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Methods for the Detection of *Listeria monocytogenes* in Foods

Results of an Inter-laboratory study

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An inter-laboratory comparison of three methods for the detection of Listeria monocytogenes in three food types is described. The three methods assessed included the IDF method for milk and milk products, the modified USDA method for meat and meat products, and an unpublished composite method incorporating a pre-enrichment step for recovery of stressed listeriae.

Results from this trial indicate that when three different food matrices (snack salami, cream cheese and porridge oats) were tested, all three methods detected L. monocytogenes in 90% or more of samples inoculated with 1×10^2 cells/25g and 1×10^4 cells/25g. Two false positive results were obtained: by one laboratory when the snack salami sample was examined using the IDF method and by another with cream cheese examined with the composite method. In addition, another laboratory which examined the salami reported 8 false negative results: 2 with the IDF method, 4 with the USDA method and 2 with the composite method. It should be noted however that this laboratory reported the presence or absence of L. monocytogenes finally on the result of the CAMP test which gave incorrect results.

Two laboratories encountered some problems with the cream cheese sample when using the composite method, where samples inoculated at both 1×10^2 and 1×10^4 cells per 25g were positive after 24h of enrichment but then negative after 48 h. The 6-8h pre-enrichment period used in the composite method would seem too long for use with some food samples, as enhancement of non-listeriae affected the selectivity of the isolation medium.

Results from this inter-laboratory study indicate that the IDF and USDA methods are preferable for detection of L. monocytogenes in a variety of foods. In particular, the use of buffered enrichment broths (as in the USDA method) is advocated to aid recovery of stressed cells in some foodstuffs.

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Introduction

Over recent years, *Listeria monocytogenes* has become increasingly recognised as an important foodborne pathogen[2,8,16]. Published research on foodborne listeriosis has been extensive, much having been focused on improving the methods for detection of the organism in foodstuffs. In addition, a symposium dedicated to *Listeria* methodology was organised by the Association of Analytical Chemists in 1987[1].

The ubiquitous nature of *Listeria*, resulting in its presence in a wide variety of food types, has provided a constant challenge to the Food Examiner, particularly when processed foods become contaminated or when the organism is sub-lethally injured. Many isolation media have been investigated[3,4,9] using selective agents to suppress the growth of competitive foodborne flora. Direct plating procedures however, do not recover reliably the organism and typically are used in conjunction with a prior enrichment procedure. Several enrichment procedures have also been evaluated[14]. The earliest was a cold enrichment procedure, where the ability of *Listeria* to grow at chill temperatures was used as a means of enhancement and recovery. However, this method is slow to produce results and has largely been replaced by the use of selective agents that decrease the time required to isolate the organism. The final stages of *Listeria* detection by conventional methods still require the use of confirmatory procedures, typically comprising the Gram stain, motility test, carbohydrate fermentations and haemolysis reactions. Due to the time-consuming nature of these conventional methods, alternative approaches involving monoclonal antibodies and DNA probes are increasingly being reported[18,19,21]. It is however, still the conventional cultural techniques that are adopted as standard or reference procedures.

A provisional International Standard for the detection of *L. monocytogenes* has been published by the International Dairy Federation (IDF)[10]. The International Organisation for Standardisation (ISO) has also adopted this method and published it as an ISO Standard for milk and milk products[11]. Prior to this publication members of ISO were requested to provide validation data on suitable methods as compared with the IDF method, in an attempt to provide a consensus on an internationally acceptable method. Several collaborative studies of the most commonly utilised procedures for the detection of *L. monocytogenes*, (the Food and Drug Administration (FDA) procedure and United States Department of Agriculture (USDA) procedure), have been published [20,22,24,25].

The MAFF Food Science Laboratory (FScL) assessed, as part of its on-going programme, three methods for the detection of *L. monocytogenes*. These included the IDF method, the modified USDA method and a composite method proposed by FScL incorporating the best features of several other methods - for example the inclusion of recovery agents, non-selective pre-enrichment for damaged cells, and dual selective enrichment in buffered broths. The results of the performance of each of the three methods, when used to examine three artificially contaminated food types, are reported.

Collaborative Trial Organisation

Participants

Twenty nine UK laboratories participated in the collaborative trial. They included 7 Government Laboratories, 4 PHLS Laboratories, 2 Research Association Laboratories, 1 food industry Laboratory and 15 Public Analyst Laboratories.

Sample preparation

Samples for this collaborative trial were prepared by the Leatherhead Food Research Association.

Preliminary Studies

Selection of food types

The following foods were selected for preliminary studies prior to the trial: frankfurters; snack salamis; Swiss style muesli; organic porridge oats; crispy muesli; natural country bran and cream cheeses. Based on preliminary data obtained for total viable count, Enterobacteriaciae and Listeriae, the snack salami, organic porridge oats and cream cheese were selected for inoculation/survival studies.

Selection of strains of *L. monocytogenes* for artificial inoculation of samples:

The following cultures were used for inoculation studies:

Code	Serotype	Source
L5434	1/2	salami
L5692a	1/2	salami
L4378a	4b	pate
L5619a	4b	cooked ham
BL87/53	4 (Difco serum)	Vacherin mont d'or (French)
BL88/2	4 (Difco serum)	Vacherin mont d'or (Swiss)
BL88/8	1 (Difco serum)	Mozarella cheese
BL88/14	1 (Difco serum)	Raw milk used in Mozarella manufacture

The four meat strains were used to inoculate the snack salami and the dairy strains were used to inoculate the cream cheese. The inoculum for the porridge oats comprised a mixture of the other two inocula. All strains were grown in Tryptone Soy broth (Oxoid) at 10°C until a stationary phase culture was obtained. Each inoculum was prepared by mixing equal volumes of the stock cultures and these were maintained at 4°C and enumerated daily to confirm their stability.

Survival studies

The cocktails of *L. monocytogenes* were diluted in maximum recovery diluent (Oxoid) and inoculated into the products at a target level of 10^6/g (1% inoculum). Samples were stored at 4°C and 25°C and monitored for up to 4 days for growth, survival or death. Results are shown in Table I.

On the basis of the counts obtained, snack salami, porridge oats and cream cheese A were confirmed as suitable for use in the trial.

Absence of *L. monocytogenes* in control uninoculated samples

Sufficient material for the trial was purchased from local retailers, using material from a single batch where possible. Uninoculated controls all came from a single batch. Samples for the trial, for reference and some extras were weighed aseptically into sputum pots (25g per pot) and stored at 4°C until required. For each food type, 60 pots were removed at random, bulked into 6 lots of 10 samples and tested for the presence of listeriae by the FDA method[15]. Listeriae were not detected in any sample.

Inoculation of test samples

Target inoculum levels were 1×10^2 and 1×10^4 cells per 25g sample. To allow for slight die-off after inoculation, stock cultures were diluted in maximum recovery diluent to give calculated levels of 2×10^2 or 2×10^4 cells per 0.25ml of culture dilution. 0.25ml was added to each pot and distributed evenly over the food sample. Colony counts on the inocula were made, to confirm that targeted levels had been achieved. Data on inoculum levels achieved for the trial and stability data are shown in Tables IIa and IIb.

Methods of Analysis being Collaboratively Tested

Laboratory Protocols

Protocols for each of the three methods to be assessed were dispatched to participants in advance of the trial to allow familiarisation with the methodology. A flow diagram of the 3 procedures is given in Figure 1. One modification was made to the published IDF protocol for the purposes of this trial, which involved incubation of the Oxford selective agar at 30°C instead of 37°C as prescribed in the IDF standard. It has been suggested by Curtis *et. al.*.[6] that the selective agents cefotetan and fosfomycin present in Oxford agar can inhibit the growth of *L. monocytogenes* at 37°C. One modification was also made to the published USDA method of McLain & Lee [17] in that Modified Oxford agar (MOX) was substituted by Oxford agar as used in the other two methods.

The confirmatory procedures used were identical for all three methods assessed and were as prescribed in the IDF standard. The unpublished composite method is described in full in Appendix 1.

Samples examined by participants

The 29 participating laboratories were sub-divided into 3 groups. Each group examined only one food type by each of the three methods prescribed.

Results and Discussion

The isolation rates achieved with the three methods are shown in Tables III - V and summarised in Tables VI - VIII.

IDF Method

When cream cheese and porridge oats were examined, *Listeria monocytogenes* was detected successfully in 100% of samples using the IDF method which incorporated a 48h selective enrichment in unbuffered modified Listeria enrichment broth (MLEB) followed by isolation on Oxford agar at 30°C for 48h. Some reduction in the efficacy of this method was observed with the snack salami, where three false negative results were reported, two of which had been inoculated at the lower level and one at the higher level. It is possible that either other organisms present in this fermented product, or a preservative effect, affected the ability of MLEB to recover *L. monocytogenes*. One laboratory reported a single false positive result with this sample type, using the IDF method, where *L. monocytogenes* was detected in an uninoculated sample. As the batch of sausage used for this trial was shown to be free from listeriae and no other false positive results were reported, it is probable that the sample was cross-contaminated during examination by the laboratory.

Modified USDA method

The USDA method differs from the IDF method in that, as published, it incorporates a two-stage selective enrichment procedure using different buffered broths followed by isolation on Modified Oxford agar (MOX, containing moxalactam). For the purposes of this trial however the published procedure was modified slightly in that MOX agar was substituted by Oxford agar as used in the other two methods being assessed.

The USDA method performed equally as well if not slightly better than the IDF method. No false positive or negative results were reported with the cream cheese or porridge oats sample but four false negative results (both inoculum levels) were reported by a single laboratory when the USDA method was used to examine the snack salami sample. However, this laboratory based their final decision on whether *L. monocytogenes* was present in the sample on the basis of incorrect Christie/Atkins/Munch-Peterson (CAMP) test results in spite of obtaining correct results for all other confirmatory tests.

Composite method

This method incorporated a pre-enrichment step in a non-selective broth, supplemented with agents reported to enhance the recovery of *L. monocytogenes*. The use of a two-stage selective enrichment procedure, where broths are plated out after both 24 and 48h, was assessed in this trial in order to find whether or not such measures are necessary to enhance recovery of *L. monocytogenes* from foodstuffs. However, this method did not perform as well as either the IDF or USDA methods. Although the composite method still detected 90% or more of inoculated samples, some inconsistencies were observed. No false positive or negative results were reported with the porridge oats sample but two laboratories reported false negative results with the snack salami sample at both high and low inoculum levels. Additionally, one laboratory reported that two samples of snack salami inoculated at both high and low levels were positive for *L. monocytogenes* after 48h incubation of the enrichment broths but not after 24h.

Contrasting results were observed with the cream cheese sample. Two laboratories reported false negative results after plating out the enrichment broth after 48h incubation where the sample had previously been positive after incubation for only 24h. This phenomenon has previously been noted when non-buffered enrichment broths are utilised and overgrowth of the authochthonous flora of the sample may have a deleterious effect on the listeriae[7]. An associated decrease in *p*H of the broth may damage cells and prevent recovery[5]. In this case, however, the phenomenon occurred in buffered enrichment broth and this has not previously been reported. It is possible that the lactic acid bacteria present in the cream cheese sample were able to produce inhibitory agents (bacteriocins) which prevented recovery of listeriae[13].

Most laboratories reported an increase in the growth of both typical and atypical colonies on Oxford agar when the composite method was used to examine, in particular, the dried food and cream cheese product. This is not unexpected as the method included a combined pre-enrichment and recovery stage in order to assess whether this step improved the detection rate of listeriae. In fact, recovery did not appear to be enhanced even when lower levels of *L. monocytogenes* (10^2 cells/25g) were present in samples. However, the simultaneous enhancement of contaminating flora interfered with the selectivity of the Oxford agar and thus prolonged the confirmatory procedures. This is a disadvantage that has been previously recognised when non-selective enrichment media are used to enhance the recovery of foodborne pathogens.

Confirmatory procedures

With regard to the confirmatory procedures used, these were identical for each method. Some options however, were given to participants for 3 of the tests: motility testing (agar or wet mount techniques permitted); Henry illumination (may be ommitted) and haemolysis testing (blood agar or microwell techniques permitted)[12]. The majority of laboratories used a

motility agar rather than a wet mount suspension for the determination of motility. Only 3 laboratories used Henry illumination to identify typical listeria colonies on Tryptone Soya Yeast Extract Agar, (TSYEA), and 3 laboratories used a microwell technique in preference to blood agar for the determination of haemolytic activity. One laboratory attempted the microwell technique concurrently with the normal blood agar technique and reported favourable results, with haemolysis being more clearly visible than on blood agar plates, which require careful preparation if optimum results are to be achieved.

Many laboratories continued to report difficulties with interpretation of the CAMP test, notwithstanding the laboratory which reported several false negative results, based on incorrect results obtained from this test. One laboratory had also dispensed with the use of the Rhodococcus equi control, when carrying out the CAMP test.

The requirement for washing of erythrocytes for use in haemolysis tests as prescribed in the IDF protocol was also questioned by several participants as centrifugation sometimes resulted in the haemolysis of the erythrocyte suspension, necessitating use of a fresh suspension of blood cells which was time-consuming and expensive.

Conclusion

Results from this inter-laboratory study indicate that all three methods could be used to successfully detect *L. monocytogenes* in 90% or more of artificially contaminated samples. However, the composite method was more time-consuming than either the USDA or IDF methods and results indicated that false negatives could be obtained with certain food types if the enrichment broth was incubated for 48 h. Incorporation of a pre-enrichment step did not seem to enhance the recovery of *L. monocytogenes* from the foods tested when 100 or more cells/25g sample were present. Pre-enrichment of some foodstuffs may also interfere with the selectivity of the isolation medium due to simultaneous enhancement of competing flora in the food sample. The most favourable results were obtained with the modified USDA method and the use of buffered enrichment broths is advocated. There is still concern regarding the reliability of the CAMP test, which continues to be recommended as a confirmatory procedure in international standards.

It is not possible to determine from this trial whether the efficacy of these three methods is equal when lower numbers of stressed cells of *L. monocytogenes* (i.e. <10 cells/25g) are present in a sample and this requires further investigation.

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References

1. Anon. (1988) Symposium on Listeria methodology. J. Assoc. Anal. Chem., **71**, 3,643-683.
2. Anon. (1991) National Advisory Committee on Microbiological Criteria for Foods: Listeria monocytogenes Working Group. Int. J. Food Microbiol., **14**, 3/4, 183-246.

3. Cassidy, P. Brackett, R. and Beuchat, L. (1989a) Evaluation of ten selective direct plating media for enumeration of *Listeria monocytogenes* from hams and oysters. Food Microbiol., **6**, 113-125.
4. Cassidy, P. Brackett, R. and Beuchat, L. (1989b) Evaluation of three newly developed plating media to enumerate *Listeria monocytogenes* in foods. Appl. Environ. Microbiol., **55**, 6 1645-1648.
5. Conner, D.E.,Scott, V.N. and Bernard, D.T. 1990 Growth inhibition and survival of *Listeria monocytogenes* as affected by acidic conditions. J.Food Protect. 53,**8**,652-655
6. Curtis, G.D. (1989) Selective agents for listeria can inhibit their growth. Letters in Appl. Microbiol., **8**, 169-172
7. Dallas, H.L., Tran, T.T., Poindexter, C. and Hitchins, A.D. (1990) Competition of food bacteria with *Listeria monocytogenes* during enrichment. P. 40 In Abstracts of the 90th Annual Meeting of the American Society for Microbiology, Washington, D.C. Anaheim, California, May 13-19 1990, p. 284.
8. Farber, J.M and Peterkin, P. (1991) *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Reviews, **55**, 3 476-511.
9. Golden, D., Brackett,R. and Beuchat, L. (1990) Efficacy of direct plating media for recovering *Listeria monocytogenes* from foods. Int. J. Food Microbiol., **10**, 143-156.
10. International Dairy Federation (1990) Milk and Milk products Detection of *Listeria monocytogenes*. Provisional IDF Standard No. 143:1990. IDF, Square Vergote 41, B-1040, Brussels.
11. International Organisation for Standardisation standard No. 10560 Milk and milk products. Detection and enumeration of *Listeria monocytogenes*. International Organisation for Standardisation, Geneva, Switzerland
12. in't Veld, P.H. and de Boer, E. (1991) Recovery of Listeria monocytogenes on selective agar media in a collaborative study using reference samples. Int. J. Food Microbiol., **13**, 295-300.
13. Klaenhammer, T.R. (1988) Bacteriocins of lactic acid bacteria. Biochimie, **70**, 337-349.
14. Lammerding, A. and Doyle, M. (1989) Evaluation of enrichment procedures for recovering *Listeria monocytogenes* from dairy products. Int.J. Food Microbiol., **9**, 249-268.
15. Lovett, J., Francis, D.W and Hunt, J.M. (1987) *Listeria monocytogenes* in raw milk: Detection, incidence and pathogenicity. J. Food Protect., **50**, 188-192.
16. Lund, B. (1990) The prevention of foodborne listeriosis. Brit. Food J., **92**, 4, 13-22.
17. McLain, D. and Lee, W. (1989) FSIS method for the isolation and identification of *Listeria monocytogenes* from processed meat and poutlry products. USDA Laboratory Communication No. **57**, 1989, USDA, FSIS Microbiology Division, Beltsville, MD.
18. Niederhauser, C., Candrian, U., Hofelein, C., Jermini, M., Buhler, H. and Luthy, J. (1992) Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. Appl. Environ. Microbiol., **58**, 1564-1568.
19. Noah, C., Ramos, N. and Gipson, M.V. (1991) Efficiency of two commercial ELISA kits compared with the BAM culture method for detecting *Listeria monocytogenes* in naturally contaminated foods. J. Assoc. Anal. Chem., **74**, 5, 819-821.

20. Northolt, M.D. (1989) Recovery of *Listeria monocytogenes* from dairy products using the TNCB-TNSA method and the draft IDF methods. Neth. Milk Dairy J., **43**, 299-310.

21. Walker, S.J., Archer, P. and Appleyard, J. (1990) Comparison of Listeria-Tek ELISA kit with cultural procedures for the detection of Listeria species in foods. Food Microbiol., **7**, 335-342.

22. Warburton, D.W., Farber, J.M., Armstrong, A., Caldeira, Hunt, T., Messier, S., Plante, R., Tiwari, N.P. and Vinet, J. (1991a) A comparative study of of the FDA and USDA methods for the detection of *Listeria monocytogenes*. Int. J. Food Microbiol., **13**, 2, 105-118.

23. Warburton, D.W., Farber, J.M., Armstrong, A., Caldeira, R., Tiwari, N.P., Babiuk, T., Lacasse, P. and Read, S. (1991b) A Canadian comparative study of modified versions of the FDA and USDA methods for the detectin of *Listeria monocytogenes*. J. Food Protect., **54**, 9, 669-676

24. Warburton, D.W., Farber, J.M., Powell, C., Tiwari, N.P., Read, S., Plante, R., Babiuk, T., Laffey, P., Kauri, T., Mayers, P., Champagne, M., Hunt, T., LaCasse, P., Viet, K., Smando, R. and Coates, F. (1992) Comparison of methods for optimum detection of stressed and low levels of *Listeria monocytogenes*. Food Microbiol., **9**, 127-145.

25. Yu, L.S. and Fung, D.Y. (1991) Evaluation of the FDA and USDA procedures for enumerating *Listeria monocytogenes* in ground beef. Food Microbiol., **8**, 69-74.

Table I

Survival of *L. monocytogenes* in different artificially inoculated foods.

Sample	Temp oC	Log_{10}/g 0h	Log_{10}/g 1h	Log_{10}/g 24h	Log_{10}/g 4d
Snack Salami	4	6.21	6.08	6.21	6.09
Single Cheese	4	6.03	5.90	6.11	7.15
Organic Oats	4	6.20	6.17	5.97	5.83
Cream Cheese A	4	6.03	6.03	5.83	5.87
Cream Cheese B	4	6.03	6.17	6.17	6.15
Cream Cheese C	4	6.03	6.02	5.92	5.91
Soft Cheese	4	6.03	5.93	5.89	5.73
Crisp Muesli	4	6.20	6.16	5.99	5.63
Snack Salami	25	6.21	6.08	5.20	3.08
Single Cheese	25	6.03	5.90	>8	-
Organic Oats	25	6.20	6.17	5.92	5.28
Cream Cheese A	25	6.14	6.19	6.15	6.17
Cream Cheese B	25	6.14	6.17	6.91	7.71
Cream Cheese C	25	6.14	6.12	6.10	6.07
Soft Cheese	25	6.14	6.14	6.18	5.99
Crisp Muesli	25	6.16	6.17	5.57	5.58

Table IIa

Inocula (log_{10}) achieved per 25g test sample

Food Type	Target Level	Inoculum achieved
Cream cheese A	2.3	2.33
Cream cheese A	4.3	4.22
Organic Oats	2.3	2.08
Organic Oats	4.3	4.26
Snack Salami	2.3	2.31
Snack Salami	4.3	4.31

Table IIb

Stability of *L. monocytogenes* inocula (log_{10}) during storage

Food Type	Target Level	Mean level achieved[a)]
Cream cheese A	4	4.54
Organic Oats	4	4.02
Snack Salami	4	3.88

[a)] *Listeria* colony counts made on 5 replicates of each food type inoculated at the higher level after storage at 4°C for 5d.

Table III

Detection of *L. monocytogenes* using the IDF method

Laboratory code	Food Type	Inoculum Level /25 g				
		0	1×10^2	1×10^2	1×10^4	1×10^4
1	Snack Salami	ND	+	+	+	+
2	Snack Salami	ND	ND	+	+	ND
3	Snack Salami	ND	+	+	+	+
4	Snack Salami	ND	+	+	+	+
5	Snack Salami	ND	+	+	+	+
6	Snack Salami	ND	+	+	+	+
7	Snack Salami	+	+	+	+	+
8	Snack Salami	ND	+	+	+	+
9	Snack Salami	ND	+	+	+	+
10	Snack Salami	ND	ND	+	+	+
11	Cream cheese	ND	+	+	+	+
12	Cream cheese	ND	+	+	+	+
13	Cream cheese	ND	+	+	+	+
14	Cream cheese	ND	+	+	+	+
15	Cream cheese	ND	+	+	+	+
16	Cream cheese	ND	+	+	+	+
17	Cream cheese	ND	+	+	+	+
18	Cream cheese	ND	+	+	+	+
19	Cream cheese	ND	+	+	+	+
20	Cream cheese	ND	+	+	+	+
21	Oats	ND	+	+	+	+
22	Oats	ND	+	+	+	+
23	Oats	ND	+	+	+	+
24	Oats	ND	+	+	+	+
25	Oats	ND	+	+	+	+
26	Oats	ND	+	+	+	+
27	Oats	ND	+	+	+	+
28	Oats	ND	+	+	+	+
29	Oats	ND	+	+	+	+

ND not detected; + *L. monocytogenes* detected.

Table IV

Detection of *L. monocytogenes* using the USDA method

Laboratory code	Food Type	Inoculum Level /25 g 0	1×10^2	1×10^2	1×10^4	1×10^4
1	Snack Salami	ND	+	+	+	+
2	Snack Salami	ND	ND	ND	ND	ND
3	Snack Salami	ND	+	+	+	+
4	Snack Salami	ND	+	+	+	+
5	Snack Salami	ND	+	+	+	+
6	Snack Salami	ND	+	+	+	+
7	Snack Salami	ND	+	+	+	+
8	Snack Salami	ND	+	+	+	+
9	Snack Salami	ND	+	+	+	+
10	Snack Salami	ND	+	+	+	+
11	Cream cheese	ND	+	+	+	+
12	Cream cheese	ND	+	+	+	+
13	Cream cheese	ND	+	+	+	+
14	Cream cheese	ND	+	+	+	+
15	Cream cheese	ND	+	+	+	+
16	Cream cheese	ND	+	+	+	+
17	Cream cheese	ND	+	+	+	+
18	Cream cheese	ND	+	+	+	+
19	Cream cheese	ND	+	+	+	+
20	Cream cheese	ND	+	+	+	+
21	Oats	ND	+	+	+	+
22	Oats	ND	+	+	+	+
23	Oats	ND	+	+	+	+
24	Oats	ND	+	+	+	+
25	Oats	ND	+	+	+	+
26	Oats	ND	+	+	+	+
27	Oats	ND	+	+	+	+
28	Oats	ND	+	+	+	+
29	Oats	ND	+	+	+	+

ND not detected; + *L. monocytogenes* detected.

Table V

Detection of *L. monocytogenes* using the composite method

Laboratory code	Food Type	Inoculum Level /25 g				
		0	1×10^2	1×10^2	1×10^4	1×10^4
1	Snack Salami	ND	+	+	+	+
2	Snack Salami	ND	ND	+#	ND	+#
3	Snack Salami	ND	+	+	+	+
4	Snack Salami	ND	+	+	+	+
5	Snack Salami	ND	+	+	+	+
6	Snack Salami	ND	+	+	+	+
7	Snack Salami	ND	+	+	+	+
8	Snack Salami	ND	+	+	+	+
9	Snack Salami	ND	+	+	+	+
10	Snack Salami	ND	ND	+	ND	+
11	Cream cheese	ND	+	+	+	+
12	Cream cheese	ND	+	+	+	+
13	Cream cheese	ND	+	+	+	+
14	Cream cheese	ND	+	+	+	+
15	Cream cheese	ND	+/ND$	+	+	+
16	Cream cheese	ND	+	+	+	+
17	Cream cheese	ND	+	+	+	+
18	Cream cheese	ND	+	+	+	+
19	Cream cheese	ND	+	+	+	+
20	Cream cheese	ND/+#	+/ND$	+/ND$	+/ND$	+
21	Oats	ND	+	+	+	+
22	Oats	ND	+	+	+	+
23	Oats	ND	+	+	+	+
24	Oats	ND	+	+	+	+
25	Oats	ND	+	+	+	+
26	Oats	ND	+	+	+	+
27	Oats	ND	+	+	+	+
28	Oats	ND	+	+	+	+
29	Oats	ND	+	+	+	+

ND not detected; + *L. monocytogenes* detected;

sample +ve after 48h enrichment;

$ sample -ve after 48h enrichment when previously +ve.

Table VI

Summary of total scores of each method for detection of *L. monocytogenes* in snack salami

Method	Negative Controls		Positive Samples		Combined Score	
	No. tests	+ve (%)	No. tests	+ve (%)	No. tests	+ve (%)
IDF	10	1 (10)	20	19 (95)	40	37 (92.5)
USDA	10	0 (0)	20	18 (90)	40	36 (90)
Composite	10	0 (0)	20	18 (90)	40	36 (90)

Table VII

Summary of total scores of each method for detection of *L. monocytogenes* in cream cheese

Method	Negative Controls		Positive Samples		Combined Score			
	No. tests	+ve (%)	No. tests 1×10^2/25g	+ve (%)	No. tests 1×10^4/25g	+ve (%)	No. tests	+ve (%)
IDF	10	0 (0)	20	20 (100)	20	20 (100)	40	40 (100)
USDA	10	0 (0)	20	20 (100)	20	20 (100)	40	40 (100)
Composite	10	1 (10)*	20	20[a] (100)	20	20[b] (100)	40	40 (100)

* false positive result reported after 48h enrichment;

[a] 3 false negative results reported after 48h enrichment

[b] 1 false negative result reported after 48h enrichment

Table VIII

Summary of total scores of each method for detection of *L. monocytogenes* in oat

Method	Negative Controls		Positive Samples		Combined Score			
	No. tests	+ve (%)	No. tests 1×10^2/25g	+ve (%)	No. tests 1×10^4/25g	+ve (%)	No. tests	+ve (%)
IDF	9	0 (0)	18	18 (100)	18	18 (100)	36	36 (100)
USDA	9	0 (0)	18	18 (100)	18	18 (100)	40	36 (100)
Composite	9	0 (0)	18	18 (100)	18	18 (100)	40	36 (100)

APPENDIX 1

Composite Method for the Detection of *Listeria monocytogenes*

1. Scope and Field of Application

This standard specifies procedures recommended for the detection of *Listeria monocytogenes* in foods and in particular for those foodstuffs likely to contain stressed or process-damaged cells.

2. Definitions

For the purpose of this recommended method the following definitions apply:

2.1 *Listeria* spp: Microorganisms which form typical colonies on a solid selective medium and which display the morphological, physiological and biochemical characteristics described when tests are carried out in accordance with this standard.

2.1.1 *Listeria monocytogenes*: A *Listeria* species which is considered as pathogenic and which can be differentiated from other, non-pathogenic species occurring in foods by specific biochemical characteristics.

2.2 Detection of *Listeria monocytogenes*: Determination of the presence or absence of this microorganism, in a specified mass or volume, when tests are carried out in accordance with this standard.

3. Principle

In general, the detection of *Listeria* spp. necessitates at least five successive stages as in **3.1** to **3.5**.

3.1 Pre-enrichment in resuscitative non-selective liquid medium

The pre-enrichment medium is inoculated with the test portion of the sample and incubated at 30°C for 6-8 h.

3.2 Primary enrichment in selective liquid medium

After pre-enrichment for the specified time, the pre-enrichment medium (**3.1**) is supplemented with selective agents and re-incubated at 30°C for a further 18 and 40 h.

3.3 Secondary enrichment in selective liquid medium.

After 18 h and 40 h of primary enrichment, 0.1 ml of the primary enrichment broth (**3.2**) is subcultured to 10 ml secondary enrichment broth and incubated at 30°C for 24 h.

3.4 Isolation and presumptive identification

The isolation medium is inoculated with the secondary enrichment medium (**3.3**). The isolation medium is incubated at 30°C and examined after 48 h to check for the presence of colonies which, from their appearance, are considered to be presumptive *Listeria* spp.

3.5 Confirmation of identity

Colonies of presumptive *Listeria* spp. (**3.4**) are sub-cultured onto a non-selective solid medium for confirmation of identity by means of appropriate morphological, physiological and biochemical tests.

4. Culture Media and Reagents

4.1 Basic materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the culture media, dehydrated basic components or complete dehydrated media are used. The manufacturer's instructions shall be rigorously followed. Where particular manufacturer's are specified this does not infer superiority over other commercial sources but is merely for uniformity.

The chemical products used for the preparation of the culture media and reagents shall be of recognised analytical quality.

The water used shall be distilled or deionised water, free from substances that might inhibit the growth of microorganisms under the test conditions.

When agar is specified, the amount used should be varied according to the manufacturer's instructions to give media of suitable firmness.

Measurements of *p*H shall be made using a *p*H meter, measurements being referred to a temperature of 25°C. Adjustments, if necessary, are made by adding either 1 M hydrochloric acid or 1 M sodium hydroxide solution.

If the prepared culture media and reagents are not used immediately, they shall, unless otherwise stated, be stored in the dark at a temperature between 2 and 5°C for no longer than 1 month, conditions which do not produce any change in their composition.

4.2 Culture media

4.2.1 Pre-enrichment broth (UVM1, Oxoid CM863)

4.2.1.1 Base

	g/l
Proteose peptone	5.0
Tryptone	5.0
'Lab-Lemco' powder	5.0
Yeast extract	5.0
Sodium chloride	20.0
Di-sodium hydrogen phosphate	12.0
Potassium dihydrogen phosphate	1.35
Aesculin	1.0
	*p*H 7.2 ± 0.2

4.2.1.2 Base supplement

	g/500ml base
Magnesium sulphate (anhydrous)	2.5
Ferrous sulphate $7.H_20$	0.15
Water	10 ml

Combine 2.5 g magnesium sulphate and 0.15g ferrous sulphate in 10 ml water. Warm gently to dissolve. Filter sterilise through a 0.22 μm membrane filter.

4.2.1.3 Complete medium

Preparation using commercially available medium: Suspend 27.2 g of Oxoid CM863 in 500 ml water. Dispense in 225 ml volumes. Autoclave at 121°C for 15 min and cool. Add 4.5 ml salt solution (**4.2.1.2**) per 225 ml medium. Some precipitate may form when this medium is incubated.

4.2.2 Primary enrichment broth

4.2.2.1 Supplement (Oxoid SR142)

	mg/500 ml
Nalidixic acid	10.0
Acriflavine HCl	6.0

Preparation: Immediately before use, aseptically add 2 ml sterile distilled water to a vial of Oxoid SR142. Invert gently to dissolve. 0.9 ml is added per 225 ml medium (**4.2.1.1**).

4.2.3 Secondary enrichment broth (Fraser broth, Oxoid CM859)

4.2.3.1 Base

	g/l
Proteose peptone	5.0
Tryptone	5.0
Lab-lemco powder	5.0
Yeast extract	5.0
Sodium chloride	20.0
Di-sodium hydrogen phosphate	12.0
Potassium dihydrogen phosphate	1.35
Aesculin	1.0
Lithium chloride	3.0
	*p*H 7.2 ± 0.2

Preparation: Suspend 28.7g of base (CM859) in 500 ml water. Sterilise by autoclaving at 121°C for 15 min. Cool to 50°C.

4.2.3.2 Supplement (Oxoid SR156)

Ferric ammonium citrate	0.25 g
Nalidixic acid	10.0 mg
Acriflavine hydrochloride	12.5 mg

Preparation: Immediately before use, re-constitute a vial of SR156 with 5 ml of ethanol/sterile water (1:1). Mix well.

4.2.3.3 Complete medium

Add 5ml re-constituted supplement (**4.2.3.2**) aseptically to 500 ml broth base (**4.2.3.1**). Distribute in 10 ml volumes in sterile containers.

4.2.4 Isolation medium (Oxford agar, Oxoid CM 856 + SR 140)

4.2.4.1 Agar base

	g/l
Columbia Agar Base (Oxoid CM331 see note)	39.0
Aesculin	1.0
Ferric ammonium citrate	0.5
Lithium chloride	15.0

Note - Composition of Columbia blood agar base:

	g/l
Special peptone	23.0
Starch	1.0
Sodium chloride	5.0
Agar	10.0

4.2.4.2 Supplement for 500 ml medium (Oxoid SR140)

Cycloheximide	200.0 mg
Colistin sulphate	10.0 mg
Acriflavin	2.5 mg
Cefotetan	1.0 mg
Fosfomycin	5.0 mg
Ethanol	2.5 ml
Water	2.5 ml

Preparation: Prepare exactly according to manufacturer's instructions.

4.2.4.3 Preparation of complete medium

Suspend 27.75 g Oxoid CM856 agar base in 500 ml distilled water. Boil to dissolve the agar then sterilise by autoclaving at 121°C for 15 min. Cool to 50°C and aseptically add supplement SR140 as prescribed by manufacturer (**4.2.4.2**). The *p*H of the final medium should be 7.0 ± 0.2. Distribute in quantities of about 15 ml in sterile Petri dishes and allow to solidify. Base may be prepared in advance and stored at 4°C until required.

4.2.5 Solid culture medium Tryptone Soya Yeast Extract Agar (TSYEA)

Tryptone soy broth	30 g
Yeast extract	16 g
Agar	12 to 18 g
Water	1000 ml

Preparation: Dissolve the dehydrated components or complete dehydrated medium in the water by boiling. Adjust the *p*H so that after sterilisation it is 7.3 ± 0.1. Transfer quantities of about 6 ml of the solid culture medium in tubes. Sterilise the tubes at 121°C for 15 min. Allow to set in a sloping position.

For the preparation of agar plates sterilise the solid culture medium in flasks of suitable capacity. Distribute the medium while still liquid in quantities of about 15 ml in sterile Petri dishes and allow to solidify.

4.2.6 Liquid culture medium Tryptone Soya Yeast Extract Broth (TSYEB)

Tryptone soy broth	30 g
Yeast extract	16 g
Water	1000 ml

Preparation: Dissolve the dehydrated components or the complete dehydrated medium in the water by heating gently. Adjust the *p*H so that after sterilisation it is 7.3 ± 0.1. Transfer the TSYEB in quantities of about 6 ml in tubes. Sterilise for 15 min at 121°C.

4.2.7 Blood agar (not required if microwell haemolysis technique used)

Blood agar base No 2

(Oxoid CM271, see note)	40 g
Water	1000 ml
Washed sheep red blood cells	70 ml

Note - Composition of blood agar base No 2:

Proteose peptone	15 g
Liver digest	2.5 g
Yeast extract	5 g
Sodium chloride	5 g
Agar	12 g

Preparation: Prepare washed sheep red blood cells by centrifuging defibrinated sheep blood (Oxoid SR51) at 900 × g for 30 min, aseptically removing the supernatant liquid and resuspending the pellet in sterile 0.85% saline solution to the original volume. If the centrifuged suspension has haemolysed, a fresh suspension of washed blood cells must be prepared. Dissolve the dehydrated blood agar base in the water by boiling. Adjust the *p*H so that after sterilisation it is 7.0 ± 0.1. Transfer to tubes or flasks of not more than 500 ml capacity. Sterilise the blood agar base for 15 min at 121°C. Cool the medium to 45°C. Add the washed sheep red blood cells and mix well. Distribute the medium in quantities of about 20 ml in sterile Petri dishes and allow to solidify.

4.2.8 Brain heart infusion broth (Oxoid CM225)

	g/l
Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Dextrose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
	*p*H 7.4 ± 0.2

Preparation: Prepare exactly according to the manufacturer's instructions. Distribute in 10 ml screw-capped containers and autoclave at 121°C for 15 min.

4.2.9 PBS Buffer (Oxoid BR14a)

	g/l
Sodium chloride	8.0
Potassium chloride	0.2
Disodium hydrogen phosphate	1.15
Potassium dihydrogen phosphate	0.2
	*p*H 7.3 ± 0.1

Preparation: Prepare exactly according to the manufacturer's instructions. Dispense in 10 ml volumes and autoclave at 115°C for 10 min.

4.2.10 Carbohydrate utilisation broth (Difco 0227-01-6)

4.2.10.1 Base

Proteose peptone	10 g
Beef extract	1 g
Sodium chloride	5 g
Bromocresol purple	0.02 g
Water	1000 ml

Preparation: Dissolve the components in the water by gentle heating. Distribute into tubes in quantities such that after sterilisation 9 ml will remain. Sterilise at 121°C for 15 min. Adjust the *p*H so that after sterilising it is 6.8 ± 0.2 at 25°C.

4.2.10.2 Carbohydrates

a)Rhamnose	5 g
Water	100 ml
b) Xylose	5 g
Water	100 ml

Preparation: Dissolve separately each carbohydrate in 100ml water.

Sterilise by filtration through a 0.22 µm filter.

4.2.10.3 Complete medium

For each carbohydrate, add aseptically 1 ml of the carbohydrate solution (**4.2.10.2**) to 9 ml base medium (**4.2.10.1**).

4.2.11 Motility medium (Difco 0105-01-3)

Casein peptone	20.0 g
Meat peptone	6.1 g
Agar	3.5 g
Water	1000 ml

Preparation: Dissolve the components in the water by boiling. Adjust the *p*H so that after sterilisation it is 7.3 ± 0.2. Dispense in tubes in quantities of about 5 ml. Sterilise for 15 min at 121°C.

4.2.12 CAMP (Christie/Atkins/Munch-Peterson) test

Very thin double-layered sheep blood agar plates are required for this test.

4.2.12.1 Base

Blood agar base No 2 (see 4.2.7)	40 g
Water	1000 ml

Preparation: Dissolve the dehydrated base in the water by boiling. Adjust the *p*H so that after sterilisation it is 7.0 ± 0.1. Transfer to tubes or flasks of not more than 500 ml capacity. Sterilise for 15 min at 121°C. Cool to 45°C.

4.2.12.2 Sheep blood medium

Basal layer medium (**4.2.12.1**)	100 ml
Washed sheep red blood cells (see **4.2.7**)	7 ml

Preparation: Add the washed cell suspension to the sterilised, molten base (**4.2.12.1**) cooled to 45°C.

4.2.12.3 Complete medium

Distribute the basal medium (**4.2.12.1**) in sterile Petri dishes in quantities of about 10 ml and allow to solidify. Pour a very thin layer of sheep blood medium (**4.2.12.2**) using no greater than 3 ml per plate. Allow to solidify in an even layer. If the blood is added to dishes containing the basal medium which have been prepared in advance, it may be necessary to warm the dishes by placing in an incubator at 37°C for 20 min before pouring the blood layer. Dry plates before use.

4.2.12.4 CAMP reaction cultures

A weakly ß-haemolytic strain of *Staphylococcus aureus* (eg NCTC 1803) and a strain of *Rhodococcus equi* (eg NCTC 1621) are required to undertake the CAMP test. Not all strains of *Staphylococcus aureus* are suitable for the CAMP test.

Maintenance

Maintain stock cultures of *S.aureus, R.equi, L. monocytogenes, L.innocua* and *L.ivanovii* by inoculating TSYEA slopes (**4.2.3**), incubating at 37°C for 24-48 h, or until growth has occurred and storing at 4°C. Sub-culture at least once per month.

5. Apparatus and Glassware

Usual microbiological laboratory equipment, and in particular:

5.1 Apparatus

5.1.1 Apparatus for dry sterilisation (oven) or wet sterilisation (autoclave)

Apparatus that will enter into contact with the culture media, the dilution fluid or the sample, except for apparatus that is supplied sterile (particularly plastic apparatus), shall be sterilised either

- by being kept at 170 to 175°C for not less than 1 h in an oven;
  or
- by being kept in contact with saturated steam at 121°C for not less than 15 min in an autoclave.

An autoclave is also necessary for the sterilisation of culture media and reagents. It shall be capable of being maintained at 121°C.

5.1.2 Incubator, capable of being maintained at 30°C ± 1°C.

5.1.3 Incubator, capable of being maintained at 37°C ± 1°C

5.1.4 Incubator, capable of being maintained at 25°C ± 1°C

5.1.5 Water-baths, capable of being maintained at 45 and 37°C ± 1°C

5.1.6 Blending equipment.

One of the following shall be used:

a) a rotary blender, operating at a rotational frequency between 8000 and 45000 min^{-1}, with glass or metal bowls fitted with lids, resistant to the conditions of sterilisation.

b) a peristaltic type blender (Stomacher Model 400), with sterile plastic bags.

Note - The bowls or plastic bags should have sufficient capacity to allow the sample to be properly mixed with the appropriate amount of diluent. In general, the volume of the container should be equal to about twice the volume of the sample plus diluent.

5.1.7 Loops, of platinum-iridium, nickel-chromium or plastic of diameter approximately 3 mm.

5.1.8 Inoculating needle, of platinum-iridium, nickel-chromium or plastic.

5.1.9 *p*H-meter (for measuring the *p*H of prepared media and reagents), having an accuracy of calibration of 0.1 *p*H unit at 25°C.

5.1.10 Refrigerator (for storage of prepared media and reagents), capable of being maintained at 2 to 5°C.

5.1.11 Source of beamed white light.

5.1.12 Mirror, flat or concave, for optional Henry illumination

5.1.13 Tripod for illuminating Petri dishes

5.1.14 Phase-contrast microscope with oil immersion objective.

5.1.15 Sterile round-bottom microtitre plates for alternative haemolysis test

5.1.16 Automatic pipette capable of dispensing 100 µl)

5.2 Glassware

The glassware shall be resistant to repeated sterilisation.

5.2.1 Culture bottles or flasks, for sterilisation and storage of culture media and incubation of liquid media.

5.2.2 Test tubes, approx. 16 mm in diameter and 125 mm in length or McCartney bottles.

5.2.3 Measuring cylinders, for preparation of the complete media.

5.2.4 Graduated pipettes, of nominal capacities 25, 10 and 1 ml, graduated respectively in divisions of 0.5, 0.5 and 0.1 ml.

5.2.5 Sterile Petri dishes.

5.2.6 Microscope slides/coverslips.

6. Procedure

See the safety precautions in clause 9

6.1 Inoculation of pre-enrichment medium

6.1.1 Add 225 ml pre-enrichment medium (**4.2.1.3**) to 25g test sample in a stomacher bag. Blend for 2 min. Transfer aseptically to suitable screw-capped container and incubate at 30°C for 6-8h.

6.2 Supplementation of pre-enrichment broth

6.2.1 After 6-8h incubation add 0.9 ml Oxoid SR142(**4.2.2.1**) to each 225 ml portion of pre-enrichment broth (**6.1.1**). Swirl gently to mix. Re-incubate at 30°C for 18-40h e.g total 24h and 48h incubation.

6.3 Secondary enrichment and primary plating

6.3.1 After both 24 and 48 h incubation, subculture 0.1 ml of supplemented pre-enrichment broth (**6.2.1**) to 10 ml Fraser broth (**4.2.3.3**). Incubate at 30°C for 24h.

6.4 Isolation and presumptive identification

6.4.1 After 24h incubation, streak a loopful of the secondary enrichment culture (**6.3.1**) onto the surface of an Oxford agar plate (**4.2.4.3**) so that well isolated colonies will be obtained.

6.4.2 Incubate the plate inverted at 30 ± 1°C for 48 h.

6.4.3 Examine the plate for the presence of colonies typical of *Listeria* spp. (colonies surrounded by dark brown or black haloes).

6.5 Confirmation

6.5.1 Selection of colonies for confirmation

From each plate of isolation medium (Oxford agar, **4.2.4.3**) select three typical or suspect colonies or, if there are fewer than three such colonies, select all for confirmation.

6.5.2 Incubation

Streak the selected colonies onto the surface of TSYEA plates (**4.2.5**) in a manner which will allow well isolated colonies to develop. Incubate the plates at 37°C for 24 h or until growth is satisfactory. The thinness of the agar medium (15 ml/plate) is important for good Henry illumination.

Examine the plates using beamed white light, powerful enough to illuminate plates well, striking the bottom of the plate at a 45 degree angle (see Fig. II). When examined in this obliquely transmitted light from directly above the plate, colonies of *Listeria* spp. exhibit a blue colour and granular appearance.

Note: *The use of Henry illumination is optional. If it is not used proceed as described from* ***6.4.3****.*

If TSYEA plates do not show ample, well-isolated, typical colonies, restreak a colony and proceed as described above.

6.5.3 Catalase reaction

Pick a typical colony and suspend it in a drop of 3% hydrogen peroxide solution on a slide. Cover with a coverslip to prevent aerosol formation. *Listeria* spp. are catalase positive demonstrated by the formation of gas bubbles.

6.5.4 Morphology and staining properties

6.5.4.1 Prepare a wet mount by suspending a typical colony in 0.85% saline and examine using an oil immersion phase contrast objective. After 24 hours' incubation, *Listeria* spp. appear as slim, short rods. **Characteristic tumbling motility is best exhibited by cultures grown at 25-30°C.** Always compare to a known culture. Cocci, large rods, or rods with rapid, swimming motility are not *Listeria* spp. As an additional test for motility, inoculate the motility medium (**4.2.11**) by stabbing and incubate at 25°C for 48 h. Examine for growth around the stab. If negative, incubate for an additional 5 days. *Listeria* spp. are motile, giving a typical umbrella-like growth pattern.

6.5.4.2 Test for Gram reaction. *Listeria* spp. are Gram-positive.

6.5.5 Haemolysis

If the morphological and physiological characteristics, and catalase reaction indicate the possibility of *Listeria* spp., inoculate blood agar plates (**4.2.7**) to determine the haemolytic reaction.

Dry the agar surface well before use. Draw a grid on the plate bottom, marking 20-25 spaces/plate. Take a typical colony from the TSYEA (**4.2.5**) plate and stab one space for each culture, using an inoculating needle. Simultaneously stab positive and negative control cultures (*L.monocytogens, L.ivanovii* and *L.innocua*).

After 48 h incubation at 37°C, examine the test strains and controls. *L. monocytogenes* shows narrow, slight zones of clearing (ß-haemolysis): *L.innocua* should show no clear zone around stab. *L.ivanovii* usually shows wide, clearly delineated zones of ß-haemolysis. Hold plates up to a bright light to compare test cultures with controls.

6.5.5.1 Haemolysis using microwell technique.

As an alternative to the preparation of blood agar plates for the determination of haemolytic activity, a microwell method may be used.

Prepare a 2% sheep erythrocyte suspension by washing i.e centrifuging and resuspending, sterile defibrinated sheep blood three times in PBS buffer, *p*H 7.3 (**4.2.9**). From this suspension pipette 100 µl in duplicate into wells of a microtitre plate (round bottom). To the erythrocyte suspension add 100 µl of a culture of suspect listeriae

grown at 37°C for 48h in Brain Heart Infusion broth (**4.2.8**, Oxoid CM225). Incubate the microtitre plate for 45 min at 37°C followed by incubation for 2h at 4°C. The presence of haemolysins are shown by a homogeneous red liquid. A clear supernatant with a layer of red blood cells on the bottom of the well indicate no haemolytic activity. Reference strains of *L. monocytogenes* and *L. innocua* should be run concurrently with this test.

6.5.6 Further biochemical confirmation

For these assays use a culture in TSYEB (**4.2.6**) corresponding to the typical colony used for the haemolysis reaction (**6.5.5**). Pick the typical colony and suspend in a tube containing TSYEB. Incubate for 24 h at 37°C.

6.5.6.1 Carbohydrate utilisation

Inoculate the carbohydrate fermentation broths (**4.2.10.3**) each with one loopful of the TSYEB culture (**6.5.6**). Incubate for up to 7 days at 37°C, although positive reactions (acid formation indicated by a yellow colour) occur mostly within 24-48 h.

6.5.6.2 CAMP test

Streak the *S.aureus* and *R.equi* cultures in single lines across the blood agar plate (**4.2.12.3**) so that the two cultures are parallel and diametrically opposite (see Fig. I). A thin, even inoculum is required. This can be obtained by using an inoculating needle or a loop held at right angles to the agar. Streak the test strain in a similar fashion at right angles to these cultures so that the test culture and reaction cultures do not touch but at their closest are about 1-2 mm apart. Several test strains may be streaked on the same plate.

Simultaneously, streak control cultures of *L. monocytogenes*, *L.innocua* and *L.ivanovii*. Incubate the plates at 37°C for 18-24 h.

Positive reactions are indicated by an enhanced zone of ß-haemolysis at the intersection of the test strain with either the *S.aureus* and *R.equi* culture. However, the appearance of positive results varies with the reaction culture. A positive reaction with *R.equi* is seen as a wide (5-10 mm) 'arrow-head' of haemolysis. Small (about 1 mm) zones of weak haemolysis around the intersection of the test and *R.equi* cultures are negative reactions. A positive reaction with *S.aureus* is seen as a small rounded zone of enhanced haemolysis extending only about 2 mm from the test strain and within the weakly haemolytic zone due to growth of the *S.aureus* culture. Large zones of haemolysis around the *S.aureus* culture do not occur.

6.6 Interpretation of morphological and physiological properties and of biochemical reaction

All *Listeria* spp. are small, Gram-positive rods (only with 24 h old cultures) that demonstrate motility in wet mount and in the motility medium. They are catalase positive. *L. monocytogenes* utilises rhamnose but not xylose.

L. monocytogenes, *L.ivanovii* and *L.seeligeri* (weak) produce ß-haemolysis in blood agar stabs and show positive reactions in the microwell test. Remove the colony to examine the haemolysis underneath the colony. Of the three haemolytic *Listeria* spp. only L. monocytogenes fails to utilise xylose and is positive for rhamnose utilisation.

L. monocytogenes and *L. seeligeri* show a positive CAMP reaction with *S.aureus* but not with *R.equi*. *L.ivanovii* reacts with R.equi but not with *S.aureus*. The other *Listeria* spp. show negative CAMP reactions with both reaction cultures.

7. Control Cultures

In order to check the ability of the enrichment and identification media to support the growth of *Listeria monocytogenes* a dilution of a reference culture should be introduced into a control flask of the enrichment medium (see **6.1**). Add 10-100 *L. monocytogenes* cells per flask. Proceed with the control flasks as for the test cultures to demonstrate that the positive control culture is recovered.

8. Expression of Results

In accordance with the interpretation of the results, report the presence or absence of *Listeria monocytogenes* in the test portion, specifying the mass in grams, or the volume in millilitres, of the sample tested.

9. Safety Precautions

9.1 The procedures described shall only be carried out in laboratories with suitable facilities and under control of a qualified microbiologist.

9.2 These procedures shall not be performed in quality control laboratories, or in food manufacturing or processing premises, where there is a risk of contamination of the environment.

9.3 Full bacteriological precautions shall be taken at all times whilst carrying out the procedure specified in this Standard. Particular attention shall be given to the sterilisation of used equipment and media after testing suspect samples and prior to disposal or reuse.

Note - For further and more detailed safety precautions reference is made to ISO 7218, Microbiology - General Instructions for microbiological examinations, in general and the clauses 3, 4 and 7 in particular.

10. Test Report

The test report shall show the method used and the results obtained. It shall mention all operating details not specified in this method or

regarded as optional, together with details of any incidents likely to have influenced the result.

The test report shall include all the information necessary for the complete identification of the sample.

4.2.3.3 Complete medium

Add 5ml re-constituted supplement (**4.2.3.2**) aseptically to 500 ml broth base (**4.2.3.1**). Distribute in 10 ml volumes in sterile containers.

4.2.4 Isolation medium (Oxford agar, Oxoid CM 856 + SR 140)

4.2.4.1 Agar base

	g/l
Columbia Agar Base (Oxoid CM331 see note)	39.0
Aesculin	1.0
Ferric ammonium citrate	0.5
Lithium chloride	15.0

Note - Composition of Columbia blood agar base:

	g/l
Special peptone	23.0
Starch	1.0
Sodium chloride	5.0
Agar	10.0

4.2.4.2 Supplement for 500 ml medium (Oxoid SR140)

Cycloheximide	200.0 mg
Colistin sulphate	10.0 mg
Acriflavin	2.5 mg
Cefotetan	1.0 mg
Fosfomycin	5.0 mg
Ethanol	2.5 ml
Water	2.5 ml

Preparation: Prepare exactly according to manufacturer's instructions.

4.2.4.3 Preparation of complete medium

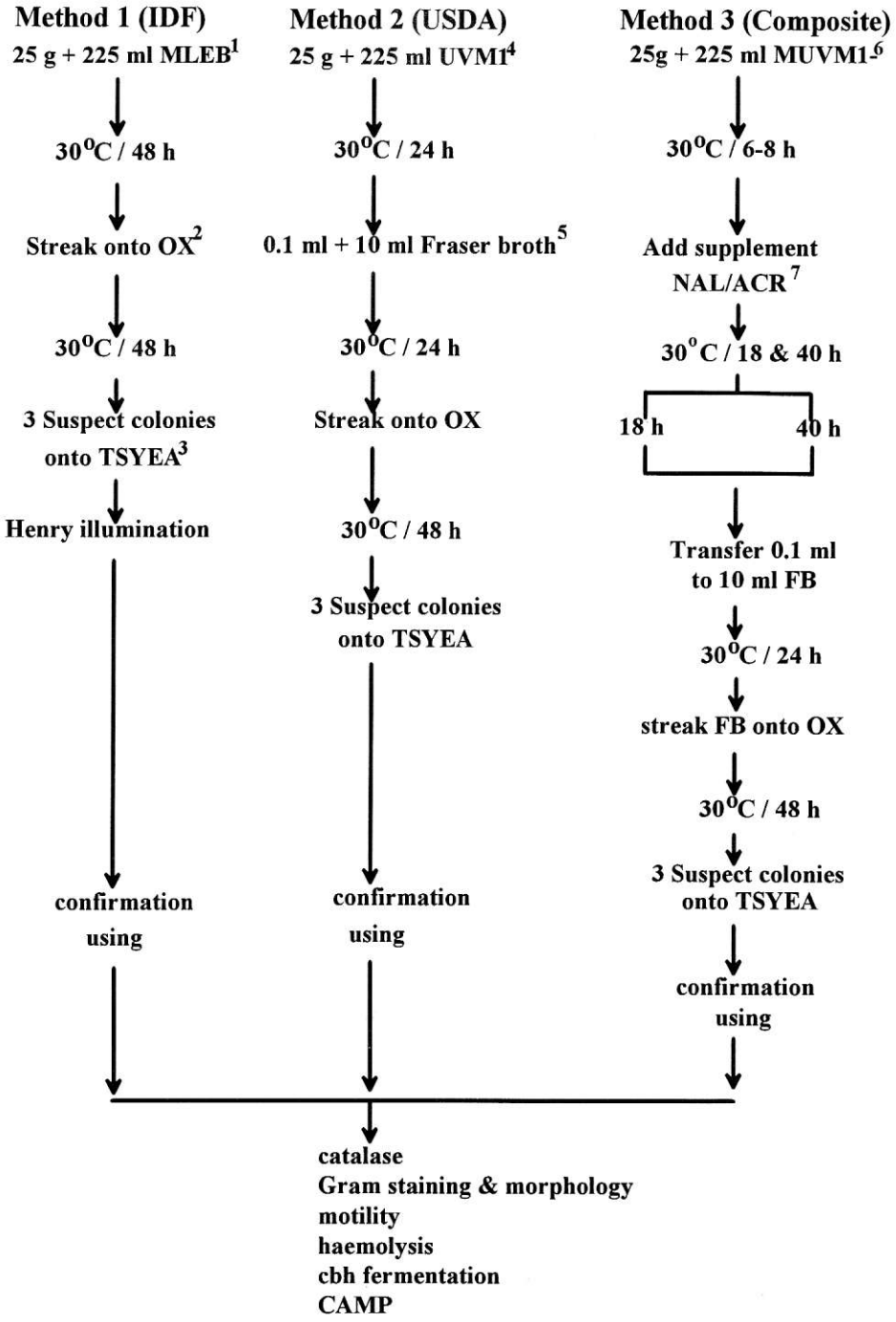
Suspend 27.75 g Oxoid CM856 agar base in 500 ml distilled water. Boil to dissolve the agar then sterilise by autoclaving at 121°C for 15 min. Cool to 50°C and aseptically add supplement SR140 as prescribed by manufacturer (**4.2.4.2**). The *p*H of the final medium should be 7.0 ± 0.2. Distribute in quantities of about 15 ml in sterile Petri dishes and allow to solidify. Base may be prepared in advance and stored at 4°C until required.

4.2.5 Solid culture medium Tryptone Soya Yeast Extract Agar (TSYEA)

Tryptone soy broth	30 g
Yeast extract	16 g
Agar	12 to 18 g
Water	1000 ml

Preparation: Dissolve the dehydrated components or complete dehydrated medium in the water by boiling. Adjust the *p*H so that after sterilisation it is 7.3 ± 0.1. Transfer quantities of about 6 ml of the solid culture medium in tubes. Sterilise the tubes at 121°C for 15 min. Allow to set in a sloping position.

Figure 1
Schematic of Methods for the Detection of *Listeria monocytogenes:* Collaborative Trial



Key to Schematic diagram:

1. *Modified Lovett enrichment broth*
2. *Listeria isolation medium (Oxford formulation)*
3. *Tryptone soya yeast extract agar*
4. *University of Vermont primary enrichment broth*
5. *Fraser broth*
6. *Modified University of Vermont primary enrichment broth without selective supplements*
7. *Nalidixic acid/acriflavine HCl supplements*

J. Assoc. Publ. Analysts, 29, 253-262

MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

No. V 30

Method for the Detection of Thermotolerant *Campylobacter* in Foods

Correspondence on this method may be sent to S. Scotter, Statutory Methods (Chemistry and Microbiology) Department, Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Food Safety Directorate, Norwich Research Park, Colney, Norwich NR4 7UQ

1. Scope and Field of Application

This method provides general guidance on the detection of thermotolerant *Campylobacter*. It is applicable to products for human or animal consumption.

2. Definition

For the purposes of this method thermotolerant *Campylobacter* are Gram-negative, curved bacilli type micro-organisms forming characteristic colonies on solid selective media at 37°C and 42°C and which have the described biochemical characteristics under the test conditions specified.

3. Principle

In general, the detection of thermotolerant *Campylobacter* requires the following stages:

3.1 Enrichment in a liquid medium

Inoculation of a defined test portion into selective enrichment broth (Park and Sanders medium). Incubation at 32°C for 4h followed by incubation at 37°C for 2h followed by incubation at 42°C for 40-42h under microaerophilic conditions.

3.2 Plating onto selective solid media

Using the cultures obtained in **3.1**, inoculation of solid selective media campylobacter blood-free agar (CCDA); nutritive blood agar (NBG) inoculated using the differential filtration method.

Incubation at 37°C for 48h (CCDA) and 42°C for 40-42h (NBG) under microaerophilic conditions to detect the presence of thermotolerant *Campylobacter*.

3.3 Confirmation

Subculture of presumptive thermotolerant *Campylobacter* and confirmation using appropriate biochemical tests.

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4. Culture Media and Reagents

4.1 Basic components

In order to improve reproducibility of results, dehydrated media should be used wherever possible. Commercially available reagent preparations/supplements may also be used. The manufacturers' instructions shall be strictly adhered to.

Chemical reagents shall be of recognised analytical grade. Water used shall be distilled or deionised, free of substances likely to be inhibitory to the growth of micro-organisms.

Culture media, if not used immediately after preparation, may be stored between +1°C and +5°C in the dark for not longer than 2 weeks, unless otherwise stated.

Full details of the composition and preparation of each medium are given in Appendix 2.

4.2 Liquid enrichment broth:

4.2.1 Park and Sanders medium, (**2.1**)

4.3 Solid selective media:

4.3.1 Campylobacter blood-free medium (CCDA, **2.3**)

4.3.2 Nutritive blood agar (NBG, **2.4**)

4.4 Biochemical reagents and identification media:

4.4.1 Mueller Hinton agar (**2.5**)

4.4.2 Oxidase reagent (**2.6**)

4.4.3 Hydrogen peroxide (3% *V/V*) (**2.7**)

4.4.4 Nalidixic acid disc (30 µg)

4.4.5 Triple sugar iron agar (**2.8**)

4.4.6 Sodium hippurate solution (**2.9**)

4.4.7 Ninhydrin solution (3.5%) (**2.10**)

5. Apparatus

All apparatus or glassware coming into contact with the samples shall be sterilised before and after use by either wet heat at 121°C for 15 min or by dry heat at 170-175°C for not less than 60 min.

Usual microbiology equipment and in particular:

5.1 Water baths capable of maintaining temperatures of 32 ± 1°C; 37 ± 1°C; 42 ± 1°C; 45 - 50°C. (Air incubators capable of maintaining equivalent temperatures may be used if water baths are not available).

5.2 *p*H meter accurate to ± 0.1 *p*H unit at 25°C.

5.3 Sterile glass wide-necked flasks of suitable capacity (small headspace) for enrichment broths.

5.4 Petri dishes 90 - 100 mm of glass or plastic.

5.5 Sterile nitrocellulose filter membranes, diameter ca 47 mm, pore size 0.65 µm (available from Millipore, Watford; Whatman, Maidstone).

5.6 Sterile forceps.

5.7 Platinum-iridium loop or single use plastic loop ca 3 mm diameter.

5.8 Suitable apparatus for culturing in a microaerophilic atmosphere of 5% O_2, 10% CO_2 plus H_2 or N_2.

5.9 Sterile pasteur pipettes.

5.10 Phase contrast microscope.

Procedure

6.1 Inoculation of enrichment broth

Place a quantity of test portion in a volume of the enrichment medium (**4.2.2**) so as to obtain a 1 in 10 dilution. Blend or stomach the sample to mix.

6.2 Enrichment

Incubate the inoculated enrichment broth (**6.1**) in a water bath at 32°C for 4h and then add aseptically the antibiotic solution B (**2.1.4**) at a concentration of 5%. Transfer the broth to a water bath at 37°C for a further 2h. After this time transfer the broth to a water bath at 42°C for 40-42h.

6.3 Plating out onto solid selective agar

After the enrichment broth has been incubated for the required time, plate out as follows to obtain isolated colonies:

6.3.1 Inoculate the surface of campylobacter blood-free agar (**4.3.1**) with a loopful (**5.7**) of culture from the Park and Sanders enrichment broth (**6.1**).

Incubate under microaerophilic conditions (**5.8**) at 37°C for 48h.

6.3.2 At the same time as **6.3.1**, place a nitrocellulose filter membrane (**5.5**) aseptically with forceps (**5.6**) onto the surface of NBG agar (**4.3.2**) ensuring no air bubbles are trapped between the surface of the agar and the membrane. Using a sterile Pasteur pipette (**5.9**), add 8 drops of Park and Sanders enrichment broth (**6.1**) to the surface of the membrane. After 30 min at room temperature (when the broth has passed through the membrane) remove the membrane with sterile forceps and discard.

Incubate under microaerophilic conditions (**5.8**) at 42°C for 48h (or up to 5d if necessary).

6.4 Presumptive identification

Examine the plates for the presence of characteristic colonies of thermotolerant *Campylobacter*. *EXAMINE PLATES WITHIN 2H OF REMOVAL FROM MICROAEROPHILIC CONDITIONS:*

- on CCDA agar colonies are grey/creamy, moist and may be spreading.
- on NBG agar colonies are small, greyish translucent and irregular in shape.

6.5 Confirmation

6.5.1 Pick off at least 5 characteristic colonies from each agar plate. If there are less than 5 use all the colonies.

6.5.2 Subculture each colony onto Mueller Hinton agar (**4.4.1**) so as to obtain pure cultures of discrete colonies. Incubate at 42°C for 24h under microaerophilic conditions (**5.8**). Use the pure cultures for the biochemical tests.

6.5.3 Biochemical tests

Carry out the following biochemical tests:

6.5.3.1 Gram stain

6.5.3.2 Growth at 25°C

Streak a presumptive colony onto Mueller Hinton agar (**4.4.1**) and incubate under microaerophilic conditions (**5.8**) at 25°C for 48h. Record the presence or absence of growth.

6.5.3.3 Aerobic growth at 37°C

Streak a presumptive colony onto Mueller Hinton agar (**4.4.1**) and incubate at 37°C aerobically for 48h. Record the presence or absence of growth.

6.5.3.4 Oxidase test

Using a glass rod, smear a well isolated colony onto filter paper moistened with oxidase reagent (**4.4.2**). Development of a blue coloration within 30s denotes a positive result. A commercially available equivalent may be used.

6.5.3.5 Catalase test

Deposit a colony from the Mueller Hinton agar (**4.4.1**) into 3% hydrogen peroxide (**4.4.3**) on the surface of a clean slide. The test is negative if no bubbles appear.

6.5.3.6 Sensitivity to nalidixic acid

Place a disc of nalidixic acid (30 µg) (**4.4.4**) on to the primary streak of a pure culture on Mueller Hinton agar (**4.4.1**). Incubate at 37°C for up to 48h. Observe sensitivity (presence of zone of inhibition around disc) or resistance (growth up to and around antibiotic disc).

6.6 Where confirmation to species level is required the following additional tests may be carried out.

6.6.1 H_2S test

Using the colonies isolated in **6.5.1** inoculate TSI agar using a wire by stabbing the butt of TSI slopes (**4.4.5**). Incubate at 42°C for 24h (or up to 5d if necessary) in a microaerophilic atmosphere (**5.8**). Blackening of the butt of TSI denotes a positive result. (This medium may also be used for determination of sugar fermentation reactions when required).

6.6.2 Hippurate hydrolysis test

Using the colonies isolated in **6.5.1** avoiding the introduction of any agar, inoculate 0.5ml of sodium hippurate solution (**4.4.6**) with a loop.

Shake to mix and incubate for 2h in a water bath at 37°C. Add 0.2ml of 3.5% ninhydrin solution (**4.4.7**) and incubate for a further 10 min at 37°C. A deep violet colour indicates a positive reaction; a light violet colour a negative reaction.

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. Interpretation of Results

Thermotolerant *campylobacters* give the following results:

morphology	small curved rods
motility	+ (characteristic)
Gram reaction	-
growth at 25°C	-
growth in air at 37°C	-
oxidase	+
catalase	+
nalidixic acid	S (*C. laridis R*)

Of the thermotolerant campylobacters, the most frequently isolated are *C. coli, C. jejuni* and *C. laridis*. The following additional biochemical tests may be used to differentiate between them:

	C. jejuni	*C. coli*	*C. laridis*
H_2S	-	(*)	-
Nalidixic acid	S	S	R
Hippurate hydrolysis	+	-	-

* - slight blackening of synerese water after 5d

9 Expression of Results

According to the results of confirmatory procedures, state the presence or absence of thermotolerant *campylobacter* in the test portion of the product.

10 References

10.1 Park, C.E. & Sanders, G.W., 1989. A sensitive enrichment procedure forthe isolation of *Campylobacter jejuni* from frozen foods. In Proceedings of Fifth International Workshop on Campylobacter Infections ed. Ruix-Palacios, G.M., Calva, E. and Ruix-Palacios, B.R. Puerto Vallarta, Mexico: National Instute of Nutrition, Mexico.

10.2 Scotter, S.L., Humphrey, T.J. and Henley, A. 1993. Methods for the detection of thermotolerant campylobacters in foods: results of an inter-laboratory study. J. Appl. Bact. **74**, 155-163.

10.3 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 Assurance

1.1 Limit of Detection

Although no minimum detection limit has been determined for this method, in a collaborative trial levels of ca 2 cfu per 10g of sample were able to be detected.

APPENDIX 2

Culture Media

2.1 Park and Sanders broth

2.1.1 Basal Medium (Difco Brucella broth (0495-01-1) plus sodium pyruvate and sodium citrate)

	g/l
tryptone	10.0
peptone P (Oxoid)	10.0
glucose	1.0
yeast extract	2.0
sodium citrate	1.0
sodium chloride	5.0
sodium bisulphate	0.1
sodium pyruvate	0.25
water	1000 ml

Dissolve the constituents in water, heating if required. Adjust the *p*H if necessary, so that after sterilisation it is 7.0 ± 0.1 at 25°C. Divide the medium into suitable quantities for autoclaving at 121°C for 15 min. Cool to 50°C.

2.1.2 Sterile lysed horse blood

2.1.3 Antibiotic solution A

vancomycin	0.1g
trimethoprim lactate	0.1g
Brucella broth (**2.2**)	50 ml

Dissolve the constituents in the Brucella broth (**2.2**) and filter sterilise through a 0.22 μm filter.

2.1.4 Antibiotic solution B

cefoperazone	0.032g
cycloheximide	0.100g
Brucella broth (**2.2**)	50 ml

Dissolve the constituents in the Brucella broth (B.2) and filter sterilise through a 0.22μm filter. (This solution is subsequently added to the enrichment broth during incubation at a concentration of 5%.)

2.1.5 Complete medium without antibiotic solution B

basal medium (**2.1.1**)	950 ml
lysed horse blood (**2.1.2**)	50 ml
antibiotic solution A (**2.1.3**)	5 ml

Add the blood aseptically to the basal medium and then the antibiotic solution A. Mix thoroughly. Divide the medium aseptically into 90 ml portions (or volume suitable to produce 1 in 10 dilution of test material) into suitable containers (small headspace).

2.2 Brucella broth (Difco Brucella broth (0495-01-1) plus sodium citrate)

	g/l
tryptone	10.0
peptone P	10.0
glucose	1.0
yeast extract	2.0
sodium citrate	1.0
sodium chloride	5.0
sodium bisulphite	0.1
water	1000 ml

Dissolve the constituents in water, heating if necessary. Adjust the pH if required so that after sterilisation it is 7.0 at 25°C. Divide the medium in quantities of 50 ml for the preparation of antibiotic solutions A and B for use in Park and Sanders broth.

Sterilise at 121°C for 15 min.

2.3 *Campylobacter* blood-free medium (CCDA) (Commercially available Lab M 112 or equivalent)

2.3.1 Basal medium

	g/l
peptone	25.0
bacteriological charcoal	4.0
sodium chloride	3.0
sodium desoxycholate	1.0
ferrous sulphate	0.25
sodium pyruvate	0.25
agar	12-15
water	1000 ml

Dissolve the constituents in water by boiling. Adjust the *p*H if necessary so that after sterilisation it is 7.4 ± 0.1 at 25°C. Divide the medium into suitable portions for sterilising at 121°C for 15 min. Cool to below 50°C.

2.3.2 Antibiotic solution (commercially available Lab M X112)

	Wt (mg)	final conc. mg/l
cefoperazone	80	32
amphoteracin	25	10
water		5 ml

Dissolve antibiotics in the water and filter sterilise through 0.22µm filter.

2.3.3 Complete medium

Basal medium (**2.3.1**)	500 ml
Antibiotic solution (**2.3.2**)	1 ml

Add the antibiotic solution aseptically to the cooled basal medium. Mix well and dispense 15 ml amounts into sterile petri dishes. Allow to set.

2.4 Nutritive blood agar (Oxoid Nutrient Broth No 2 plus agar or equivalent).

2.4.1 Basal medium

	g/l
meat extract	10
peptone	10
sodium chloride	5
agar	12-15
water	1000

Dissolve the constituents in water by boiling. Adjust the *p*H if necessary so that after sterilisation it is 7.4 at 25°C. Sterilise by autoclaving at 121°C for 15 min.

2.4.2 Sterile lysed horse blood

2.4.3 FBP supplement

ferrous sulphate hydrated	2.5g
sodium pyruvate	2.5g
sodium metabisulphite	2.5g
water	10 ml

Dissolve the constituents in water. Filter sterilise through 0.22μm filter.

2.4.4 Complete medium

basal medium (**2.4.1**)	950 ml
lysed horse blood (**2.4.2**)	50 ml
FBP supplement (**2.4.3**)	1 ml

Add the blood aseptically to the cooled basal medium followed by the FBP supplement, mix thoroughly. Pour ca 15 ml in to sterile petri dishes and leave to set. Immediately before use dry the plates to remove surface water.

2.5 Mueller Hinton agar (commercially available)

2.5.1 Complete medium

	g/l
meat infusion	6.0
casein hydrolysate	17.5
starch	1.5
agar	12-15
water	1000 ml

Dissolve the constituents in the water, by boiling. Adjust the *p*H so that after sterilisation it is 7.4 at 25°C. Divide the medium into suitable capacity for sterilisation at 121°C for 15 min. Cool to 50°C. Dispense ca 15 ml into sterile petri dishes and leave to set. Immediately prior to use, dry the plates to remove surface water.

2.6 Oxidase reagent

1% aqueous solution of *N, N* tetramethyl-*p*-phenelenediamine oxalate. Make up immediately before use. Development of a blue coloration denotes a positive result. Commercially available equivalents may be used.

2.7 Hydrogen peroxide solution (3% *V/V*)

2.8 Triple sugar iron agar (commercially available)

	g/l
meat extract	3.0
yeast extract	3.0
peptone	20.0
sodium chloride	5.0
lactose	10.0
sucrose	10.0
glucose	1.0
iron (III) citrate	0.3
sodium thiosulphate	0.3
phenol red	0.024
agar	12-15
water	1000 ml

Dissolve the constituents in water by boiling. Adjust the pH if necessary so that after sterilisation it is 7.4 at 25°C. Divide the medium in 10 ml quantities in tubes of suitable capacity. Sterilise at 121°C for 15 min. Leave to set in a sloped position so as to obtain a butt of 2.5 cm.

2.9 Sodium hippurate solution

sodium hippurate	0.5g
water	50 ml

Dissolve the sodium hippurate in the water. Sterilise by filtration through 0.22µm filter. Dispense aseptically in 0.5 ml amounts and store at ca -20°C.

2.10 Ninhydrin solution

ninhydrin	1.75g
acetone	25 ml
butanol	25 ml

Dissolve the ninhydrin in the acetone/butanol mixture. Store refrigerated for no longer than 1 week.

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MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

No. V 31

Method for the Determination of the Total Revivable Colony Count by Inoculation on a Solid Medium

Correspondence on this method may be sent to Susan Scotter and Roger Wood, Statutory Methods (Chemistry and Microbiology) Department, Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Food Safety Directorate, Norwich Research Park, Colney, Norwich NR4 7UQ

1. Scope and Field of Application

This media specified in this protocol are suitable for the determination of the total revivable colony count of natural mineral waters at 22°C and 37°C

2. Definition

For the purpose of this method, viable microorganisms are all aerobic bacteria, yeasts and moulds capable of forming colonies in on the media specified under the test conditions described.

3. Principle

The determination of the revivable total colony count in natural mineral water is based on inoculation by spreading on the surface of a specified culture medium in Petri dishes, measured volumes of the sample or dilutions of the sample. One set of plates is incubated at 37°C for 24 h, and another set at 22°C for 72 h. The number of colony-forming-units (cfu) per ml of the sample is calculated from the number of colonies formed in or on the medium.

4. Culture Media and Diluents

4.1 Diluents

Use one of the following diluents:

4.1.2 Peptone saline solution (**I, 1.1**)

4.1.3 Quarter strength Ringer's solution (**I, 1.2**)

4.2 Media

4.2.1 Yeast Extract Agar (Oxoid) (**I, 2.1**)

4.2.2 Plate Count Agar (**I, 2.2**)

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5. Apparatus

Usual microbiology equipment, including:

5.1. Incubators, capable of being maintained at 37 ± 1°C and 22 ± 1°C.

5.2. Colony-counting equipment, with a method of illumination against a dark background, a lens and, preferably, a mechanical or electronic digital counter.

6. Procedure

6.1 Preparation and Inoculation

Take a 1 ml sample of the natural mineral water and make a 10 and a 100 fold dilution. Spread 0.1 ml of the dilutions over the dry surface of the solid agar medium (**4.2**). Inoculate at least two plates for every dilution used for incubation at each temperature.

6.2 Incubation and Examination

Invert the plates and incubate one set at 37 ± 1°C for 24 ± 2 h; incubate the other set of plates at 22 ± 1°C for 72 ± 3 h. Examine the plates as soon as they are removed from the incubators. Reject any plate with confluent growth.

6.3 Counting of Colonies

Count the colonies present on each plate, if necssary with magnification and the aid of a counting device (**5.2**). Determine the average number of colonies from the pairs of plates from each dilution, each plate ideally containing between 25 and 300 colonies.

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

Since each colony is assumed to have arisen from one microorganism or from a single aggregate of microorganisms, the result is expressed as the number of cfu in a specified reference volume of the sample (generally 100 ml or 1 ml) according to the following formula:

$$C_s = \frac{N_i}{(n_1 V_1 F_1) + (n_2 V_2 F_2) + \ldots.(n_n V_n F_n)}$$

where:

C_s = the number of cfu in the reference volume, V_s, of the sample
N_i = the sum of colonies on all plates counted
n_1 = the number of plates counted for a particular dilution (F_1)
V_1 = the test volume dilution F_1 in plate 1
F_1 = the dilution used for the test portion V_1
(F = 1 for an undiluted sample, F = 0.1 for a ten-fold dilution, etc.)
V_s = the reference volume chosen to express the concentration of the micro-organisms in the sample

NOTE - The final count thus obtained is the weighted average of the counts from each plate.

9. References

9.1 Scotter, S.L, Lewis, S.J., McIntosh, L., Waddell, J. and Wood, R. 1993. Evaluation of microbiological methods for natural mineral waters. Part II : Revivable total colony count - Collaborative trial. J. Assoc. Public Anal. 1993, **29 (3)**. 143-163

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 I

Diluents and Media for Determination of Revivable Total Colony Counts

It is recommended that media be prepared from dehydrated basic materials or commercially available dehydrated media. The media shall be prepared according to the manufacturer's instructions. Media may be stored at between 0°C and 5°C for no longer than 1 month, unless otherwise stated.

All chemicals used in the preparation of culture media and diluents shall be of analytical quality unless otherwise stated. All water used shall be glass-distilled or deionised.

1. Diluents

1.1 Peptone saline solution

Peptone	1.0g
Sodium chloride	8.5g
Water	1000ml

Preparation: Dissolve the ingredients in 950 ml water. Adjust the pH with NaOH or HCl so that after sterilisation it is 7.0 ± 0.1. Make up to 1000 ml and dispense in 10 ml volumes. Sterilise at 121°C for 15 min.

HCl so that after sterilisation it is 7.00.1. Make up to 100ml and dispense in 10 ml volumes. Sterilise at 121°C for 15 min.

1.2 Quarter strength Ringer's solution

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride, anhydrous	0.12 g
Sodium bicarbonate	0.05 g
Water	1000 ml

Preparation: Dissolve the ingredients and dispense in 10 ml volumes. Sterilise at 121°C for 15 min.

2. Culture Media

2.1 Yeast Extract Agar (Oxoid)

Peptone	5 g
Yeast Extract	3 g
Agar	15 g
Water	1000 ml

Preparation: Dissolve the ingredients in the water by boiling. Adjust the *p*H if necessary so that after sterilisation it is 7.2 0.2 at 25°C. Sterilise at 121°C for 15 min.

2.2 Plate Count Agar

Tryptone	5.0 g
Yeast Extract	2.5 g
Dextrose	1.0 g
Agar (Oxoid No 1)	9.0 g
Water	1000 ml

Preparation: Dissolve the ingredients in the water by boiling. Adjust the *p*H if necessary so that after sterilisation it is 7.2 ± 0.2 at 25°C. Sterilise at 121°C for 15 min.

APPENDIX II

Analytical Quality Control

General principles of analytical quality control are outlined in protocol V0 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 collaborative trial data. r should normally be taken as $\log_{10}$ 0.75 at 22°C and $\log_{10}$ 1.41 at 37°C for yeast extract agar and $\log_{10}$ 0.60 at 22°C and $\log_{10}$ 1.13 at 37°C for plate count agar.

A2 Reproducibility

The absolute difference between two test results obtained under reproducibility conditions should not be greater than the reproducibility, R, deduced from collaborative trial data. R should normally be taken as $\log_{10}$ 1.15 at 22°C and $\log_{10}$ 1.88 at 37°C for yeast extract agar and $\log_{10}$ 1.13 at 22°C and $\log_{10}$ 2.04 at 37°C for plate count agar.

A3 Limit of Detection

The limit of detection was not established for this method but is considered to be not less than 100 cfu/ml.

A4 Statistical Data Derived from Inter-Laboratory Tests

TABLE 1

Statistical Analysis of Numbers of LOG_{10}CFU/ML in Natural Mineral Water using Yeast Extract Agar.

Sample Code	1	1	2	2
Incubation Temperature (°C)	22	37	22	37
Number of Laboratories retained after eliminating outliers	15	15	16	17
Number of Laboratories eliminated as outliers	0	0	1	0
Number of results accepted after eliminating outliers	30	30	32	34
LEVEL OF ORGANISM				
Mean observed value $\bar{x}$	4.35	3.61	5.27	3.61
REPEATABILITY				
Standard Deviation S_r	0.27	0.50	0.15	0.32
Relative Standard Deviation $RSD_r(\%)$	6.17	13.96	2.94	8.95
Repeatability r $[2.8 \times S_r]$	0.75	1.41	0.43	0.90
REPRODUCIBILITY				
Standard Deviation S_R	0.41	0.67	0.36	0.58
Relative Standard Deviation $RSD_R(\%)$	9.47	18.62	6.81	16.14
Reproducibility R $[2.8 \times S_R]$	1.15	1.88	1.01	1.63

TABLE 2

Statistical Analysis of Numbers of LOG_{10}CFU/ML in Natural Mineral Water using Plate Count Agar.

Sample Code	1	1	2	2
Incubation Temperature (°C)	22	37	22	37
Number of Laboratories retained after eliminating outliers	17	17	16	16
Number of Laboratories eliminated as outliers	0	0	0	0
Number of results accepted after eliminating outliers	34	34	32	32
LEVEL OF ORGANISM				
Mean observed value $\bar{x}$	4.32	3.50	5.14	3.36
REPEATABILITY				
Standard Deviation S_r	0.22	0.40	0.22	0.40
Relative Standard Deviation RSD_r(%)	4.91	11.36	4.20	12.02
Repeatability r [$2.8 \times S_r$]	0.59	1.11	0.60	1.13
REPRODUCIBILITY				
Standard Deviation S_R	0.41	0.61	0.38	0.73
Relative Standard Deviation RSD_R(%)	9.37	17.52	7.44	21.75
Reproducibility R [$2.8 \times S_R$]	1.14	1.72	1.07	2.04

Key to Tables I and 2

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

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MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

No. V 32

Method for the Examination of Natural Mineral Waters - Detection of *Escherichia coli*

Correspondence on this method may be sent to S. Scotter, Statutory Methods (Chemistry and Microbiology) Department, Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Food Safety Directorate, Norwich Research Park, Colney, Norwich NR4 7UQ

1. Scope and field of Application

This method allows the detection and enumeration of *Escherichia coli* (*E. coli*) in natural mineral waters after filtration through a membrane, subsequent culture on a differential lactose medium and calculation of their numbers in a sample.

In practice this method would normally be used to provide an indication of recent faecal pollution.

2. Definition[a]

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

2.1 Presumptive *E. coli*: Organisms capable of forming colonies aerobically at 44 ± 0.5°C on a selective and differential lactose culture medium with the production of acid within 24 h, and which also produce gas from lactose and indole from tryptophan at 44 ± 0.5°C

[a] for further information on this organism see para 9.

3. Principle

The detection of *E. coli* in a volume of natural mineral water (usually 250 ml) is based on the filtration of the sample through a membrane filter with a pore size which will retain bacteria. The membrane is placed on either a selective lactose agar culture medium or on an absorbent pad saturated with a liquid medium containing lactose and incubated for 4 h at 30°C followed by 18 ± 2 h at 44°C and examined for the presence of *E. coli*. Characteristic colonies formed on the membrane are subcultured and subjected to confirmatory tests for gas and indole production.

4. Culture Media, Diluent and Reagents

It is recommended that media be prepared from dehydrated basic materials or commercially available dehydrated media. The media shall be prepared according to the manufacturer's instructions and selective agents added, as supplements, at the given concentrations. Media may be stored in the dark at between 0 and 5°C for no longer than 1 month,

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unless otherwise stated. Care must be taken to avoid any changes during storage.

All chemicals used in the preparation of culture media and diluents shall be of analytical quality unless otherwise stated.

4.1 Membrane Lauryl Sulphate Broth

Peptone	40 g
Yeast extract	6 g
Lactose	30 g
Phenol red (0.4% *m/V* aqueous solution)	50 ml
Sodium lauryl sulphate - specially pure	1 g
Distilled water	1000 ml

Preparation: Add the ingredients to the water and mix gently to avoid froth. The final *p*H of the sterile medium should be 7.4 to 7.5 and it may be necessary to adjust the *p*H to about 7.6 before sterilisation to achieve this. Distribute into screw-capped bottles and autoclave at 115°C for 10 min. If Membrane Lauryl Sulphate Agar is required 10-20 g of agar[a] should be added and dissolved by heating prior to sterilisation.

[a] depending on gel strength of the agar

4.2 Confirmatory Media

4.2.1 Lactose Peptone Water (for gas production)

Peptone	10 g
Sodium chloride	5 g
Lactose	10 g
Phenol red (0.4 per cent m/*V* aqueous solution)	2.5 ml
(or Andrade's indicator, prepared as below)	10 ml
Distilled water	1000 ml

Preparation: Dissolve the ingredients in the water and adjust to pH 7.5. Add the phenol red indicator and distribute in 5 ml volumes into test tubes containing inverted Durham tubes. (Alternatively, adjust to *p*H 6.8-7.0, and add the Andrade's indicator.) Autoclave the plugged tubes at 110°C for 10 min. Test for sterility by incubation at 37 ± 1°C for 24 h.

4.2.1.1 Andrade's Indicator

This is prepared by dissolving 0.5 g of acid fuchsin in 100 ml of distilled water. Add 17 ml of sodium hydroxide solution (1 mol/litre) and leave at room temperature overnight. The solution should be straw coloured the following morning. If it is at all brownish, add a little more sodium hydroxide solution and allow to stand again. This solution is strongly alkaline, and consequently media to which it is added should be adjusted previously to a *p*H of about 6.8.

4.2.2 Tryptone Water (for indole reaction)

Certain peptones which give satisfactory results in tests at 37°C are not satisfactory for the indole test at 44°C. Tryptone has been found satisfactory and is recommended.

Tryptone	20 g
Sodium chloride	5 g
Distilled water	1000 ml

Preparation: Dissolve the ingredients in the water and adjust to *p*H 7.5. Distribute in 5 ml volumes into suitably sized plugged test tubes or universal bottles, and autoclave at 115°C for 10 min.

4.2.3 Laurel Tryptose Mannitol Broth with Tryptophan

Tryptose	10 g
Mannitol	5 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2.75 g
Potassium dihydrogen phosphate	2.75 g
Sodium lauryl sulphate	0.1 g
L(-) Tryptophan	0.2 g
Distilled water	1000 ml

Preparation: Add the tryptose, mannitol, sodium chloride, phosphates and tryptophan to the water and heat to dissolve. Add the sodium lauryl sulphate and mix gently to avoid frothing. Adjust to *p*H 6.8 ± 0.2. Distribute in 5-ml volumes in tubes containing an inverted Durham tube. Plug the tubes and autoclave at 115°C for 10 min.

4.3 Reagents

4.3.1 Kovac's Reagent for Indole

Para-dimethylaminobenzaldehyde	5 g
Amyl Alcohol (free from organic bases)	75 ml
Hydrochloric acid (ρ = 1.18 g/ml)	25 ml

Preparation: Dissolve the aldehyde in the alcohol. Add the concentrated acid with care. Protect from light and store at 4 ± 1°C.

Note: The reagent should be light yellow to light brown in colour; some samples of amyl alcohol are unsatisfactory, and give a dark colour with aldehyde.

4.4 Diluent

4.4.1 Peptone Saline Solution

Peptone	1.0 g
Sodium chloride	8.5 g
Distilled water	1000 ml

Preparation: Dissolve the ingredients and dispense in convenient volumes. Autoclave at 121°C for 15 min.

5. Apparatus and Glassware

The apparatus required is normal microbiological laboratory equipment, and in particular:

5.1 Incubators or water baths capable of being maintained at 30 ± 1°C and 44 ± 0.5°C.

5.2 Membrane filtration apparatus consisting of:

5.2.1 Sterile filtration units with vacuum flask tubing, a moisture trap flask and a vacuum source;

5.2.2 Sterile forceps;

5.2.3 Sterile membrane filters, approximately 47 mm diameter, with a pore size equal to or less than 0.45µm.

6. Procedure

6.1 Filtration and Incubation

Filter a volume (not normally <250 ml) of natural mineral water in the following manner:

With sterile forceps, aseptically remove the membrane filter. Centre the membrane grid side up or face-up on the filter holder base. Place a filter funnel on to the assembly and secure it.

With vacuum on, add 20-30 ml of sterile diluent (**4.4.1**). Release vacuum. Aseptically transfer the specified volume from a well-mixed sample of natural mineral water to the funnel. Apply the vacuum and filter the entire contents. Rinse the funnel with 20-30 ml of sterile diluent twice, applying the vacuum continuously. Turn off the vacuum immediately after the last rinse has passed through the filter. Remove the filter funnel and with sterile forceps remove the membrane filter from the base.

If a solid isolation medium is chosen roll the membrane filter onto the agar surface in the Petri dish, making sure that air is not trapped between the membrane and the agar surface. If an air bubble is observed, the membrane should be raised and again rolled onto the agar to eliminate the air.

Alternatively, if a liquid isolation medium is chosen, roll the membrane filter in a similar manner onto a sterile pad soaked with this medium. The same precaution to avoid air bubbles should be taken. Incubate each membrane for 4 h at 30 ± 1°C followed by 10-24 h at 44 ± 0.5°C.

6.2 Examination of Membranes

After incubation examine the membranes and count as presumptive *E. coli* organisms all colonies, irrespective of size, which show the following characteristics.

6.2.1 On Membrane Lauryl Sulphate Broth or Agar (**4.1**): a yellow colour extending to the membrane.

6.3 Confirmatory Tests

It must be noted that the colonies on membranes are only presumptive *E. coli.* Since gas production is not detected, it is important to carry out confirmatory tests.

6.3.1 Gas and Indole Production

To confirm the membrane results, subculture ten typical colonies or 20 per cent of all typical colonies (whichever is the greater number) to separate tubes of lactose peptone water (**4.2.1**) and to separate tubes of tryptone water (**4.2.2**) and incubate at 44 ± 0.5°C for 24 h. Alternatively each colony of presumptive *E. coli* may be subcultured to separate tubes of the prescribed single step media (**4.2.3**) and incubated at 44 ± 0.5°C for 24 h. Look for:

(a) production of gas;

(b) development of a red colour at the surface of the culture after the addition of 0.2-0.3 ml of Kovac's reagent (**4.3.1**).

These reactions confirm the presence of presumptive *E. coli*.

6.3.2. Typical colonies may be subcultured onto nutrient agar and incubated at 30°C for up to three days before continuing with confirmation steps as described above.

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. Calculation of Results

Calculate the proportion of all typical colonies subcultured in 6.3.1 which were confirmed as presumptive *E. coli*. Using this number calculate the number of presumptive *E. coli* obtained in **6.2**.

Example: assuming nine out of ten colonies are confirmed as being *E. coli*, the proportion of all typical colonies subcultured in 6.3.1 which can be regarded as presumptive *E. coli* is thus 90%. Therefore the number of presumptive *E. coli* forming colonies in **6.2** may be calculated as = 0.9 × the total number of typical colonies counted.

9. Further Microbiological Information Relevant to the Coliform Group of Organisms

Presumptive *E. coli* are Gram-negative, non-sporing, oxidase-negative, rod-shaped bacteria, which are capable of aerobic and facultatively anaerobic growth in the presence of bile-salts (or other surface-active agents with similar growth-inhibiting properties), and which are also able to ferment lactose with the production of acid (or aldehyde), and gas within 48 h when incubated at a temperature of 44°C.

Presumptive *E. coli* which also give a positive result in the methyl red test and can decarboxylate *l*-glutamic acid, but which are not able to produce

acetyl methyl carbinol, utilise citrate as the sole source of carbon or to grow in potassium cyanide broth, may be regarded as *E. coli*.

10. References

10.1 Lewis, S.J., McIntosh, L., Scotter, S.L., Waddell, J. and Wood, R. 1988. Evaluation of methods of analysis for natural mineral waters: Part 1 - Escherichia coli : Collaborative trial. J. Assoc. Public Analysts, **26**, 33-47.

10.2 International Standards Organisation 1990, Water Quality - Detection and enumeration of coliforms organisms, thermotolerant coliform organisms and presumptive *Escherichia coli* - Part 1 : membrane filtration method. ISO 9308-1.

10.3 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 Assurance

General principles of analytical quality control are outlined in protocol V.0 of the series[3].

Precision data from this trial have been calculated from an absolute minimum number of results and in this case are considered to be better than that which would normally be expected.

A1. Repeatability

The absolute difference between two test results obtained under repeatability conditions should not be greater than the repeatability r, deduced from collaborative trial data summarised in Table 1. At about 60 cells/250 ml water, r may be taken as $\log_{10}$ 0.05. This precision corresponds to an overall relative standard deviation of repeatability (RSD_r) of about 1%

A2 Reproducibility

The absolute difference between two test results obtained under reproducibility conditions should not be greater than the reproducibility R, deduced from collaborative trial data summarised in Table 1. At about 60 cells/250 ml water, R may be taken as $\log_{10}$ 0.38. This precision corresponds to an overall relative standard deviation of reproducibility (RSD_R) of about 8%

A3 Limit of Detection

This limit has not been established, but collaborative trial data suggests that the method can detect at least 60 *cfu E. coli* / 250 ml water.

A4 Statistical Data Derived from the Results of Interlaboratory Tests

Participants in the trial examined two 250 ml samples of natural mineral water spiked with river water containing naturalised *E. coli.* A summary of the statistical data is tabulated below. Results are expressed as $\log_{10}$.

TABLE 1

Statistical Analysis of Numbers of E. Coli in Natural Mineral Water

Number of Laboratories retained after eliminating outliers	5
Number of Laboratories eliminated as outliers	1
Number of results accepted after eliminating outliers	10
LEVEL OF ORGANISM	
Mean observed value $\bar{x}$	1.72
REPEATABILITY	
Standard Deviation S_r	0.02
Relative Standard Deviation RSD_r(%)	1.07
Repeatability r [$2.8 \times S_r$]	0.05
REPRODUCIBILITY	
Standard Deviation S_R	0.14
Relative Standard Deviation RSD_R(%)	8.00
Reproducibility R [$2.8 \times S_R$]	0.38

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

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MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

No. V 33

Method for the Detection of *Pseudomonas aeruginosa* in Natural Mineral Waters by Liquid Enrichment

Correspondence on this method may be sent to S. Scotter, Statutory Methods (Chemistry and Microbiology) Department, Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Food Safety Directorate, Norwich Research Park, Colney, Norwich NR4 7UQ

1. Scope and Field of Application

This protocol describes a method for the isolation of *Pseudomonas aeruginosa* from natural mineral waters by enrichment in a liquid medium.

It is recommended for use with other waters where the expected number of *Pseudomonas aeruginosa* is low, e.g. bottled waters, or the water contains a relatively high level of residual disinfectant (e.g. swimming pools).

2. Definition

For the purpose of this method the following definition applies:

Pseudomonas aeruginosa: Micro-organisms capable of growth and producing a water soluble, fluorescent pigment in media containing asparagine and ethanol. They also produce characteristic colonies when grown on an agar medium containing milk at 42°C. Some strains are non-pigmented.

3. Principle

Measured volumes of the water sample are added to a selective medium in containers and incubated under the conditions given for the medium.

3.1 Detection

Examination of the containers for either the presence of a water-soluble fluorescing pigment under ultraviolet irradiation, or for growth.

3.2 Confirmation

Subcultures are made from each container showing growth or fluorescence onto plates of milk agar medium. After incubation, the plates are examined for typical colonies of *Pseudomonas aeruginosa*.

3.2.1 Non-pigmented and atypical strains

Subcultures are made from each container onto the surface of a solid agar plate and incubated. Pure cultures are obtained by further

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subculture onto plates of the same agar medium as required. Each pure culture is finally tested for certain bio-chemical characteristics (**9.1**).

4. Culture Media and Reagents

Use reagents of analytical reagent quality in the preparation of culture media unless otherwise specified. Prepare media using glass-distilled water, or water of equivalent quality.

Alternatively, commercially available dehydrated media can be used. The media shall be prepared according to the manufacturer's instructions and selective agents added, as supplements at the given concentrations.

4.1 Culture media

It is essential that the culture medium used be suitable for the type of water to be analysed and the purpose of the analysis. Use the following medium for the determination of presumed *Pseudomonas aeruginosa*.

4.1.1 Asparagine broth with ethanol (Drake's medium 10)

4.1.1.1 Composition

	Single strength	Concentrated
DL-asparagine	2 g	3.2 g
L-proline	1 g	1.6 g
Anhydrous dipotassium hydrogen phosphate	1 g	1.6 g
Magnesium sulphate heptahydrate	0.5 g	0.8 g
Anhydrous potassium sulphate	10 g	16 g
Ethanol	25 ml	40 ml
Water	to 1,000 ml	to 1,000 ml

4.1.1.2 Preparation

Dissolve all the constituents in the water and proceed in either of the following ways:

Sterilise the ethanol by filtration through a cellulose acetate or nitrate membrane of average pore size 0.22 µm and then add it aseptically to the medium after autoclaving and cooling. Adjust the *p*H to 7.2 ± 0.2. Store in screw-capped bottles in the dark at room temperature for up to a maximum of 3 months.

4.2 Confirmatory medium

4.2.1 Milk agar with cetrimide

4.2.1.1 Composition

Skim milk powder	100 g
Yeast extract broth (see below)	250 ml
Agar	15 g
Hexadecyltrimethylammonium bromide (cetrimide)	0.3 g
Water	to 750 ml

Yeast extract broth:

Bacteriological yeast extract	3 g
Bacteriological peptone	10 g
Sodium chloride	5 g
Water	to 1000 ml

4.2.1.2 Preparation of medium

Prepare the yeast extract broth by dissolving all the constituents in the distilled water by steaming. Adjust the *p*H to between 7.2 and 7.4. Sterilise by autoclaving at 121°C for 20 min.

Mix the sterile yeast extract broth, cetrimide and agar, and steam this mixture until the agar has dissolved. In a separate glass container, add the skim milk powder to the distilled water and mix, preferably with a magnetic stirrer, until the powder has completely dissolved. Autoclave both solutions separately at 121°C for 5 min. To prevent caramelisation of the milk, take care to follow these instructions. Cool the solutions to between 50°C to 55°C, aseptically add the milk solution to the agar medium and mix well.

4.2.1.3 Preparation of agar plates

Distribute 15 ml portions of the final agar medium into sterile Petri dishes. Allow the medium to solidify in the plates. Dry the plates. Store at 4°C ± 1°C for a maximum of 1 month.

5. Apparatus

Conventional microbiological laboratory equipment, including:

5.1 Glassware

All glassware shall be sterilised at 170°C ± 5°C for 1 h in a dry oven or at 121°C for 15 min in an autoclave before use.

Use sterile Petri dishes with a diameter of either 90 mm or 100 mm.

5.2 Containers, capable of holding 500 ml volume with head space and through which fluorescence can be viewed.

5.3 Incubators, capable of being maintained at 37°C ± 1°C and 42°C ± 0.5°C.

5.4 Ultraviolet lamp emitting light of wavelength 360 nm ± 20 nm used with facility for excluding natural light.

6. Procedure

6.1 Inoculation

Add 250 ml from each sample to an equal volume of the concentrated medium (**4.1.1.2**) . Larger volumes may be examined if desired.

6.2 Incubation

Incubate the containers at 37°C ± 1°C for 48 h. Examine for growth and fluorescence under an ultraviolet lamp in either a darkened room or apparatus designed to exclude all visible light.

6.3 Confirmation

6.3.1 Milk agar with cetrimide

Subculture a loopful of culture medium from each container showing either fluorescence or growth onto 2 milk agar plates (**4.2.1.3**). Incubate one milk agar plate at 42°C ± 0.5°C for 24 h and the other at 37°C ± 1°C for 24h. Examine the 42°C plates for growth, and the 37°C plates for pigment production and casein hydrolysis (clearing of the milk medium around the colonies) and record the reactions as shown in Table 1.

Table 1

***Pseudomonas aeruginosa* reactions**

Reaction Mode	Typical	Atypical	
	(1)	(2)	(3)
Casein hydrolysis	+	+	+
Growth at 42°C	+	+	+
Fluorescence (under UV irradiation only	+	+	-
Pyocyanine (blue-green) pigment	+	-	-

+ = positive reaction, - = negative reaction

(2 & 3) *Other bacteria can sometimes give atypical reactions. In such instances the procedure described in 6.3.3 should be followed.*

NOTE - Pigment production in the culture medium may be inhibited by the growth of bacteria other than Pseudomonas aeruginosa. In such cases, the milk agar plates should be exposed to daylight at room temperature for a minimum of 4h before they are examined for pigment production.

6.3.2 Detection

All containers of the culture medium exhibiting either growth or fluorescence, which yield colonies (after subculture on milk agar plates) that produce either reaction (1) or (2) (see table 1 in **6.3.1**) shall be regarded as positive for the presence of *Pseudomonas aeruginosa*.

*NOTE - Others identified as non-pigmented or atypical Pseudomonas aeruginosa by the procedure in **6.3.3** may be included also.*

6.3.3 Non-pigmented strains

*NOTE - As a further step, it is possible to obtain confirmation of non-pigmented strains. If required, a suitable method is to take a loopful of culture medium and transfer it to a milk agar plate (**4.2.1.3**). The plate is incubated at a temperature of 37°C ± 1°C for 24 h. A well-isolated colony is selected and final confirmation is obtained by testing for certain biochemical characteristics (see annex A). Commercially available identification kits may be used.*

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

Express the results qualitatively by stating that *Pseudomonas aeruginosa* were present or absent in 250 ml of water sample.

9. Further information about *Pseudomonas aeruginosa*

9.1 *Pseudomonas aeruginosa* is the type species of the genus *Pseudomonas*.

It is a Gram negative, non-sporing rod which is oxidase and catalase positive. It is capable of growth at 42°C but not at 4°C; it usually produces a water soluble fluorescing pigment (98% of strains) and exhibits oxidative metabolism as indicated by the Hugh and Leifson test. It generally reduces nitrate beyond the stage of nitrite and produces ammonia from the breakdown of acetamide.

Gelatin is liquefied, casein is hydrolysed, but starch is not hydrolysed. The pigment pyocyanine (blue-green) is produced by more than 90% of strains.

10. References

10.1 International Standards Organisation 1988. Water Quality - Detection and enumeration of *Pseudomonas aeruginosa* Part 1 Method by enrichment in a liquid medium. ISO Standard No. 8360-1 1988.

10.2 Mutimer, R., Scotter, S. and Wood, R. 1993. Detection of *Ps. aeruginosa* in natural mineral waters - Results of an inter-laboratory study. J. Assoc. Publ. Anal. 29, **165**, 1993.

APPENDIX 1
Analytical Quality Assurance

A1 Limit of Detection

Although no limit of detection was determined for this method, in collaborative trial 10 cfu/250ml natural mineral water could be detected.

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The Annual Report of the Council of The Association of Public Analysts for 1992

Presented at the Swallow Royal Hotel, South Normanton, Derbyshire on 8th May 1993 by the Honorary Secretary, Mr M. Barnett.

Introduction

This report reviews the activity of the Council of the Association of Public Analysts and members of the Association during the year ending December 31st, 1992 and discusses events that influence the Association and its members. This year has been marked by the establishment of further scientific contacts within Europe, proposals for the reintroduction of the Journal of the Association, the continued development of the programme of training of Public Analysts and their staff, and the preparation of the Association's policy document which addresses " The Future of Food Law Enforcement in the United Kingdom.". This document presents the views of the Association and takes account of the European Food Control Directive, the proposed Additional Measures Directive and likely changes in the structure of Local Government.

The Council of the Association meets of five occasions each year and members of Council represent the Association on the Analytical Methods Committee of the Royal Society of Chemistry (R.S.C.), the Food Law Enforcement Practitioners forum, (FLEP), and committees of the Local Authorities Co-ordinating Body on Trading Standards, and act as scientific advisors to the Association of County Councils and the Association of Metropolitan Authorities. Members of the Association sit on the M.Chem.A. Examination Board, of the R.S.C. and represent on committees of CEN, the European Committee for Standardisation and on Codex Alimentarius and on committees and working parties of the Ministry of Agriculture, Fisheries and Food (MAFF)

Public Analysts

The first formal organisation for analytical chemists in the United Kingdom, the Society of Public Analysts, was formed in 1874, and with the expansion in analytical science eventually became the Society of Public Analysts and Other Analytical Chemists. This body continued until 1953 when after much discussion Public Analysts determined to regain control of their own affairs. The organisation then divided into the Association of Public Analysts on the one hand and the Society for

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Analytical Chemistry on the other, the latter eventually being absorbed into the R.S.C.

The Mastership in Chemical Analysis (M.Chem.A.), the statutory post graduate qualifying examination for Public Analysts, is administered and awarded by the Royal Society of Chemistry. This qualification embraces analytical science including chemistry and microbiology and technological and legal aspects of foods, agricultural materials, environmental matters and other issues and involves an assessment of the candidates ability to interpret scientific findings and to place these interpretations into a legal framework.

Public Analysts are identified in the Food Safety Act 1990, the Road Traffic Act 1988, and other legislation such as the Materials and Articles in Contact with Food Regulations 1987, the Natural Mineral Waters Regulations 1985 and regulations made under the Agriculture Act which were published in 1991. Most recently the qualification M.Chem.A. has been recognised in Council Directive 89/48/EEC which is concerned with the equivalence of higher educational diplomas relating to professional activity.

There are 34 Public Analysts' laboratories in the United Kingdom and typically between 10 and 50 scientific and support staff are employed in each of these.

Annual Conference 1992

The Annual Conference of the Association which was held at the Swallow Royal Hotel, Bristol, on 30th April and 1st May, 1992 had the theme "Science for Public Protection - The future within Europe ". The programme was in three sessions; "the Needs of Reorganised Local Government "," Food Enforcement Science the UK Position " and "Food Science the European Approach ". During the first session guest speakers included Dr. S.Benn, Mr. R.Manley and Mr. D.Boon and these complimented deliveries by members of the Association Mr. K.T.Chisnall and Mr. G.Keen. Mr. C.Cockbill of the Ministry of Agriculture, Fisheries and Food (MAFF), and Miss G.Fletcher-Cooke of the Department of Health (DoH) presented the roles of their respective central government departments. A view from the food industry was presented by Mr.A.Turner. Miss P.Brooke discussed the need for an integrated approach to the subject. In the final session Mrs. P.Green MEP, Dr.A.Mossel of Directorate General III of the European Commission and Dr.A.Preuss of the Food Chemists Society, Germany described the European approach.

Education and Training

Last year it was reported that the trustees of the APA Educational Trust (a trust established in 1988, registered charity: number 328086) had appointed a training officer.

The Training Officer, M.F.Godfray, has this year organised two one day meetings and the Annual Training School of the Association. The first one day meeting was held in London on the subject of "Departmental Management" and dealt with a variety of topics which all Public Analysts have to manage. The other meeting, in Worcester, was on the subject of Food Factory Inspection and covered Food Law and Codes of Practice, Items to be included during an Inspection Visit, and the assessment of factory testing and analysis facilities. In April the Annual Training School of the Association was held at the University of Reading in the Food Studies Building. It was well attended and subject matters included microbiology, entomology, microscopy, food technology and certificate writing.

Laboratories have received questionnaires which have been designed to provide the Training Officer with the information necessary to continue a successful training programme.

In recent years the regulations that govern the M.Chem,A. examination have undergone revision which has resulted in a change to the format of the written papers and the practical examination. A system of counselling for registered candidates has also been introduced . The role of the counsellors, who are invariably, senior Public Analysts, is to guide registered candidates in their preparation for the examination. In order to inform of these developments and to update on the examination standards a meeting was held in October, this being sponsored jointly by the R.S.C. and the Training Committee of the A.P.A., where members of the M.Chem A. board were able to address counsellors on these topics.

A Policy Document

This year a major activity has been the production of a policy document entitled " The Future of Food Law Enforcement in the United Kingdom." This activity was initiated as an outcome of the Conference of 1992 in order that the Association could address legislation such as the European Food Control Directive (Council Directive 89/397/EEC) and the proposed Additional Measures Directive and their anticipated implementation and also the forthcoming review of Local Government which will most probably lead to single tier or unitary authorities.

A great deal of effort has been expended by a small group of the Associations Honorary Officers under the President's Chairmanship to

produce this document. The document seeks a complete review of the U.K. Food Enforcement system with the creation of a unified structure of all disciplines involved taking into full account the science based nature of food law enforcement. (note - this policy document was approved by an Extraordinary General Meeting of the Association held in February 1993 and it will be available in May 1993.)

The Statistics of Official Samples

There were significant variations in these statistics when compared to those published last year in the annual report for the Association for the year 1992 and these were highlighted in the Monthly Bulletin of the Association. For example the number of formal Food Safety Act (1990) samples analysed in Public Analyst's laboratories was recorded by the Association as 19,184 whereas that number recorded as being Official Samples in the Official returns of MAFF to the commission was over twice this, being 46,976. The discrepancy between the two sets of data is of concern to members since it indicates that more than one standard exists for the definition of an official sample.

The Journal of the Association

D.R.Tomkins has recently become editor of the journal and has reported regularly to Council his plans for the distribution of the journal, the finances of publication and the format of the journal. Papers currently being considered for publication have been submitted from members of the Association and from scientists at the Food Science Laboratories of MAFF. This latter source will include collaboratively tested methods of analysis, work to which Public Analysts and the food industry make a major contribution. It is anticipated that these methods will be welcomed by colleagues throughout the E.C.

Environmental Matters

Proposals, which were published during the year in the form of revisions to the Control of Asbestos at Work Regulations 1987 and to the Approved Codes of Practice which are concerned with these regulations and with work with asbestos coating, insulation and insulation boards received comment form those members' laboratories engaged in this work. Changes will include the introduction of "wet stripping" as an alternative to techniques used to remove asbestos in a dry and often friable state from installations, and the recommendation that sampling on site shall be carried out by those accredited for that purpose. This

accreditation for sampling will not be restricted to laboratories accredited for the corresponding analysis of airborne asbestos fibre densities.

Members' laboratories continue to play a major role in the sampling and analysis of private water supplies and of bottled Natural Mineral Waters. Advice is provided concerning the quality of water for human consumption after remedial action to the water supply system has been carried out.

Similarly, the scientific expertise of many laboratories is applied in the fields of occupational hygiene and monitoring of land fill site containing waste materials.

Professional liaisons

Over the last year presidential duties have included major activities in liaison with governmental and professional bodies both national and international.

Liaison with the Public Health Laboratory Services (PHLS) and the Department of Health has been an essential step precipitated by the introduction of the Food Examiner in to the Food Safety Act 1990. Progress has been made over the year which it is hoped will result in a closer ties between professions with the possibility of extension into joint training regimes and common microbiological methodology.

Liaison has also taken place regularly with the Royal Society of Chemistry, the President, the Honorary Secretary, and the Co-ordinator of Scientific Affairs representing the Association and during this liaison a key role is played by Dr.S.Benn of the RSC.

At International level an informal group of European Food Law Practitioners (FLEP), established in October 1990 to achieve close co-operation between professional practitioners in member states and EFTA countries, continues to meet to identify common problems and to develop mutual confidence between enforcement systems. The president has represented the Association at this forum from its inauguration and attended two meeting in the Netherlands during 1992. The first meeting was concerned exclusively with the achievement of mutual recognition and transparency between laboratory quality systems. The second was a plenary meeting at which the work of other groups dealing with import/export, manufacturing quality systems, and food frauds of common concern were discussed. The meeting was addressed on behalf of the commission by Dr.E.Gaerner from Directorate General III, with particular reference to the Additional Measures Directive Proposals and the development of the E.C directed co-ordinated sampling programme, which is to be in operation for first time during 1993.

n The Food Control Directive 89/397/EEC has received supplementary principles in the form of a proposed Additional Measures Directive. This sets out not only the criteria for official laboratories but also the qualification requirements of those who carry out factory inspection. The Association continues to prepare methods of chemical analysis and microbiological examination in the form of a manual of peer reviewed work entitled " Validated Enforcement methods service " (VEMS). This it is anticipated will contribute to the EN45001 accreditation requirements of future official laboratories.

The Co-ordinator of Scientific Affairs, E.B.Reynolds has continued to represent the Association jointly with the President on the steering Committee of FAPAS, the Food Analysis Performance Assessment System (of MAFF). Other committees of MAFF that have APA representation are the Food Advisory Committee, the steering group on Chemical Aspects of Food Surveillance and the Food Authenticity Working Party. International fora in the form of technical working parties of CEN have also received representation. In addition to members of council, R.A.Evans, A.F.Parker, M.F.Godfray, and J.Fulstow have been active on these committees and working parties.

Conclusions

1992 has been a year of considerable activity and has seen continual progress in establishing the contribution that Public Analysts are able to make consumer health and protection.

During 1993 the European Food Control directive will be supplemented by the additional measures directive which will establish in more detail some aspects of the Control Directive. The Association will therefore need to address these requirements in detail once finally established.

Annual Statistics of the Public Analysts Scientific Service 1992

Prepared and collated by Mr. R.S. Stevens

Introduction

These statistics are produced with some misgivings, because by concentrating on numbers of samples received, they overlook other aspects of the professions role which are of equal importance. These include interpretative skill, expertise in food law, expert witness provision and scientific consultancy in many fields. Such attributes, while difficult to quantify, make the difference between a collection of test houses (even accredited test houses) and a vital, coherent, public service which is based on but certainly not restricted to laboratories.

This report is drawn from a summary of information supplied by members of the Association, who operate the UK's Public Analyst Laboratories. The level of response was as high as last year, with only three laboratories unable to respond before the deadline.

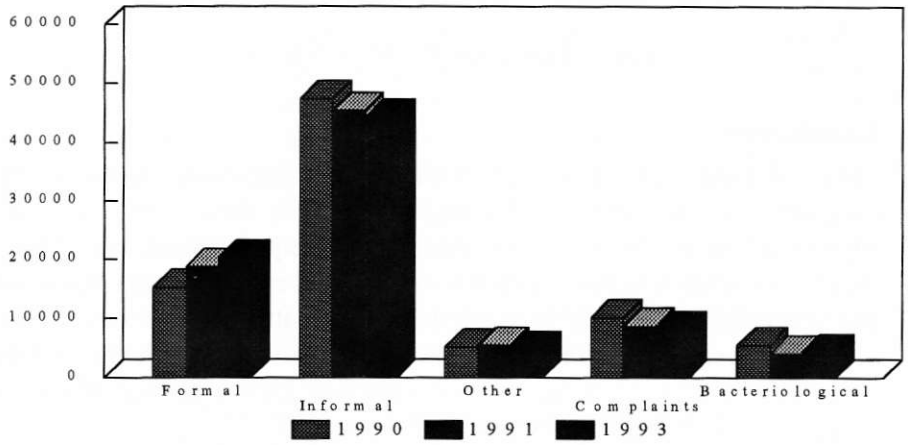
The data presented relates only to the work carried out for local authorities: work for industry and private clients is not included. The full numerical data are presented in table 1, while individual areas of work are explored below.

Food Work

The total returns for food samples are considered first; the changes relative to 1990 and 1991 are shown in chart 1.

Formal sampling continued to rise, whilst informal sampling and consumer complaint referrals fall. The overall sampling rate is 4% lower at 1.18 (1.22 in 1992) per 1000 population. However, the effect of pooling national data is to smooth out quite substantial variations from one area of the country to the next. Chart 2 shows in bar graph form the sampling rate recorded at each laboratory, which should be compared with the recommended minimum sampling rate of 2.5 per 1000 population.

Chart 1



Several points are clear from this

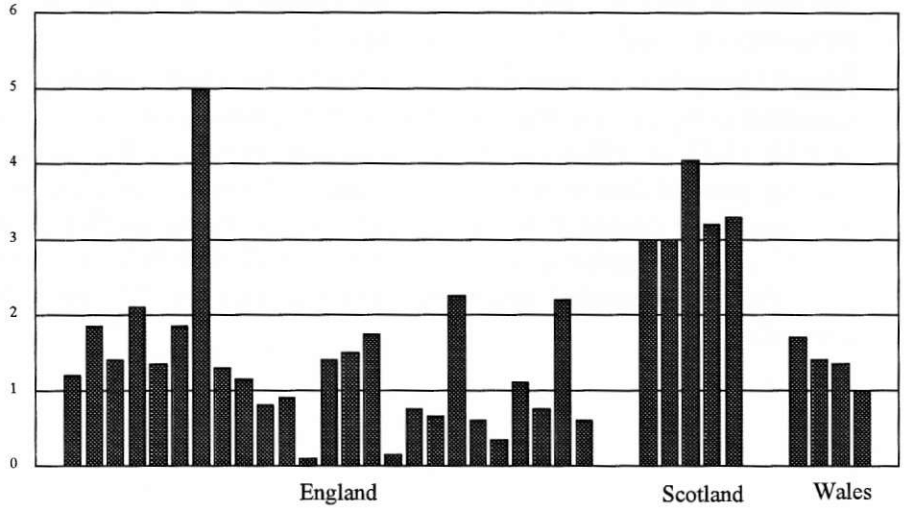
1) Scotland is the only part of the country where sampling rates to the laboratories are uniformly above the minimum target.

2) Only one laboratory in England receives the minimum target level of samples

3) If the effect of that one laboratory on the overall statistics is removed, the average in England falls to only 1.10 samples per 1000 population.

Chart 2

Planned Samples per 1000 population analysed at each laboratory



Factory Inspection

Time spent on factory inspections by staff under laboratory control is up from 206 days in 1991 to 1188 days in 1992, an increase of 570%. Even so, only a minority of laboratories are currently involved in this aspect of enforcement.

Non Food Work

In decreasing order of sample numbers, the principle activities were:

Pollution monitoring
Water quality
Atmospheric samples
Asbestos
Consumer Safety work
Agriculture Act
Miscellaneous work

The last category was a very varied selection, including radiation monitoring, toxicology, microwave oven testing, COSHH assessments, paint investigations, Trade Descriptions support, Building materials and Contract supply monitoring.

Accreditation

Fourteen member laboratories now hold NAMAS accreditation for food analysis, including microbiology, and a number of laboratories include in their accredited scope work areas such as water, asbestos, occupational hygiene monitoring and toys safety. Virtually all Public Analyst laboratories participate in externally assessed Performance Schemes providing an independent check on their work.

Conclusions

The downward trend in samples submitted for enforcement purposes under the Food Safety Act is disturbing, currently at less than 50% of the suggested minimum target level of 2.5 samples per 1000 population. The small increase in the proportion of official samples is welcomed.

The increase in Public Analyst involvement with the factory inspection process as recommended in the Codes of Practice is also encouraging though still falling well below the anticipated levels were these requirements to be applied uniformly throughout the country.

Local authorities' continuing needs for reliable scientific services to be provided are reflected in the maintenance of overall levels of demand for a wide range of work in support of UK and EC legislation.

The Association continues to improve it's standards of accreditation and service quality in line with European requirements and in particular in the

interests of the consumers it services. 40% of member laboratories now hold accreditation for food analysis, with another 46% in preparation, with accreditation targeted within two years.

Annual Statistics for Local Authority work 1992

Summary of Returns		Total of Returns	Calculated to 56 millions
Total Number of Samples received in 1992		287,253	295,992
Including:			
Foods - Formal	(Planned Sampling)	19,766	20,367
Foods - Informal	" "	43,016	44,325
Foods - Home authority	" "	1,577	1,625
Foods - Imported	" "	1,663	1,714
Foods - Complaint		7,903	8,143
Foods - Bacteriological		4,147	4,273
Foods - Total		86,985	89,631
Milks (All types)		8,215	8,465
Drinking Waters		35,954	37,048
Mineral Waters		398	410
Swimming Pool Waters		5,008	5,160
Pollution - Waters, effluents, tip leachates		45,781	47,174
Atmospheric Samples		31,840	32,809
Soil Samples		3,266	3,365
Workplace Monitoring		4,544	4,682
Asbestos - Identification and Fibre Counts		20,514	21,138
Feeding Stuffs - Agriculture Act		2,684	2,766
Feeding Stuffs - Medicines Act		308	317
Fertilisers		420	433
Toys Safety		4,051	4,174
Cosmetic Safety Regulations		764	787
Other Consumer Safety Work		4,613	4,753
Any work not covered above		30,814	31,751
Time spent on Factory Inspections (days)		1,152	1,188
Sampling rate per 1000 population		1.18	1.18
Bacteriology sampling per 1000 population		0.08	0.08

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