\log_{10}$ 0.18 [14].

BS 5763 Part 5, 1981.

Colony count at 30°C (surface plate method)

This method differs from the pour plate technique described above in that the test sample is spread onto the surface of the agar as opposed to mixing with the agar medium in the petri dish. For this reason a smaller inoculum is used for examination (0.1 ml) and the sensitivity of this method is therefore decreased. The incubation conditions and counting medium are otherwise as for the pour plate technique.

The precision of this method was generally better than for the pour plate technique; however better precision was observed again with the low count sample (mean $\log_{10}$ 3.64) where r = $\log_{10}$ 0.24 (S_r 0.09) and R = $\log_{10}$ 0.42 (S_R0.15). Little difference was obtained for repeatability and reproducibility for sample means of $\log_{10}$ 6.13 and 7.72 (Table 7). In comparison, the precision of the surface plate method obtained with liquid egg was better than that obtained with natural mineral waters.[15]

Some participants reported that the spread plates were more difficult to count because of problems associated with spreading colonies which could mask other colony types on the plate. It was also noted that the egg inoculum tended to soak very rapidly into the agar and so in order to spread it sufficiently to achieve discrete colonies it should be spread immediately after inoculation.

BS 5763 Part 4, 1990.

Detection of *Salmonella*.

This method for the detection of *Salmonella* spp. necessitates four stages; pre-enrichment in a non-selective broth (buffered peptone water), enrichment in two selective media (Rappaport-Vassiliadis and selenite cystine), plating out onto two selective agar media (brilliant green phenol red agar and xylose lysine desoxycholate agar) and confirmation using biochemical tests and serology. The method is qualitative and participants were required to demonstrate the presence or absence of *Salmonella* in 2 samples (positive and negative).

No false negative results were reported with this method; however, one laboratory was unable to confirm the presence of *Salmonella* in one of the duplicates for the positive sample and therefore reported their result as inconclusive. In contrast, a total of 6 false positive results (16%) were reported by 5 laboratories (4 laboratories identified *Salmonella* in only one duplicate). Laboratory 12 again reported an inconclusive result for one of the negative samples.

Participants reported some difficulties with this method. The main criticism being that the confirmation procedures are time-consuming and labour intensive when there are large numbers of colonies involved. Also, the detection of acid production in the triple sugar iron agar for confirmation of carbohydrate utilisation tended to be masked by hydrogen sulphide production (blackening of the medium) by the

Salmonellae. Participants also reported that testing for the H antigen using the semi-solid agar was not successful as either a weak reaction or no reaction was observed because it was almost impossible to free the inoculum used to test for agglutination from the semi-solid agar which interfered with the reaction. Notwithstanding these criticisms of the method, 16% false positive results is unacceptable and attributable to poor laboratory practice. Those laboratories who reported such results should re-examine their in-house quality assurance procedures.

BS 5763 Part 7, 1983.
Enumeration of *Staphylococcus aureus* by colony count technique

This method requires the inoculation of the surface of a solid selective medium (Baird Parker) with a specified quantity (0.1 ml) of the test material (or an appropriate dilution). Plates are incubated at 37°C for 24 - 48 h. The number of *S. aureus* per g of sample would normally be calculated from the number of typical and atypical colonies obtained on the plates which were confirmed using the coagulase test. For the purposes of this trial however, participants were required to determine only the presence or absence of *S. aureus* in the 3 samples by confirming the presence of presumptive colonies using the coagulase test. One of the samples was negative for *S. aureus,* one was positive for *S. aureus* and one contained *Staphylococcus* species which were not *S. aureus*.

A total of 7 false positive results (9.2%; all one duplicate only) were reported. Four of the false positive results (11%) were reported for the sample that contained *Staphylococcus* species other than *S. aureus* (Table 9). Six false negative results (16%) were reported; one laboratory failed to confirm *S. aureus* in either of the duplicates.

Although participants generally reported few problems with this method, the high rate of false positive and false negative results are a cause for concern. Some laboratories reported difficulties in deciding whether a colony was typical or not and thus whether it should be picked for confirmation. This problem has previously been found with BP agar and it is now well recognised that *S. aureus* do not always produce the typical black, shiny, convex colonies with a halo and egg yolk precipitate and therefore a representative number of both typical and atypical colonies should be picked for confirmation. It is probable that the high number of false negative results is due in part to not picking sufficient colonies for confirmation. The high rate of false positive results is again a cause for concern and further work is warranted to confirm the reliability of the coagulase test.

BS 4285 Part 3,
Microbiological examination for dairy purposes, Section 3.10 *Staphylococcus aureus*. Sub-section 3.10.2 Detection.

This method is designed for the detection of low numbers of *S. aureus* where an enrichment broth (Giolitti-Cantoni) is inoculated with a specified volume (1 ml) of test sample. For the purposes of this trial, the same sample types as used for the colony count method for *S. aureus* (Table 9) was used for this method. A total of 16 false positive results (22%) were reported, 5 for the sample containing no staphylococci and 11 for the sample containing 'non-*aureus*' staphylococci (Table 10). Four of the laboratories had previously reported false positive results using the colony count method. Four false negative results (11%) were reported, again 2 laboratories had previously reported false negative results using the colony count method. The even higher level of false positive results reported using this method is, in part, believed to be due to the problems associated with using a water agar plug to cap the Giolotti-Cantoni (GC) broth. The method states that the water agar should be poured over the top of the inoculated GC broth. However, in practice this resulted in aerosols of GC broth splashing back into the water agar thereby contaminating this broth which was then used to seal all other samples. This procedure is not recommended and the water agar should be either gently pipetted into the tube of GC broth or individual aliquots of water agar made up sufficient for overlaying each tube of GC broth. Subculturing from the GC broth onto BP agar plates was also difficult when the water agar plug was used. The instructions in the method require that prior to subculturing onto the selective agar, the water agar plug should be cut into sections permitting it to sink to the bottom of the tube making subculture more easy. In practice however, this was not found to be the case and the plug remained on the surface of the broth.

One laboratory also reported that the culture of *S. aureus* used in their quality control procedures became atypical on BP agar after passing through an enrichment period in GC broth in that it did not produce clear zones. This anomaly requires further investigation and may have contributed to the false negative results.

BS 5763 Part 10, 1986.
Enumeration of Enterobacteriaceae (colony count method)

This method requires the inoculation of two plates of a selective agar (violet red bile glucose agar, VRBGA) with a specified quantity of the test sample (1 ml) or appropriate dilutions thereof. The VRBGA medium is then overlayed with the same to create an atmosphere more optimal for the growth of Enterobacteriaceae. Plates are then incubated at 37°C for 24 h and the number of Enterobacteriaceae per ml of sample is determined from the number of confirmed colonies per plate.

Unfortunately, although this method was quantitative, it was not possible to determine the precision characteristics from the data returned by

participants because the batch of liquid whole egg used to prepare the trial samples was subsequently found to be contaminated with naturally occurring Enterobacteriaceae. None had been detected in the pre-trial samples by the laboratory carrying out sample preparation. These results were therefore considered void and this method will have to be assessed at a later date.

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Appendix I

Organisms used for production of egg reference materials.

Staphylococci spp.

Staph. aureus NCTC 4136
Staph. aureus PB14 (LFRA isolate)
Staph. saprophyticus (PB3 LFRA isolate)

Salmonella spp.

S. enteritidis PT4 P167807
S. enteritidis PT4 P125678

Kindly supplied by Dr B. Rowe, Division of Enteric Pathogens, Central Public Health Laboratory, Colindale.

S. typhimurium 7M-5522
S. typhimurium 77-19478

Kindly supplied by Dr P. McClure, AFRC, Institute of Food Research, Reading Laboratory, Reading.

Enterobacteriaceae

Citrobacter freundii NCTC 6266
Klebsiella pneumoniae AG308 (LFRA isolate)
Escherichia coli NCTC 9001
Proteus vulgaris NCTC 4175
Hafnia alvei NCTC 6578
Enterobacter cloacae NCTC 9394

Cultures were maintained on frozen storage beads (Protect) and resuscitated in Trypticase Soya broth (TSB, Oxoid) incubated at 37°C for 18-24 h. Cocktail mixtures were prepared by combining the diluted TSB bead cultures and inoculating the samples with 50 µl of the pooled suspension.

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The Determination of 3-Methylhistidine In Meat Products Collaborative Trial

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The results of a collaborative trial carried out in 15 U.K. laboratories to determine the level of protein-bound 3-methylhistidine (N^{τ}-methylhistidine) in meat products are reported. The analyte is a potential marker for actin and myosin proteins, and therefore for fat-free collagen-free (FFCF) meat. The prootocol involves the hydrolysis of a washed acetone powder of the sample, followed by the preparation of an acid-stable fluorescamine derivative of the 3-methylhistidine present; this specific derivative is isolated by high performance liquid chromatography (HPLC) and quantified fluorimetrically.

Initially, ten standard solutions of 3-methylhistidine were analysed by HPLC alone; recoveries between 102% and 108% were observed over a wide range of concentrations (0.2 to 50mg/l). Then, using the full protocol, seven meat products of known composition were analysed, each in blind duplicate. The overall results were consistent with satisfactory recoveries of beef (96-100%) and pork (88-93%)

The precision of the method was less acceptable. The overall relative standard deviation (RSD, "Coefficient of Variation") was 17-22% with standard solutions containing Img/l and above. With the meat products, RSDs of 16-40% were observed at levels of 64-169 ug/g. The value of 3-methylhistidine as a quantitative marker for FFCF meat is limited, and this method cannot be accepted as an official procedure at present. Nevertheless, the results indicate that it has at least as firm a basis as the hydroxyproline method often used to estimate collagen and connective tissue in meat products.

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Introduction

The Meat Products and Spreadable Fish Product Regulations (1984)[1] came into operation on 1st July 1986. These Regulations specify minimum meat and lean meat contents for a number of meat products. The responsibility for enforcing these Regulations will principally fall on Food Authorities and their appointed Public Analysts. Proposals have also been made for self-regulation within the Food Industry. To enforce the Regulations, effective analytical procedures are required. Amongst such procedures is a robust index of lean meat protein. In addition to thermal and chemical stability, the index is required to resist degradation from high pressures (during extrusion) and leaching (during washing, curing and cooking). Lawrie and his co-workers proposed the substituted aminoacid 3-methylhistidine (N^{τ}-methylhistidine) for this purpose over two decades ago[2]. More recently, an American report[3] has noted that "particular attention" should be paid to 3-methylhistidine assay development, which illustrates interest on both sides of the Atlantic.

3-Methylhistidine is virtually exclusive to the animal kingdom, where it exists in both soluble form (e.g. as the dipeptide balenine, i.e. β-alanyl-3-methylhistidine[4]) and as a constituent aminoacid in a number of proteins. The majority of this protein-bound 3-methylhistidine is to be found in the two principal myofibrillar proteins actin and myosin[5,6]. Low levels of 3-methylhistidine have been reported in other proteins (e.g. histones[7]), but these do not contribute significantly to the total muscle titre of this aminoacid. A matter of potentially greater importance with respect to the use of 3-methylhistidine as an index of meat protein in meat products is its variable occurrence in the myosin fraction of the myofibrillar protein. This was first noticed by Trayer et al.[8]; the low titre of 3-methylhistidine in beef cheek meat found by Jones et al[9] was identified by White and Lawrie[10] as being caused by the low level of 3-methylhistidine in the myosin of the *Masseter* and *Malaris* muscles. Nevertheless, total protein-bound 3-methylhistidine has been found to be useful, and its levels in several anatomical parts from different meat species (beef, pork, lamb, rabbit and chicken) have been reported[9-15], with a view to establishing a conversion factor for fat-free, connective tissue-free meat. This paper reports the results of a collaborative trial of a method for protein-bound 3-methylhistidine. Its design ensured that the variability of the myosin-bound 3-methylhistidine was unimportant, because all the meat products were prepared exclusively from *Longissimus dorsi,* in which it is believed that one mole of histidine is specifically methylated per mole of myosin heavy chain. The 3-methylhistidine component of actin, however, appears to be constant throughout the beef carcass[11], also at a level of one mole per mole (specifically at residue no 73). This has prompted the investigation of actin-bound 3-methylhistidine as a more consistent index for lean muscle meat[16], which was also based on the analytical method tested here.

Design of the Collaborative Trial

Method for the Determination of 3-Methylhistidine

The analytical method for the determination of 3-methylhistidine tested in this collaborative trial (Appendix I) is based on the published procedure of White and Lawrie[17]. This in turn developed from a specific derivitisation technique discovered by Nakamura and Pisano[18] and the work of Jones, Shorley and Hitchcock[12,13]. The latter combined the derivitisation with a separation by high performance liquid chromatography (HPLC), and applied their method to meat and meat products.

After the methodology had been established as suitable for "in-house" use at Nottingham University, a protocol was drawn up in collaboration with AD Jones of Unilever Research (Colworth House, Sharnbrook) and the authors. This protocol (Appendix I) was circulated to interested parties, of whom a number agreed to participate.

Complementary Determinations

The meat product samples were also analysed for the following analytes, using the appropriate BS 4401 procedures: moisture, fat, ash, Kjeldahl nitrogen and hydroxyproline[19]; in addition, the Kjeldahl nitrogen content of the acetone powder prepared from each sample (Appendix I, section 6.1) was determined. Collagen levels were calculated from the observed hydroxyproline concentrations using the factor 7.25.

One effect of this comprehensive analysis was to enable the 3-methylhistidine content to be expressed in terms of fat-free collagen-free (FFCF) meat. Since the precision of this parameter cannot be greater than that of the content in terms of total sample as received, this paper is concerned only with the latter simpler parameter. The results of the complementary analyses are not reported here in detail, though some of the conclusions are mentioned in the Discussion below.

Collaborative Trial Phase 1 (Standard Solutions)

The trial was divided into two phases. Initially, the participants were to analyse a series of 3-methylhistidine standard solutions (ranging from 0.2 to 50 μg/ml in 0.10 mol/l hydrochloric acid) to determine if their apparatus possessed the necessary sensitivity, a problem which had been identified by White and Lawrie[17].

In the first phase, the unknown solutions of pure 3-methylhistidine were to be diluted with 0.10 mol/l hydrochloric acid (if necessary), and 0.1ml aliquots neutralised with 0.1ml of 0.10 mol/l sodium hydroxide. These 0.2ml samples were then to be analysed exactly as described for the 0.2ml sample of neutralised hydrolysate (Appendix I, section 6.3). If successful, the participants were to proceed to analyse a series of model meat products; recipes (Table I) were based on beef or pork, and included a dried soup powder and a meatless soyaburger. All samples were coded and presented as "blind" duplicates to the participants.

Table I

Composition of meat products analysed

Sample[a]	Code[b]	Minced Beef[c]	Minced Pork[c]	Fat[d]	Rusk[e]	Soya Flour[f]	Meat Content[g]
Minced Beef	A/N	100	0	0	0	0	100
Beefburger	C/F	80	0	5	15	0	80
Beef Sausage	E/J	50	0	20	30	0	50
Powdered Soup[h]	B/M	59[g]	0	2.9	0	0	59
Minced Pork	H/K	0	100	0	0	0	100
Pork Sausage	G/D	0	60	10	30	0	60
Soyaburger	I/L	0	0	20	60	20	0

[a] All products, except the soup powder, were mixed in a bowl chopper.

[b] Blind duplicates.

[c] *Longissimus dorsi* muscle was used for all meat preparations.

[d] Refined lard was used in all products except soup powder, where suet was used.

[e] Hydrated rusk (water:dry rusk 2:1).

[f] Hydrated soya flour (water:defatted dry soya flour 2:1).

[g] Equivalent (wet) meat content, calculated from the recipe.

[h] Mock turtle soup, after Binstead and Devay ("Soup Manufacture, Canning, Dehydration and Quick Drying" 3rd Edn, Food Trade Press Ltd, London, p237). Beef[c] was minced, weighed, dried in a freeze drier, reweighed and ground in a liquidiser; its observed water content was 71.18%. This dried ground beef was used in the following recipe: wheat flour, 27.3%; dried ground beef, 17.0%; onion powder, 13.7%; carrot powder, 11.4%; tomato powder, 10.8%; salt, 9.1%; monosodium glutamate, 2.9%; beef fat (suet), 2.9%; hydrolysed vegetable protein powder, 2.3%; yeast extract powder, 2.3%; ground thyme, 0.2%; ground bay leaves, 0.07%; ground coriander, 0.06%; ground paprika, 0.05%; ground white pepper, 0.05%. The ingredients were mixed and passed through a 0.5mm mesh on an ultracentifugal mill; the resultant fine powder was again mixed before distribution.

Collaborative Trial Phase 2 (Meat Samples)

In the second phase, one acetone powder was to be prepared from each meat product sample, and one hydrolysate prepared from each acetone powder; this provided a single result for each sample, i.e. a "blind" duplicate observation from each standard solution and each meat product.

For the chromatographic analysis, the recommended injection sequence within each run was: standards (2.0, 1.5, 1.0, 0.5 mg/ml); samples A, B, C, D, E, F, G; standards (2.0, 1.5, 1.0, 0.5 mg/ml); samples H, I, J, K, L, M, N; standards (2.0, 1.5, 1.0, 0.5 mg/ml). The calculation of observed 3-methylhistidine levels in the meat products was to be based on the average of the standards observed immediately before and immediately after each set of samples.

Results

Tables II to IV record the results from the first phase of the trial, listing the levels of 3-methylhistidine in each of the 10 sample solutions (5 duplicates) reported by each of the 15 laboratories. Tables V-VIII record the results from the second phase of the trial, listing the levels of 3-methylhistidine in each of the 14 meat product samples (7 duplicates; Table I) reported by each of the 14 laboratories.

Table II

Determination of methylhistidine (mg/litre) in standard solutions

Laboratory	Sample Code (0.2 mg/l)		Sample Code (1.0 mg/l)	
	7	9	5	8
1	0.24	0.19	1.06	1.10
2	0.07	0.08	0.42	0.77
3	0.40	0.40	1.10	1.10
4	0.30	0.40	1.10	1.00
5	0.25	0.24	1.40	1.36
6	0.26	0.24	0.79	1.11
7	0.21	0.20	1.25	0.88
8	0.00	0.00	0.56	0.75
9	0.50	0.22	1.09	0.91
10	0.20	0.20	1.00	1.00
11	0.26	0.24	0.94	1.00
12	0.20	0.28	1.32	1.18
13	0.30	0.30	1.00	1,00
14	0.00	0.00	1.19	1.08
15	0.17	0.24	1.14	1.02
Number of Observations Accepted	30		30	
Overall:				
Mean	0.205		1.021	
Standard Deviation	0.114		0.195	
RSD(%)	56		19	
Known (Actual) Level	0.200		1.000	
Recovery (%)	102		102	
Repeatability:				
S_r	0.0423		0.128	
RSD_r(%)	21		13	
r	0.119		0.359	
RSD_r (Horwitz)(%)	13.4		10.5	
Ho_r	1.54		1.19	
Reproducibility:				
S_R	0.118		0.215	
RSD_R(%)	57		21	
R	0.329		0.603	
RSD_R (Horwitz)(%)	20.3		16.0	
Ho_R	2.83		1.32	

Table III
Determination of methylhistidine (mg/litre) in standard solutions

Laboratory	Sample Code (5 mg/l)		Sample Code (20 mg/l)	
	1	4	3	10
1	5.82	5.54	22.64	20.99
2	4.08	3.05	15.00	14.72
3	3.10	5.00	20.20	18.40
4	4.50	4.60	17.60	16.80
5	7.23	6.49	29.10	27.80
6	5.56	2.52	21.88	21.20
7	5.94	6.17	25.10	25.38
8	4.31	6.00	22.50	25.90
9	2.45	5.76	22.20	25.00
10	5.00	6.40	23.00[a]	1.90[a]
11	4.40	4.80	22.40	19.20
12	5.80	5.80	24.40	24.00
13	4.00	5.00	16.00	19.00
14	6.56	7.85	24.90[a]	37.90[a]
15	5.05	4.97	21.04	20.70
Number of Observations Accepted	30		30	
Overall:				
Mean	5.125		21.51	
Standard Deviation	1.07		3.72	
RSD(%)	21		17	
Known (Actual) Level	5.000		20	
Recovery (%)	102		108	
Repeatability:				
S_r	1.05		1.36	
RSD_r(%)	21		6	
r	2.95		3.80	
RSD_r (Horwitz)(%)	8.26		6.65	
Ho_r	2.49		0.95	
Reproducibility:				
S_R	1.31		3.84	
RSD_R(%)	25		18	
R	3.66		10.75	
RSD_R (Horwitz)(%)	12.51		10.08	
Ho_R	2.04		1.77	

[a] Data failed Cochran's test; not used in calculations

Table IV
Determination of methylhistidine (mg/litre) in standard solutions

Laboratory	Sample Code (50 mg/l)	
	2	6
1	51.77	55.98
2	40.40	40.14
3	35.00	39.20
4	43.50	41.60
5	71.30	70.10
6	39.20	57.60
7	62.87	62.06
8	51.70	57.30
9	55.60	59.90
10	56.00	56.00
11	55.00	53.00
12	60.40	27.00
13	21.00	46.00
14	67.70	78.50
15	52.08	40.53
Number of Observations Accepted	30	
Overall:		
Mean	51.61	
Standard Deviation	11.56	
RSD(%)	22	
Known (Actual) Level	50.00	
Recovery (%)	103	
Repeatability:		
S_r	8.99	
RSD_r(%)	17	
r	25.17	
RSD_r (Horwitz)(%)	5.83	
Ho_r	2.99	
Reproducibility:		
S_R	13.19	
RSD_R(%)	26	
R	36.94	
RSD_R (Horwitz)(%)	8.84	
Ho_R	2.89	

Table V
Determination of methylhistidine (μg/g) in meat products

Laboratory	Minced Beef Sample Code		Beefburger Sample Code	
	A	N	C	F
1	116.8	99.2	95.7	93.2
2	183.0	172.0	148.0	141.0
3	142.0	133.6	105.6	106.3
4	49.0	56.9	27.5	177.0
5	138.0	140.0	99.2	110.0
6	156.4	173.8	131.7	131.3
7	136.0	128.0	202.0	89.4
8	190.0	153.0	97.6	96.5
9	151.0	153.0	123.0	64.4
10	147.0	137.0	114.0	120.0
11	144.0	149.0	131.0	129.0
12	127.0[a]	230.0[a]	85.3	80.6
13	80.5	70.9	52.3	57.0
14	130.0	121.0	70.2	97.2
Number of Observations Accepted	26		28	
Overall:				
Mean	132.7		106.3	
Standard Deviation	36.0		25.5	
RSD(%)	27		24	
Repeatability:				
S_r	10.0		37.5	
RSD_r(%)	8		35	
r	28.0		105.1	
RSD_r (Horwitz)(%)	5.06		5.23	
Ho_r	1.49		6.75	
Reproducibility:				
S_R	36.7		36.8	
RSD_R(%)	28		35	
R	102.7		105.1	
RSD_R (Horwitz)(%)	7.67		7.93	
Ho_R	3.61		4.37	

[a] Data failed Cochran's test; not used in calculations

Table VI
Determination of methylhistidine (μg/g) in meat products

Laboratory	Beef Sausage Sample Code		Powdered Soup Sample Code	
	E	J	B	M
1	57.0	52.9	77.2	70.6
2	84.5	82.3	109.0	99.1
3	69.0	70.2	87.8	80.3
4	138.0[a]	25.4[a]	187.0	177.0
5	59.5	67.9	66.7	66.7
6	66.5	78.8	91.2	94.6
7	0.0[a]	65.0[a]	132.0	120.0
8	72.7	70.0	32.6	39.4
9	71.7	76.8	104.0	94.5
10	79.2	80.1	89.1	88.3
11	75.0	72.0	109.0	94.0
12	51.3	61.4	98.4	109.0
13	21.9	21.2	[c]	[c]
14	41.1	48.0	56.2	56.5

Number of Observations		
Accepted	24	26
Overall:		
Mean	63.8	92.8
Standard Deviation	17.31	35.15
RSD(%)	27	38
Repeatability:		
S_r	4.27	7.75
RSD_r(%)	7	8
r	11.97	21.70
RSD_r (Horwitz)(%)	5.65	5.34
Ho_r	1.19	1.56
Reproducibility:		
S_R	17.57	35.58
RSD_R(%)	28	38
R	49.20	99.62
RSD_R (Horwitz)(%)	8.56	8.09
Ho_R	3.22	4.74

[a] Data failed Cochran's test; not used in calculations

[c] Exact data not reported

Table VII
Determination of methylhistidine (μg/g) in meat products

Laboratory	Minced Pork Sample Code		Pork Sausage Sample Code	
	H	K	G	D
1	148.5	119.1	82.2	90.0
2	218.0	199.0	136.0	129.0
3	143.3	197.1	74.4	80.6
4	21.2[b]	34.0[b]	44.6	22.7
5	142.0	148.0	89.3	93.5
6	173.5	216.4	121.3	117.4
7	181.0	116.0	180.0[a]	110.0[a]
8	178.0	163.0	78.5	71.4
9	165.0	152.0	85.2	87.0
10	216.0	227.0	170.0	164.0
11	175.0	193.0	97.0	102.0
12	113.0	155.0	90.1	71.6
13	137.0	172.0	28.1	49.5
14	193.0	159.0	62.1	79.9
Number of Observations				
Accepted	26		26	
Overall:				
Mean	169.2		89.1	
Standard Deviation	27.46		35.75	
RSD(%)	16		40	
Repeatability:				
S_r	24.18		8.53	
RSD_r(%)	14		10	
r	67.71		23.89	
RSD_r (Horwitz)(%)	4.88		5.37	
Ho_r	2.93		1.78	
Reproducibility:				
S_R	32.35		36.25	
RSD_R(%)	19		40	
R	90.58		101.51	
RSD_R (Horwitz)(%)	7.39		8.14	
Ho_R	2.59		5.00	

[a] Data failed Cochran's test; not used in calculations

[b] Data failed single Grubb's test; not used in calculations

Table VIII
Determination of methylhistidine (mg/litre) in meatless products

Laboratory	Soyaburger Sample Code	
	I	L
1	3.29	3.49
2	0.00	0.00
3	4.84	6.05
4	6.34[(a)]	2.42[(a)]
5	0.00	0.00
6	0.00	0.00
7	0.00[(a)]	112.0[(a)]
8	10.10[(a)]	12.80[(a)]
9	4.10	3.98
10	0.00[(d)]	0.00[(d)]
11	10.00	10.00
12	0.00[(d)]	0.00[(d)]
13	0.00[(d)]	0.00[(d)]
14	0.00	0.00

Number of Observations	
Accepted	22
Overall:	
Mean	2.08
Standard Deviation	3.31
RSD(%)	159
Repeatability:	
S_r	0.26
RSD_r(%)	13
r	0.74
RSD_r (Horwitz)(%)	9.46
Ho_r	1.34
Reproducibility:	
S_R	3.32
RSD_R(%)	160
R	9.30
RSD_R (Horwitz)(%)	14.33
Ho_R	11.14

[(a)] Data failed Cochran's test; not used in calculations

[(d)] Data reported as less than the limit of detection, and taken as zero

Statistical Analysis of the Results

The data were examined for evidence of individual aberrant systematic error ($p<0.01$) using Cochran's test and Grubbs test progressively, following procedures described in the internationally agreed Protocol for the Design, Conduct and Interpretation of Collaborative Studies[20]. Mean values and precision parameters were calculated, and are given in Tables II to VIII, which also identify rejected outliers.

Precision

The repeatability (r) and the reproducibility (R) as defined by that Protocol[20] were calculated after the removal of outliers. Corresponding values for standard deviation (S) and relative standard deviation (RSD, expressed as a percentage; i.e. "Coefficient of Variation") were also computed.

Horwitz-predicted precision parameters

There is often no validated reference/statutory method with which to compare precision criteria when assessing a method. In such cases it is useful to compare the precision data obtained from a collaborative trial with the predicted acceptable levels of precision. These latter levels, predicted by the Horwitz equation, give an indication as to whether the method is sufficiently precise for the level of analyte being measured[21].

The Horwitz predicted value was calculated from the Horwitz equation[21]:

$$RSD_R \text{ (Horwitz)} = 2^{(1 - 0.51 \times \log C)}$$

where C is the observed concentration of the analyte expressed as a decimal.

Horrat values (Ho)

The Horrat[22] value gives a comparison of the actual precision measured with the precision predicted by the Horwitz equation for a method measuring at that particular level of analyte. It was calculated from the equation:

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

An Ho_R value of greater than 2 usually indicates unacceptable interlaboratory precision, one that is too variable for most analytical purposes.

Similarly, Ho_r was calculated using the approximation

$$RSD_r(\text{Horwitz}) = 0.66RSD_R(\text{Horwitz}).$$

This assumes the approximation $r = 0.66R$, and the resultant Ho_r value was used to assess intralaboratory precision as above.

Discussion

3-methylhistidine

The accuracy of the method, when applied to standard solutions of pure analyte, is satisfactory: Tables II-IV record recoveries between 102% and

108% for a wide range of concentrations (0.2 to 50mg/l). The accuracy of analysis of meat products is more difficult to assess, since the use of samples spiked with pure 3-methylhistidine is arguably not appropriate. The observed 3-methylhistidine content of 100% beef *L dorsi* was 132.7μg/g (Table V); from this, the beef content of the corresponding products may be calculated. They are 80.1% (recipe, 80%) and 48.1% (recipe, 50%). Similarly, the calculated levels of pork in the pork products are 52.7% (recipe, 60%) and 54.8% (recipe, 59%). The recovery of beef is therefore about 96-100% and of pork 88-93%. The analysis of the blank soyaburger is also acceptably accurate (observed, 2.1μg/g, corresponding to 1.6% beef; recipe, 0%).

The precision of the method, when applied to pure solutions or to meat products, is far less satisfactory. The overall RSD observed during Phase 1 is fairly constant but undesirably high (17% to 22%) at 1mg/l and above (Tables II - IV). The method is less precise at lower levels (56% at 0.2mg/l). In Phase 2, the effects of the sample matrix and the necessary extra manipulations lead in general to an even higher overall RSD (16% to 40%) at levels of 64 to 169μg/g (Tables V - VIII); the blank is more acceptable (2.1 ± 3.3μg/g). As summarised in Table IX, the majority of calculated Horrat values exceed 2, and it must be concluded that the procedure defined in the protocol cannot be accepted as an official method at present. This reinforces previous suggestions[9,15] that the value of 3-methylhistidine as a quantitative marker is limited.

Chemical markers for meat

There are three chemical indexes relevant to the analysis of meat products[23]: nitrogen is a useful marker for fat-free meat (and for total protein)[24]; hydroxyproline is often used to estimate collagen and connective tissue[25]; 3-methylhistidine corresponds with levels of muscle (fat-free collagen-free meat). All are subject to uncertainty due to lack of precision in the determination of the index itself; the overall precision parameters are compared in Table IX, which summarises the statistical analysis of results from this collaborative trial for 3-methylhistidine (Tables V - VIII), for nitrogen and for hydroxyproline (detailed data available). The overall RSD and Horrat values in Table IX confirm that the well-established official Kjeldahl method for nitrogen content is acceptable, but that the 3-methylhistidine method criticised in this paper is significantly less imprecise than the established but unofficial hydroxyproline method. However, the poor precision of the hydroxyproline data may be due in part to the type of sample: all the products contained very low levels, corresponding to <5% connective tissue unavoidably added as part of the meat. A previous collaborative study[25] reported lower RSDs in samples containing 20% connective tissue; nevertheless, even these RSDs were higher than expected. With samples containing no added connective tissue, the precision data in

TABLE IX

Summary of overall precision of determination of three chemical indexes in 14 Laboratories

Range of selected statistical parameters observed in 12 sets of data, after the analysis (in blind duplicate) of 6 meat-containing product samples[a] for three chemical indexes.

Parameter	MeHis (total sample)[(b)]	MeHis (FFCFsample)[(c)]	Kjeldahl Nitrogen[(d)]	Hydroxyproline[d]
Overall Mean	64-169 μg/g	82-173 μg/g	1.8-3.5g/100g	0.07-0.16g/100g
Overall RSD (%)	16-40	13-40	1.8-4.7	49-57
Ho_r	1.2-6.8	1.3-6.8	0.3-1.4	4.6-9.3
Ho_R	2.6-5.0	2.2-5.1	0.5-1.2	8.3-11.1
Number of Horrat values <2	4	4	12	0
Number of Horrat Values >2	8	8	0	12

[(a)]Composition of samples listed in Table 1. Samples I and L (meatless soyaburger) are omitted from this summary.

[(b)]Observed 3-methylhistidine content expressed as μg per g of (wet) sample as received. From Tables 5-7.

[(c)]Observed 3-methylhistidine content expressed as μg per g of fat-free collagen-free meat, calculated from observed levels of 3-methylhistidine, fat and hydroxyproline in the (wet) sample as received. From unpublished detailed data.

[(d)]Observed Kjeldahl nitrogen and hydroxyproline content expressed as g/100g of (wet) sample as received, using standard methods. From unpublished detailed data.

Table IX suggests that the determination of 3-methylhistidine has at least as firm a basis as that of hydroxyproline.

It must be remembered that the interpretation of 3-methylhistidine levels as muscle meat content would involve further uncertainty due to the possible variability of the conversion factor, a problem that is familiar in meat analysis based on hydroxyproline[23,25] (for connective tissue) and even on Kjeldahl nitrogen[23,24] (for fat-free meat). Nevertheless, the determination of 3-methylhistidine (and of hydroxyproline) may well be useful in the analysis of meat products, provided that the limitations are appreciated, and especially if the robustness of the methodology can be improved. Its usefulness would be enhanced if means could be developed to determine the actin-bound 3-methylhistidine content of samples, since all actins appear to contain the same amount of the amino acid - and are more robust than myosins.

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APPENDIX I

Determination of 3-Methylhistidine in Meat and Meat Products

1. Scope and Field of Application

The method allows the determination of 3-methylhistidine (N^{τ}-methylhistidine) in meat and meat products. This analyte is a potential marker for actin and myosin proteins, and therefore for fat-free connective tissue-free meat.

2. Definition

The content of 3-methylhistidine: the content of 3-methylhistidine as determined by the method specified.

3. Principle

An acetone powder extract of the meat or meat product is prepared, hydrolysed and the free 3-methylhistidine present in the hydrolysate is converted to its acid-stable fluorescamine derivative. This derivative is isolated by high performance liquid chromatography (HPLC) and quantified fluorimetrically.

4. Reagents

Analytical grade reagents are to be used throughout; distilled water, or water of an equivalent purity, is similarly to be used.

4.1 Chloroform:methanol mixture (2:1 by volume).

4.2 Ethanol:water mixture (80:20 by volume).

4.3 Acetone.

4.4 Hydrochloric acid, 6 mol/l.

4.5 Sodium hydroxide solution, 6 mol/l.

4.6 3-Methylhistidine: standardise the solid material before use via the Kjeldahl nitrogen content.

4.7 Methanol, HPLC grade.

4.8 Acetate buffer, *p*H 4.0: prepare by dissolving 1g of sodium acetate and 2.5g of glacial acetic acid in water, and make up to 1 litre with water.

4.9 Disodium tetraborate solution, 0.2 mol/l adjusted to pH 9.0.

4.10 Fluorescamine solution, 2mg/l: freshly prepare before use by dissolving fluorescamine in methyl cyanide, HPLC grade.

4.11 Hydrochloric acid, 2 mol/l.

5. Apparatus

5.1 Mincer, fitted with a 4mm plate.

5.2 Blender, e.g. a commercial Waring blender.

5.3 Filter paper, Whatman no 541 or equivalent.

5.4 Hartley funnel.

5.5 Incubator, set at 30°C.

5.6 Grinder, eg a domestic grinder.

5.7 Hammer mill, with 2mm, 1mm and 0.5mm plates; other apparatus may be used provided that a representative homogeneous powder which will pass through a 1mm sieve is obtained.

5.8 Desiccator, containing freshly activated silica gel with a water content indicator, or an equivalent desiccant.

5.9 McCartney bottles, fitted with polypropylene caps or equivalent small vessels, capable of holding 15ml of strong acid and solvents, and maintaining a gas-tight seal at the temperatures and pressures used in this method.

5.10 Oven, thermostatically controlled at 100°C ± 1°C.

5.11 Water bath, thermostatically controlled at 80°C.

5.12 HPLC chromatograph

HPLC chromatograph, fitted with a fluorescence detector. The detector should be fitted with a blacklight (i.e. a cold source) which emits a phosphor band spectrum with mercury lines superimposed. The maximum energy emission should be around 360nm.

The primary filter should be a Corning 7-60, with transmittance 55.6% at 365nm and almost zero transmittance below 300nm and above 400nm. The secondary filter should be a Wratten no 2A.

The injection system should preferably consist of a valve fitted with a 20μl fixed-volume loop.

5.13 HPLC column

The HPLC column (and conditions of chromatography) must be selected to give optimum separation and sensitivity. Columns of dimensions 10cm × 0.49cm with the following packings are satisfactory:

(a) Waters μ-Bondapak C18; this is preferred and commercially available.

(b) Lichrosorb 5μm SI 100 ODS; this may be prepared "on site" as follows. Reflux 10g of 5μm Lichrosorb SI 100 silica for 3h in 200ml of hydrochloric acid (2mol/l); filter the material through a Whatman no 1 paper over a low vacuum, and dry overnight in an oven at 90°C. Dissolve 10g of octadecyldimethyl monochlorosilane in 50ml of toluene and 2ml of pyridine; add the silica and reflux overnight. Add 3ml of hexamethyl disilazane and continue to reflux for a further 3h. Filter off the silica onto Whatman no 1 paper over a low vacuum, and wash twice with 100ml of hexane, once with 100ml of acetone, once with 100ml of an acetone/water mixture (50:50 by volume) and finally with acetone. Dry the resultant cleaned material overnight in an oven at 80°C.

The column is packed by preparing a slurry of the coated silica in chloroform; dispersion is assisted by ultrasonication. The packing solvent is methyl cyanide, using 150ml initially upwards at 3000-4000 psi and then a further 200ml downwards at 7000psi. It is recommended that a 10cm column with an efficiency of less than 10,000 plates/m should not be used.

The chromatographic eluant is a mixture of methanol (**4.7**) and acetate buffer (**4.8**) 50:50 by volume, degassed with helium or by ultrasonication, and filtered to remove microparticulate matter. The flow rate is ideally 1.5ml/min.

6. Procedure

6.1 Acetone powder preparation

Mince the sample through a mincer fitted with a 4mm plate (**5.1**). Weigh accurately a 10g portion of the minced sample and homogenise with 200ml of chloroform/methanol mixture (**4.1**) in a Waring blender (**5.2**) for 60sec at high speed. Filter the homogenate through a previously weighed filter paper (**5.3**) using a Hartley funnel (**5.4**) over a low vacuum. Wash the residue on the paper with 200ml of the ethanol/water mixture (**4.2**), followed by 200ml of acetone (**4.3**), and dry overnight in an incubator (**5.5**). Reweigh and calculate the weight of acetone powder. Remove the precipitate from the filter paper and grind, using a domestic grinder (**5.6**). Carefully recover all the material and further attritiate to a fine powder using a hammer mill (**5.7**), passing the sample sequentially through 2mm, 1mm and 0.5mm plates. Great care must be taken to recover all possible material from the hammer mill between passes. After the final pass, collect all material and stir to further ensure homogeneity. Store in a desiccator (**5.8**).

6.2 Hydrolysis

Weigh about 60mg (*b*mg) of the acetone powder (**6.1**) into a McCartney bottle fitted with a polypropylene cap (**5.9**), add 15ml of 6mol/l hydrochloric acid (**4.4**) and hydrolyse by heating for 16h at 100°C in an oven (**5.10**). Ensure a gas-tight seal on the container. Filter the hydrolysate through filter paper (**5.3**); this filtered hydrolysate represents the sample *S*.

6.3 Derivitisation

Prepare standard aqueous solutions of 3-methylhistidine (**4.6**) containing 2.0, 1.5, 1.0 and 0.5μg/ml by serial dilution in water. Charge glass test tubes with 0.2ml of each standard solution, or 0.1ml of sample *S*. Add 0.1 ml of sodium hydroxide solution (**4.5**) to the sample tube(s) only. Add 2.3ml of 0.2mol/l disodium tetraborate solution (**4.9**) at pH 9.0 to all tubes. Vortex all tubes with a Whirlimixer. Then slowly add 2.5ml of freshly made-up fluorescamine solution (**4.10**) to each tube whilst it is being vortexed, then allowing each to stand for approximately 2min. Then add 2.5ml of 2mol/l hydrochloric acid (**4.11**), mix and transfer to a McCartney

bottle fitted with a polypropylene cap (**5.9**); seal and heat at 80°C in a water bath (**5.11**) for 1h.

6.4 Chromatography and detection

Inject 20μl of the solutions of sample or standard derivatives onto a reverse phase column, using the chromatographic conditions given (**5.13**). If possible, ensure that all solutions are thoroughly degassed with helium prior to and during use.

6.5 Complementary analyses

If proximate analyses are undertaken, use the appropriate BS 4401 procedures.

If levels of hydroxyproline are required, 1.0ml aliquots of the sample solution *S* may be neutralised to pH 6.4 with sodium hydroxide (**4.5**), and made up to 25.0ml with water for determination of hydroxyproline (Stegemann, H. and Stalder, K., Clin. Chim. Acta, 1967, 18, 267). Collagen content may be estimated by multiplying the observed hydroxyproline level by the factor 7.25.

7. Expression of Results

7.1 Calculation

Calculate the 3-methylhistidine concentration of the sample hydrolysate solution by comparing the peak heights of the HPLC chromatograms of the samples with those of the standards (**6.4**); record this concentration as *a*mg/l. Record the weight of acetone powder taken for hydrolysis (**6.2**) as *b*mg. The total volume *v*ml of the hydrolysate may be taken as 15ml (**6.2**). Calculate the ratio *c* of dried acetone powder to wet sample used for its preparation, from the weights (expressed in the same units, e.g. g) defined in section **6.1**. Then calculate the level of analyte, *d*μg of methylhistidine per g of wet sample as received, according to the formula:

$$d = acv/b.$$

*Note that it is necessary to correct for the fact that for the derivitisation (**6.3**), 0.1ml of hydrolysate was used, whereas 0.2ml of the standard solution was used.*

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