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Evaluation of Microbiological Methods of Analysis for Natural Mineral Water

Part 2

Revivable Total Colony Count - Collaborative Trial

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A collaborative trial to evaluate a number of media for enumerating the revivable total colony count (RTCC) at 37°C and 22°C in natural mineral waters (NMW) was carried out. Eighteen laboratories participated in this trial. Each participant enumerated the RTCC of two independent samples comprising blind duplicates using six different media. The method used was essentially as described in ISO 6222 : 1988, with the restrictions that a surface plating method was used and that the 37°C count was incubated for only 24 h.

The results indicated that agar-agar, agar with gelatine and 1/10 nutrient agar were unsuitable for the enumeration of micro-organisms. Colonies produced on these agars were pinpoint and translucent making counting difficult. Yeast extract agar (ISO and Oxoid) and Plate Count agar gave the most reproducible results with discrete, good sized colonies which were easy to count. Higher numbers of organisms were obtained at 22°C than at 37°C although results submitted by some analysts (not shown) suggest that 24 h incubation at 37°C is insufficient for enumerating maximum numbers of bacteria.

The European Community (EC) Council of Ministers has adopted a Directive on the approximation of the laws relating to the exploitation and marketing of natural mineral waters (NMW)[1]. This was subsequently translated into legislation for England, Wales and Scotland[2] and also for Northern Ireland[3]. Amongst the criteria prescribed in the Council Directive are microbiological requirements for waters at source which state that at source and thereafter, up to and including the point of sale, a natural mineral water shall be free from:-

- *Escherichia coli* and other coliforms in 250 ml;
- faecal streptococci in 250 ml;
- sporulated sulphite-reducing anaerobes in 50 ml;

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- *Pseudomonas aeruginosa* in 250 ml;
- parasites and pathogens

and in addition requires the determination of the revivable total colony count (RTCC) per ml of water at:

i) 20-22°C in 72 h on agar-agar or an agar gelatine mixture
and
ii) 37°C in 24 h on agar-agar.

In order to check that samples comply with the microbiological characteristics laid down in the Directive, suitable methods of analysis must be developed and adopted. The EC Commission Working Group on NMW is considering methodology based on International Standards Organisation (ISO) methods for the microbiological examination of potable waters. These have recently been proposed by the European Natural Mineral Water Trade association (GESEM) and accepted by the Codex Alimentarius Commission[4].

Methods being discussed in EC Working Groups are assessed within the UK by carrying out collaborative trials to validate them. This is the second such trial organised by MAFF to validate microbiological methods of analysis for NMW. The aims and objectives of these trials have been given in previous publications[5,6].

Development of the Methods of Analysis Under Test

The methods being evaluated in this collaborative trial were for the determination of the revivable total colony count (RTCC). The Council Directive on NMW states that the determination of the RTCC per ml of water is performed on agar-agar or on an agar and gelatine mixture[1]. There is considerable doubt whether either of these media is suitable for enumeration of micro-organisms. In this trial these media, as well as several alternatives internationally accepted as being appropriate for the enumeration of micro-organisms in water, were evaluated.

Definition

For the purpose of this trial, total revivable micro-organisms are all bacteria, yeasts and moulds capable of forming colonies on the medium specified under the test conditions.

Culture Media

As well as agar-agar (A) and agar gelatine (AG), yeast extract agar (two formulations YEA (O) and YEA (I)), plate count agar (PCA) and 1/10 nutrient agar (NA) were also evaluated for the determination of the RTCC in NMW.

ISO methodology for determination of the RTCC in potable waters[7] recommends the use of YEA containing 6g/l tryptone, 3g/l dehydrated yeast extract and 10-20g/l of agar i.e. YEA(I). This formulation differs

from that commercially available from Oxoid i.e. YEA (O), where the tryptone is replaced by 5g/l peptone. Both these formulations were therefore included in the trial. Temperatures and times of incubation were as given in the Directive 80/777/EEC. ISO methodology allows for either a surface or pour plate technique to be used. For this trial, collaborators were instructed to use the former as it was considered easier to use and less likely to stress water-borne organisms due to incorrect tempering of molten media as used for the pour plate technique.

Collaborative Trial Organisation

Participants

Eighteen laboratories (11 Public Analysts, 3 Public Health laboratories, 2 Government laboratories, 1 private laboratory and 1 water authority) participated in this trial.

Preparation and Distribution of Samples

Participants were asked to determine the RTCC in two independent samples at 37°C and 22°C using six different media. Each sample was despatched in blind duplicate 24 h in advance of the trial.

Ideally, final plate counts in the range 25-300 per plate were required. To attempt to meet these specifications it was necessary to monitor the variation of the RTCC of NMW with time at both temperatures and to investigate the effect of sub-sampling into small plastic containers. All pre-trial testing and sample preparation was carried out at the Strathclyde Regional Chemist's Department, and full details are given in Appendix I.

Trial Samples

The NMW samples were distributed to participants as two matched pairs labelled A & D (natural mineral water) and B & C (natural mineral water spiked with river water) respectively. Aliquots of both samples were tested prior to distribution to participants to determine the RTCC. Further samples were not used in the trial which already placed heavy demands on participants.

Methods

The methods participants were required to use are given in Appendices II and III.

Results

Results reported by participants were converted to a $\log_{10}$ basis to enable normal collaborative trial statistical analysis to be carried out. The

converted results are given in Tables 1 - 12. The results were examined for evidence of individual systematic error using Cochran's and Grubb's Tests (at $P<0.5$) progressively by procedures described by Horwitz (1988)[7]. Calculations for repeatability (r) and reproducibility (R), were carried out on those results remaining after the removal of outliers. This data is summarised in Table 13.

For sample 1, the reproducibility and the repeatability of the method at 22°C using agar-agar was significantly poorer ($P<0.05$) than for any of the other media. Nine of the 18 participating laboratories were unable to count colonies using this medium as they were either too small or failed to grow on the medium at all. The repeatability of the method using 1/10 nutrient agar at 22°C was significantly better ($P<0.05$) than for the other media. At 37°C, none of the media demonstrated significantly different reproducibility, however agar-agar showed significantly poorer ($P<0.05$) repeatability at this temperature and 12 of the 18 laboratories were again unable to count colonies using this agar.

For sample 2, the repeatability and reproducibility of the method at 22°C was significantly poorer ($P<0.05$) when using agar-agar and 8 of the 18 laboratories could not count colonies on this medium. There were no significant differences in repeatability and reproducibility between the other media at this temperature. For sample 2 at 37°C, agar-agar performed so poorly that 13 of the 18 laboratories were unable to count colonies using this medium similarly as colonies were either too indistinct from the medium or failed to grow at all, thus no statistical analyses could be performed.

Use of the two tailed t-test demonstrated that for sample 1 at 22°C, mean colony counts obtained on agar-agar were significantly lower ($P<0.05$) than those obtained using other media. This was also true for sample 2 at this temperature; however use of 1/10 nutrient agar also resulted in significantly higher mean colony counts on all but one (yeast extract agar, Oxoid) of the media.

At 37°C, use of agar with gelatine resulted in significantly lower ($P<0.05$) mean RTCC's for sample 1 as compared to the 1/10 nutrient agar, yeast extract agars' and plate count agar. For sample 2 at 37°C, agar-agar performed significantly worse than the other media.

Discussion

The aim of this trial was to test the suitability of the media for the determination of revivable total colony count as prescribed in the EC Directive 80/777. Other media appropriate for enumerating the RTCC of NMW were also evaluated.

The analysis of variance from data produced in this study clearly shows that agar-agar and agar with gelatine are not suitable media for enumerating micro-organisms in NMW. Most of the participants in the trial commented that colonies produced on agar-agar, agar with gelatine and to some extent 1/10 nutrient agar, were pinpoint and translucent

making counting extremely difficult and inaccurate although 1/10 nutrient agar did produce good reproducibility and repeatability figures. Data produced from previous analyses in our laboratory (unpublished) had also shown that larger numbers of the autochthonous flora in NMW were isolated on 1/10 NA.

In contrast, the yeast extract agars' and plate count agar gave well defined, discrete colonies which were easily counted. Several collaborators also noted that yeast and mould colonies tended to appear on yeast extract agar (ISO formulation) and plate count agar at 22°C.

There was no significant difference ($P<0.05$) in counts obtained on the two formulations of yeast extract agar. It was also noted that use of the spread plate technique resulted in an accumulation of colonies where the initial innoculum was placed on the agar which sometimes made counting difficult. Pour plates are routinely used by water microbiologists and are optional in ISO methodology. The sensitivity of the method could also be increased by using pour plates as a larger volume of water could be examined, i.e. 1 ml rather than 0.1 ml as used on spread plates. Use of the pour plate technique would also reduce the likelihood of unreadable plates due to confluent growth, although dilutions would be necessary to quantify levels normally found in NMW. The problem of confluent growth on spread plates could be alleviated by drying the agar for 15 to 20 minutes prior to incubation.

The Directive also states that plates should be incubated at 37°C for a period of 24 h. However several collaborators commented that if the plates were incubated for a further 24 h their results demonstrated that many more colonies grew after the initial 24 h period and counts obtained after incubation for this period of time probably do not reflect maximum numbers of revivable bacteria.

Conclusions

Data from this collaborative study demonstrates that agar-agar and agar with gelatine as stated in EC Directive 80/777/EEC are not suitable for the enumeration of the revivable total colony count in NMW. It is suggested that the specification for the enumeration of the revivable total colony count in NMW in any further EC Directive should require the use of yeast extract agar (Oxoid or ISO formulation) or plate count agar which gave the most reproducible colony counts and produced well defined easily counted colonies and that in any subsequent methods of analysis Directive the use of the pour plate technique be included as an alternative to the spread plate method.

Data not reported in this paper shows that maximum numbers of bacteria are not enumerated, even when yeast extract agar or plate count agar are used, if the revivable total colony count is carried out at 37°C for only 24 h, further studies should be carried out to substantiate these results.

The results of this trial have been communicated to the appropriate Working Group of the E.C.

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APPENDIX I

Pre-Trial Analysis

Trial Samples B & C

The RTCC at 22°C and 37°C of a bottle of commercially available NMW which had a suitable level of bacteria, was monitored over a fourteen week period preceeding the trial. All pre-trial testing was carried out on Plate Count Agar (PCA). Throughout this period the bottle was stored at laboratory temperature. The results of this are shown in Figure 1.

Two weeks into this monitoring period the NMW was sub-sampled into small plastic containers to test their suitability as containers for sample distribution. The RTCC in these plastic containers was monitored for a period of two weeks. The results are shown in Figure 2.

At 22°C, Figure 1 shows fluctuations in RTCC over the entire monitoring period, increasing dramatically just before the start of the trial. When the sub-samples were transferred to the small plastic containers the RTCC showed a sharp increase at both temperatures and thereafter remained stable. At 37°C the RTCC increased over the first part of the monitoring period. As the laboratory temperature fluctuated the RTCC declined to below $\log_{10}$ 1.0 after six weeks, but then recovered to a reasonable level in time for the trial.

Sample 2 (B and C) examined 24 h prior to the trial was found to have a RTCC of $\log_{10}$ 4.6/ml at 22°C and $\log_{10}$ 2.7/ml at 37°C on PCA. Results indicate (Table 13) that at the time of analysis the RTCC had increased to approximately $\log_{10}$ 5.1/ml at 22°C and to approximately $\log_{10}$ 3.3/ml at 37°C.

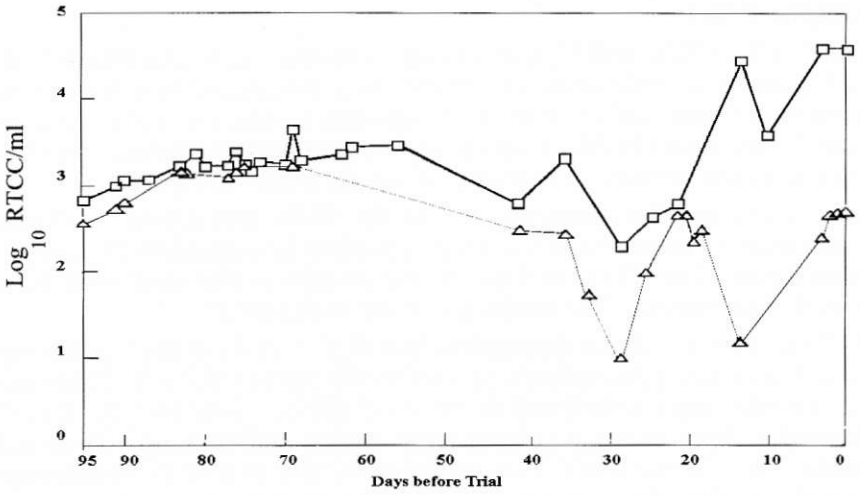
Trial Samples A & D

A bottle of NMW was spiked with a small volume of river water and the RTCC monitored at both temperatures. The RTCC remained fairly stable over a 3 week period.

Colony counts carried out on PCA 24 h prior to the trial showed that sample 1 (A and D) had a RTCC of $\log_{10}$ 3.78/ml at 22°C and $\log_{10}$ 2.5/ml at 37°C. Results indicate (Table 13) that at the time of analysis the RTCC at 22°C had increased to approximately $\log_{10}$ 4.3/ml and at 37°C the RTCC had increased to approximately $\log_{10}$ 3.5/ml.

Figure 1

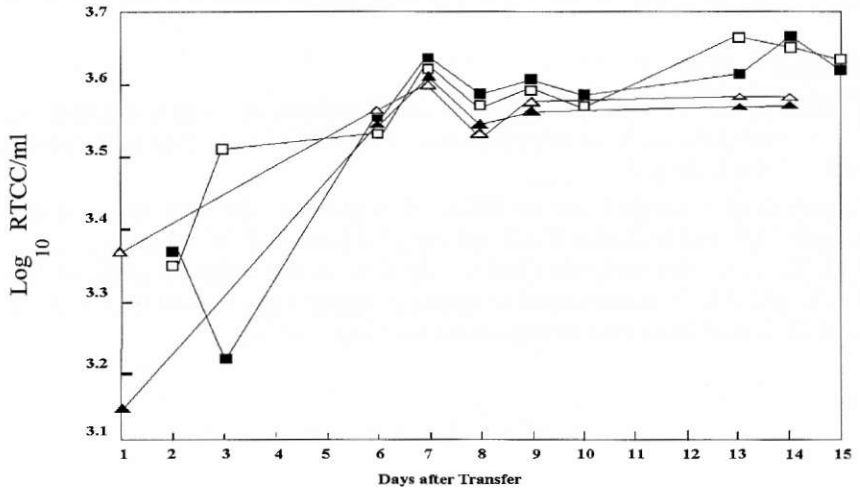
Variation with time of revivable total colony count (RTCC) in natural mineral water during storage at ambient temperature.



Legend : □ - RTCC at 22°C △ - RTCC at 37°C

Figure 2

Effect of transfer to plastic containers on numbers of organisms.



Legend : ■ - RTCC in bulk sample at 22° C □ - RTCC in bulk sample at 37° C
▲ - RTCC in subsample in plastic containers at 22 C
△ - RTCC in subsample in plastic containers at 37 C

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

1. Definition

For the purpose of this method viable micro-organisms are all aerobic bacteria, yeasts and moulds capable of forming colonies in or on the medium specified under the test conditions described.

2. 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.

3. Apparatus

Usual microbiology equipment, including:

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

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

4. Culture Media and Diluents

4.1 Diluents

Use one of the following diluents:

4.1.1 Peptone diluent (0.1%)(**III, 1.1**)

4.1.2 Peptone saline solution (**III, 1.2**)

4.1.3 Quarter strength Ringer's solution (**III, 1.3**)

4.2 Media

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

4.2.2 Yeast Extract Agar (ISO) (**III, 2.2**)

4.2.3 Plate Count Agar (**III, 2.3**)

4.2.4 Agar and Gelatine (**III, 2.4**)

4.2.5 Agar-agar (**III, 2.5**)

4.2.6 1/10 Nutrient Agar (**III, 2.6**)

5. Procedure

5.1 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.

5.2 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.

5.3 Counting of Colonies

Count the colonies present in or on each plate, if necessary with magnification and the aid of a counting device (**3.2**). Determine the average number of colonies from the pairs of plates from each dilution, each plate ideally containing between 25 and 300 colonies.

6. Calculation of Results

Since each colony is assumed to have arisen from one micro-organism or from a single aggregate of micro-organisms, 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.

7. Test Report

The test report should include:

- a statement of the numbers of cfu per ml of the sample for each media at each temperature.
- any operation(s) not specified in the method or considered optional which may have influenced the results

If there are no colonies on the plates inoculated with test volumes of the undiluted sample, express the results as less than one cfu per ml. If there are more than 300 colonies on the plates inoculated with the highest dilutions used, express the results as approximate only.

Appendix III

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 Diluent (0.1%)

Peptone	1.0g
Water	1000 ml

Preparation: Dissolve the peptone in about 950 ml of the water. Adjust the *p*H with sodium hydroxide solution [p(NaOH) = 40 g/l] or hydrochloric acid [p(HCl) = 36.5 g/l] so that after sterilisation it will be 7.0 ± 0.1. Make up to 1000 ml with water, dispense in 10 ml volumes and sterilise at 121°C for 15 min.

1.2 Peptone saline solution

Peptone	1.0g
Sodium chloride	8.5g
Water	1000ml

Preparation: Dissolve the ingredients in 950 ml water. Adjust the *p*H 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.

1.3 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 Yeast Extract Agar (ISO)

Tryptone	6 g
Yeast Extract	3 g
Agar (Oxoid No 1)	10 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.

2.3 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.

2.4 Agar with Gelatine

Agar (Oxoid No 1)	15 g
Gelatine (Oxoid)	25 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.

2.5 Agar

Agar (Oxoid No 1)	15 g
Water	1000 ml

Preparation: Dissolve the agar 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.

2.6 1/10 Nutrient Agar

Lab-Lemco powder	0.1 g
Yeast Extract	0.2 g
Peptone	0.5 g
Sodium chloride	0.5 g
Agar (Oxoid No. 1)	10 g
Water	1000 ml

Preparation: Dissolve the ingredients in the water by heating. Adjust the *p*H if necessary so that after sterilisation it will be 7.4 ± 0.2 at 25°C. Sterilise at 121°C for 15 min.

Table 1

Log_{10} colony counts obtained for sample 1 (A/D) on YEA (Oxoid)

Laboratory	Colony Count (YEA Oxoid)			
	22°C	22°C	37°C	37°C
1	4.43	3.52	4.19	3.39
2	5.18	4.64	4.27	3.36
3	4.56	4.83	2.87	3.24
4	4.49	nt	3.86	nt
5	3.55	4.38	3.24	3.71
6	4.35	4.08	4.48	3.00
7	4.78	4.62	3.99	4.42
8	4.15	4.18	4.20	4.20
9	4.71	4.72	3.71	4.51
10	5.53	nc	3.99	nc
11	4.86	4.85	4.67	3.52
12	4.33	4.24	3.98	3.32
13	4.04	4.04	4.11	3.30
14	4.46	4.39	3.03	3.00
15	4.85	nc	3.39	nc
16	4.24	4.01	2.82	3.19
17	4.58	4.26	3.89	4.38
18	3.66	3.74	2.18	2.25
Mean	4.35		3.61	
r	0.75		1.41	
SD_r	0.27		0.50	
RSD_r (%)	6.17		13.96	
R	1.15		1.88	
SD_R	0.41		0.67	
RSD_R (%)	9.47		18.62	

Legend: nt: not tested; nc: not countable;

Table 2

Log_{10} colony counts obtained for sample 1 (A/D) on YEA (ISO)

Laboratory	Colony Count (YEA ISO)			
	22°C	22°C	37°C	37°C
1	4.23	3.48	4.03	3.57
2	4.59	4.45	4.29	3.43
3	4.12	3.98	2.55	3.08
4	4.11	nt	3.85	nt
5	3.45	4.08	3.19	3.50
6	4.25	4.14	4.16	3.48
7	3.83	4.54	4.10	4.41
8	4.08	4.20	4.11	4.04
9	4.29	4.68	3.08	4.12
10	4.34	nc	2.98	3.58
11	4.44	4.48	4.26	3.54
12	4.35	4.30	4.19	4.27
13	3.93	3.84	3.40	3.26
14	4.48	4.54	4.04	3.90
15	4.74	4.82	3.03	3.11
16	4.42	4.01	3.72	3.05
17	4.53	4.13	3.99	4.33
18	3.37	3.82	2.20	3.00
Mean	4.21		3.62	
r	0.74		1.09	
SD_r	0.26		0.39	
RSD_r (%)	6.28		10.72	
R	1.03		1.59	
SD_R	0.37		0.57	
RSD_R (%)	8.77		15.65	

Table 3

Log_{10} colony counts obtained for sample 1 (A/D) on PCA

Laboratory	Colony Count (PCA)			
	22°C	22°C	37°C	37°C
1	4.18	3.46	4.04	3.53
2	5.00	4.71	4.32	3.94
3	4.29	4.25	2.83	3.01
4	4.26	nt	3.92	nt
5	3.63	3.89	3.35	3.37
6	4.33	4.12	3.10	2.93
7	4.38	4.67	4.01	4.42
8	4.18	4.15	4.00	3.85
9	5.13	4.85	3.53	4.41
10	4.42	4.66	3.26	3.66
11	4.15	4.62	4.22	3.23
12	4.29	4.28	3.55	3.37
13	4.11	3.88	3.79	2.70
14	4.46	4.43	3.57	3.61
15	4.88	4.64	4.48	3.45
16	4.32	4.61	2.61	3.28
17	4.60	4.20	3.79	3.77
18	3.55	3.68	2.10	2.05
Mean	4.32		3.50	
r	0.59		1.11	
SD_r	0.21		0.40	
RSD_r (%)	4.91		11.36	
R	1.14		1.71	
SD_R	0.41		0.61	
RSD_R (%)	9.38		17.52	

Table 4

Log_{10} colony counts obtained for sample 1 (A/D) on agar gelatine

Laboratory	Colony Count (Agar Gelatine)			
	22°C	22°C	37°C	37°C
1	3.70	3.48	4.10	3.02
2	4.79	nc	2.96	2.76
3	4.37	4.45	a	a
4	4.04	nt	3.67	nt
5	4.02	3.90	3.33	3.50
6	4.20	4.28	3.02	1.78
7	4.15	4.34	2.88	2.99
8	4.18	4.18	<1.00	<1.00
9	4.42	4.51	2.22	3.41
10	4.45	nc	a	a
11	5.07	4.53	4.49	3.43
12	4.28	4.19	2.82	2.82
13	4.48	3.71	a	a
14	4.50	4.50	3.04	3.06
15	4.74	nc	<1.00	2.74
16	4.35	4.03	2.00	2.40
17	3.28	3.63	1.90	2.46[b]
18	3.70	3.84	2.25	2.09[b]
Mean	4.15		3.00	
r	0.59		1.47	
SD_r	0.21		0.52	
RSD_r (%)	5.10		17.45	
R	1.10		1.80	
SD_R	0.39		0.64	
RSD_R (%)	9.47		21.46	

Legend: nt: not tested; nc: not countable; a : no visible colonies; [b] : denotes outlier result not used in statistical calculations

Table 5

Log_{10} colony counts obtained for sample 1 (A/D) on agar-agar

Laboratory	Colony Count (agar-agar)			
	22°C	22°C	37°C	37°C
1	3.86	4.99	4.12	2.40
2	3.67	3.31	a	1.00
3	a	a	a	a
4	2.57	nt	a	nt
5	2.53	3.00	a	a
6	2.86	2.80	3.28	1.70
7	3.56	3.69	a	a
8	nc	nc	nc	nc
9			a	a
10	a	a	a	a
11	4.40	3.33	4.38	3.59
12	3.18	2.96	<1.00	<1.00
13	3.85	3.48		
14	4.26	4.25	3.25	3.30
15	<2.00	2.62	<1.00	<1.00
16	4.17	3.38	1.70	2.55
17	a	2.02	a	a
18	1.95	3.45	a	a
Mean	3.50			
r	1.44			
SD_r	0.52		Insufficient	
RSD_r (%)	14.85		Data	
R	1.94			
SD_R	0.69			
RSD_R (%)	19.86			

Table 6

Log_{10} colony counts obtained for sample 1 (A/D) on 1/10 NA

Laboratory	Colony Count (1/10 NA)			
	22°C	22°C	37°C	37°C
1	3.90	4.11	4.11	3.30
2	4.81	4.90	4.22	3.96
3	4.76	4.99	3.17	3.38
4	>4.60	nt	3.86	nt
5	3.99	3.13[(b)]	3.68	3.36
6	4.25	3.99	3.26	2.80
7	4.54	4.66	4.17	4.51
8	4.32	4.34	4.11	4.08
9	4.85	4.99	3.29	4.38
10	4.32	4.18	3.15	3.36
11	5.04	4.91	4.41	3.53
12	4.84	4.81	4.23	4.23
13	4.26	4.30	3.79	3.08
14	4.68	4.64	3.66	3.43
15	4.64	nc	<2.00	<2.00
16	4.61	4.69	3.31	3.29
17	4.25	4.08	3.68	3.67
18	3.69	3.74	2.14	2.15
Mean	4.48		3.59	
r	0.27		0.96	
SD_r	0.09		0.34	
RSD_r (%)	2.18		9.55	
R			1.67	
SD_R	0.39		0.60	
RSD_R (%)	8.75		16.57	

Legend: nt: not tested; nc: not countable; a : no visible colonies; [(b)] : denotes outlier result not used in statistical calculations

Table 7

Log_{10} colony counts obtained for sample 2 (B/C) on YEA (Oxoid)

Laboratory	Colony Count (YEA Oxoid)			
	22°C	22°C	37°C	37°C
1	4.58	4.56	3.49	3.60
2	5.60	5.64	3.35	3.32
3	3.57	5.39[b]	3.32	3.38
4	5.70	nt	4.30	nt
5	5.30	5.16	3.33	3.31
6	5.44	5.35	4.15	3.98
7	4.66	5.25	4.54	4.53
8	5.32	5.43	3.36	3.38
9	5.48	5.28	3.64	3.32
10	5.78	5.59	3.00	3.57
11	5.53	5.33	3.34	3.44
12	5.16	5.21	4.04	2.94
13	5.52	5.26	3.67	3.30
14	5.66	5.58	3.93	4.03
15	5.39	5.76	3.57	4.82°
16	4.85	4.87	3.62	3.26
17	>5.48	5.36	4.65	4.62
18	4.74	4.74	2.45	2.34
Mean	5.27		3.61	
r	0.43		0.90	
SD_r	0.15		0.32	
RSD_r (%)	2.94		8.96	
R	1.00		1.63	
SD_R	0.36		0.58	
RSD_R (%)	6.81		16.14	

Table 8

Log_{10} colony counts obtained for sample 2 (B/C) on YEA (ISO)

Laboratory	Colony Count (YEA ISO)			
	22°C	22°C	37°C	37°C
1	4.52	4.60	3.45	3.54
2	5.54	5.69	3.37	3.34
3	5.13	5.32	2.68	3.12
4	5.70	nt	3.98	nt
5	5.10	5.00	3.65	3.51
6	4.65	4.53	3.27	2.89
7	5.24	5.10	3.76	4.32
8	5.34	5.26	3.20	3.34
9	5.11	5.34	3.40	3.38
10	5.60	nc	3.28	3.45
11	5.34	5.18	3.12	3.20
12	5.20	5.21	3.16	3.06
13	5.45	5.34	3.48	3.70
14	5.61	5.62	4.21	4.31
15	4.85	5.43	3.50	3.49
16	4.64	4.58	3.25	2.82
17	5.40	5.32	4.63	4.37
18	4.55	4.57[b]	2.06	2.34
Mean	5.12		3.40	
r	0.37		0.51	
SD_r	0.13		0.18	
RSD_r (%)	2.57		5.37	
R	1.02		1.53	
SD_R	0.37		0.55	
RSD_R (%)	7.15		16.10	

Legend: nt: not tested; nc: not countable; a : no visible colonies; [b] : denotes outlier result not used in statistical calculations

Table 9

Log_{10} colony counts obtained for sample 2 (B/C) on PCA

Laboratory	Colony Count (PCA)			
	22°C	22°C	37°C	37°C
1	4.54	4.60	3.51	3.58
2	5.71	5.71	3.76	4.20
3	5.22	5.45	2.45	2.96
4	5.70	nt	4.28	nt
5	4.53	5.18	3.64	3.35
6	4.45	4.66	2.99	2.79
7	5.61	5.03	4.25	4.30
8	5.08	5.26	2.36	3.00
9	5.33	5.46	4.25	3.44
10	5.62	4.89	3.45	3.36
11	5.53	nc	2.52	3.91
12	5.35	5.32	3.15	3.06
13	5.28	5.30	3.30	3.70
14	5.59	5.55	4.09	3.85
15	4.82	4.87	nc	3.87
16	4.98	5.15	3.47	2.61
17	5.37	5.29	4.24	4.41
18	4.59	4.69	1.34	2.13
Mean	5.14		3.36	
r	0.60		1.13	
SD_r	0.22		0.40	
RSD_r (%)	4.20		12.01	
R	1.07		2.04	
SD_R	0.38		0.73	
RSD_R (%)	7.44		21.75	

Table 10

Log_{10} colony counts obtained for sample 2 (B/C) on agar gelatine

Laboratory	Colony Count (Agar gelatine)			
	22°C	22°C	37°C	37°C
1	5.03	5.16	3.59	3.60
2	nc	nc	3.08	3.14
3	5.01	5.05	a	a
4	5.00	nt	3.36	nt
5	5.00	5.27	3.61	3.38
6	5.14	5.17	2.36	2.19
7	5.47	5.53	3.50	3.37
8	5.30	6.32[b]	<1.00	<1.00
9	5.36	5.71	4.24	3.21
10	4.63	4.84	a	a
11	>5.48	5.13	3.45	3.24
12	4.93	5.01	3.26	3.26
13	4.30	5.90[b]	a	a
14	5.39	5.26	5.06	3.79
15	4.84	nc	3.45	4.11
16	5.40	5.29	2.70	2.40
17	5.28	4.89	3.19	2.99
18	4.61	4.67	a	2.10
Mean	5.13		3.34	
r	0.38		1.05	
SD_r	0.14		0.38	
RSD_r (%)	2.68		11.24	
R	0.81		1.75	
SD_R	0.29		0.62	
RSD_R (%)	5.65		18.66	

Legend: nt: not tested; nc: not countable; a : no viable colonies; [b] : denotes outlier result removed from data

Table 11

Log_{10} colony counts obtained for sample 2 (B/C) on agar-agar

Laboratory	Colony Count (agar-agar)			
	22°C	22°C	37°C	37°C
1	5.09	5.03	3.48	3.52
2	5.03	4.89	a	a
3	a	a	a	a
4	4.15	nt		nt
5	2.86	a	a	a
6	3.29	3.25	3.20	2.91
7	5.66	5.31	a	a
8	nc	nc	nc	nc
9	a	a	a	a
10	a	a	a	a
11	4.46	4.26	<1.00	<1.00
12	4.82	4.62	<1.00	<1.00
13	3.93	5.45[(b)]	a	a
14	5.11	5.03	3.53	3.50
15	<1.00	<2.00	<1.00	<1.00
16	5.82	5.34	1.64	2.40[(b)]
17	a	a	a	a
18	4.10	3.86	a	a
Mean	4.72			
r	0.48			
SD_r	0.17		Insufficient	
RSD_r (%)	3.61		Data	
R	2.10			
SD_R	0.75			
RSD_R (%)	15.92			

Table 12

Log_{10} colony counts obtained for sample 2 (B/C) on 1/10 NA

Laboratory	Colony Count (1/10 NA)			
	22°C	22°C	37°C	37°C
1	5.28	5.26	3.49	3.41
2	5.68	5.89	4.13	4.13
3	5.41	5.34	3.34	3.28
4	5.70	nt	3.98	nt
5	5.23	5.11	3.63	3.46
6	5.30	5.36	3.29	3.23
7	5.78	5.51	4.33	4.27
8	5.48	5.40	3.67	3.95
9	5.48	5.80	3.64	3.53
10	5.34	5.56	3.51	3.38
11	5.64	5.58	3.37	3.39
12	5.48	5.45	3.43	3.27
13	5.51	5.53	3.45	3.46
14	5.63	5.50	3.94	4.12
15	5.14	nc	a	3.72
16	5.16	5.22	3.40	2.00
17	5.32	5.28	4.07	3.86
18	4.77	4.62	1.22	2.38
Mean	5.40		3.47	
r	0.29		0.93	
SD_r	0.10		0.33	
RSD_r (%)	1.93		9.62	
R	0.75		1.79	
SD_R	0.27		0.64	
RSD_R (%)	4.94		18.42	

Legend: nt: not tested; nc: not countable; a : no visible colonies; [(b)] : denotes outlier result not used in statistical calculations

Table 13

Summary of Results Obtained by Participants

Sample	Growth medium	Temperature °C	No of useable results	Mean	r	R
1	YEA (O)	22	15	4.35	0.75	1.15
1	YEA (I)	22	16	4.21	0.74	1.03
1	PCA	22	17	4.32	0.59	1.14
1	AG	22	14	4.15	0.59	1.10
1	A	22	11	3.50	1.44	1.94
1	NA	22	15	4.48	0.27	2.18
1	YEA (O)	37	15	3.61	1.41	1.88
1	YEA (I)	37	17	3.62	1.09	1.59
1	PCA	37	17	3.50	1.11	1.71
1	AG	37	10	3.00	1.47	1.80
1	A	37	6	Insufficient data		
1	NA	37	16	3.59	0.96	1.67
2	YEA (O)	22	16	5.27	0.43	1.00
2	YEA (I)	22	15	5.12	0.37	1.02
2	PCA	22	16	5.14	0.60	1.07
2	AG	22	12	5.13	0.38	0.81
2	A	22	9	4.72	0.48	2.10
2	NA	22	16	5.40	0.29	0.75
2	YEA (O)	37	17	3.61	0.90	1.63
2	YEA (I)	37	17	3.40	0.51	1.53
2	PCA	37	16	3.36	1.13	2.04
2	AG	37	12	3.34	1.05	1.75
2	A	37	5	Insufficient data		
2	NA	37	16	3.47	0.93	1.79

Key to table 13

YEA (O)	Yeast extract agar, Oxoid formulation
YEA (I)	Yeast extract agar, ISO formulation
PCA	Plate count agar
AG	Agar gelatine
A	agar-agar
NA	1/10 nutrient agar

Table 14

Key to Tables 1 to 12

SD_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
SD_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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Evaluation of Microbiological Methods of Analysis for Natural Mineral Waters

Part 3

Detection of *Pseudomonas aeruginosa* Results of a Collaborative Trial

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The results of a collaborative trial of two methods for the detection of Pseudomonas aeruginosa in natural mineral water are reported. Twenty UK laboratories participated in the trial to validate liquid enrichment and membrane filtration procedures prescribed by the International Standards Organisation for use with potable waters. The results from this trial indicate that the current membrane filtration method is unreliable for the detection of Ps. aeruginosa in natural mineral water due to unsatisfactory isolation procedures. Modifications to the technique in order to improve recovery are suggested. For detection of low numbers of Ps.aeruginosa in natural mineral water, the liquid enrichment technique is recommended but some modifications to the confirmatory procedures are suggested.

The European Council of Ministers has adopted a Directive (80/777/EEC) on the Approximation of the Laws Relating to the Exploitation and Marketing of Natural Mineral Waters (NMW)[1]. This Directive was subsequently translated into legislation for England, Wales and Scotland[2] and also for Northern Ireland[3].

The Directive 80/777/EEC prescribes microbiological standards for NMW at source and specifies:

- the absence of coliforms and *Escherichia coli* in 250 ml at 30°C and 44.4°C;
- the absence of faecal streptococci in 250 ml;
- the absence of sporulated sulphite-reducing anaerobes in 50 ml;
- the absence of *Pseudomonas aeruginosa* in 250 ml;
- the determination of total revivable colony count at 22°C and 37°C.

An EC Expert Working Group is currently considering suitable methods of analysis for NMW and under consideration are International Standards Organisation (ISO) methods for potable waters together with Groupement Europeen des Sources d'Eaux Minerales Naturelles (GESEM, a NMW trade association) methods. Methods being discussed in EC Working

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Groups are assessed within the UK by carrying out collaborative trials. Two trials of microbiological methods for NMW have already been carried out for the detection of *E. coli* (Part I)[4]; and the enumeration of total revivable colony count (Part II)[5]. This report describes the third trial organised by MAFF to validate microbiological methods of analysis for NMW and in particular for the demonstration of the absence of *Ps. aeruginosa* as required by the Directive. The aims and objectives of these trials have been given previously[6].

Development of the Method of Analysis Under Test

In the case of *Ps. aeruginosa,* both ISO and GESEM recommend similar methodologies. However, two alternative isolation procedures are described by ISO (the confirmatory procedures are identical) and are used as appropriate depending on whether high or low numbers of *Ps. aeruginosa* are expected in a sample. The ISO standards are:

- ISO 8360-1:1988 Water Quality, Detection and enumeration of *Ps. aeruginosa* by enrichment in liquid medium.
- ISO 8360-2:1988 Water Quality, Detection and enumeration of *Ps. aeruginosa* by membrane filtration.

These two methods were used in the collaborative trial.

Definition

For the purpose of this trial the following definition applies:

Ps. aeruginosa - micro-organisms capable of growth in/on Drakes media Nos. 10 and 19 and which also produce characteristic colonies when grown on milk cetrimide agar incubated at 42°C.

Collaborative Trial Organisation

Participants

A total of 20 UK laboratories participated in the trial, nineteen of which were Public Analyst laboratories and one the MAFF Food Science Laboratory, Norwich.

Preparation and Distribution of Samples

Full details of sample preparation procedures are given in Appendix I. Protocols were distributed to participants several weeks in advance of the trial to allow familiarisation with the methods.

NMW samples were prepared and cooled to *ca* 6°C on the morning of the day of distribution. They were transported in cool boxes (<10°C) and delivered to participating laboratories by overnight carrier to arrive the following morning. Participants were instructed to test all samples

immediately upon receipt but, in the case of unavoidable delay between arrival of samples and analysis, it was specified that they should be stored at 4°C.

Participants were asked to determine the presence/absence of *Ps. aeruginosa* in a total of 14 x 250ml samples of NMW. Half of the samples were tested using the liquid enrichment method (ISO 8360-1) and half using the membrane filtration method (ISO 8360-2).

Each batch of seven samples of NMW was identical and comprised four sample types, which were dispatched to participants as blind duplicates randomly coded. The sample types were as follows:

A - 250ml samples containing approximately 50 cells/250ml of a typical strain of *Ps. aeruginosa*;

B - 250ml samples containing approximately 10 cells/250ml of a typical strain of *Ps. aeruginosa* and additionally 50 cells/250ml of *Ps. cepacia;*

C - 250ml samples containing approximately 20 cells/250ml of an atypical strain of *Ps. aeruginosa* and additionally 20 cells/250ml of *Ps. cepacia;*

D - 250ml samples to which no *Pseudomonas* species were added.

Methods

The methods that participants were required to use are given in Appendix II and III.

Results

Table 1 gives the results obtained by each laboratory using the liquid enrichment technique and Table 2 for the membrane filtration technique. A summary of results with respect to false positive and false negative rates obtained for both methods is shown in Table 3.

Discussion of results

Liquid enrichment method

Of the forty samples examined containing the typical *Ps. aeruginosa* at a level of *ca* 50 cells/250 ml NMW (sample type A), thirty nine (98%) were correctly identified by participants. One laboratory failed to detect the organism in one of the blind duplicate samples. When samples containing only 10 cells *Ps. aeruginosa* /250 ml NMW in the presence of a competitor organism *(Ps. cepacia)* were examined, the detection rate fell to 95% with 2 laboratories failing to detect the organism in one of the blind duplicates. Using this method, 90% of samples inoculated with the atypical *Pseudomonas* were correctly identified. In this case, 2 laboratories failed to detect the organism in either duplicate. It should be noted however that laboratory 11 also failed to detect the organism in samples A and B. Additionally, this laboratory reported a false positive result using the liquid enrichment technique.

Participants reported that generally this method was easy to use where growth and/or fluorescence detection in the selective broth indicated the presence of presumptive *Ps. aeruginosa.* Three reactions are utilised for the confirmation of the organism: growth at 42°C, pigment production and casein hydrolysis, all of which are determined using milk cetrimide agar. Some participants reported difficulties with the preparation of this medium which in some cases required several attempts to produce a smooth agar plate. Difficulties were also encountered with interpretation of some of the biochemical reactions. In particular, pigment production was occasionally poor and required extended incubation at room temperature, yet no guidance is given in the ISO protocol as to the length of time required at room temperature. Experience would suggest that a minimum exposure time of 4h is required for pigments to be sufficiently enhanced.

Membrane filtration method

This method did not perform well in this study and detection rates were lower than for the liquid enrichment technique. For sample type A, containing the highest level of typical *Ps. aeruginosa* cells, only 85% of samples were correctly identified using the membrane filtration technique with 3 laboratories failing to detect the organism in either of the duplicate samples. Where numbers of the typical *Pseudomonas* were reduced to *ca* 10 cells/250 ml in the presence of *Ps. cepacia,* the detection rate fell to only 55% with 8 laboratories failing to detect the organism in either duplicate. Correct detections of the atypical strain were reduced further still to 45% where 9 laboratories failed to detect the atypical *Pseudomonas* in either of the duplicate samples. One laboratory also reported a false positive result using the membrane filtration method.

The protocol for this method specifies that colonies growing on the membrane showing either fluorescence or pigment production should be considered as presumptive *Ps. aeruginosa* and picked for further confirmatory procedures. However, participants reported that colony fluorescence was poor on membrane filters and that pigment production was weak or non-existent after a 48h incubation which resulted in colonies not being picked for further investigation. Confirmatory procedures were identical to those prescribed for the liquid enrichment technique and participants reported similar difficulties but to a worse degree. Casein hydrolysis was poor at 42°C and subsequently some laboratories subcultured colonies from the milk cetrimide agar and re-incubated plates at 37°C where pigment production and casein hydrolysis showed improvement. Younger colonies also tended to produce stronger fluorescence under UV light and in the authors' experience all natural light must be excluded if fluorescence is easily to be seen.

Additionally, although an option is given in both procedures as to whether the ethanol is added before or after autoclaving of the media, the aseptic addition of ethanol after autoclaving is recommended.

Conclusions

The results of this trial indicate that the isolation method for the detection of *Ps. aeruginosa* in NMW must be selected with care.

The liquid enrichment method, which relies upon growth and/or fluorescence production in a selective broth should be the method of choice for the detection of *Ps. aeruginosa* in bottled NMW where, except in exceptional circumstances, the organism will be present in low numbers.

The membrane filtration method is unreliable for the detection of this organism in 250 ml volumes using the media currently prescribed which do not demonstrate satisfactorily the biochemical characteristics (e.g. fluorescence and pigment production), required for a presumptive identification of *Ps. aeruginosa.*

The current ISO standards specify the use of a single confirmatory medium, milk cetrimide agar which is incubated at 42°C as growth at elevated temperature is a typical characteristic of *Ps. aeruginosa.* However, pigment production and casein hydrolysis are not well defined at this temperature but improve at 37°C. It may therefore be beneficial to inoculate two plates of medium for the confirmatory tests: one for the determination of growth at 42°C; and one for determination of pigment production and casein hydroysis which is incubated at 37°C.

It is possible that the membrane filtration method could be improved by incubation of the membrane in the liquid enrichment broth as opposed to incubation on a solid medium. In this way, larger volumes of water could be examined without the need for the preparation of large volumes of broth as required in the liquid enrichment technique. Whether substitution of the 0.45μm filters for 0.22μm filters improves recovery of this organism requires further investigation.

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Appendix I

Sample preparation

Water for artificial inoculation

Samples of commercially available bottled NMW were purchased on several occasions prior to the trial from a retail outlet and examined for the presence of *Ps. aeruginosa* using the liquid enrichment technique. No *Ps. aeruginosa* were detected on any occasion so, for the purposes of the trial, this source was considered to be suitable for artificial inoculation.

Selection of organisms for artificial inoculation of samples

In order to fully validate the methods under test, several organisms were considered for the use as artificial inoculants. Ideally, a range of samples were required containing not only typical strains of *Ps. aeruginosa* but additionally atypical strains which can occur in water sources but which may be 'missed' using the confirmatory procedures prescribed. Also organisms which may give similar reactions to *Ps. aeruginosa* but are of a different species should be included which would test the efficacy of the isolation/confirmation procedures prescribed.

A number of isolates from culture collections were examined for their reactions using the methods prescribed for isolation and confirmation to be used in the trial. The following organisms were selected for use:

Typical *Ps. aeruginosa* (known positive) - *Ps. aeruginosa* NCDO 1369 (type strain) was selected as the typical strain. This organism produced a green/brown pigment (pyocyanin) and/or fluorescence on the membrane filtration medium, although the degree of fluorescence and pigment production was variable. The organism produced good fluorescence in the liquid enrichment medium.

Atypical *Ps. aeruginosa* (potential false negative) - A fluorescent but non-pigmented strain was required in order to assess the efficacy of the confirmatory procedures. A total of three pyocyanin-negative strains were evaluated and all gave strong fluorescence in the liquid enrichment broth. However fluorescence was variable with the membrane filtration technique. *Ps. aeruginosa* NCIMB 10421 was selected as the atypical strain as it produced the greatest fluorescence of the three atypical strains evaluated.

Competitive/similar organism (potential false positive) - *Ps. cepacia* produces a pigment and was shown to grow in both the liquid enrichment broth and the membrane filtration medium. Although the

production of pigment on the membrane filters was weak, *Ps. cepacia* NCTC 10743 was still included in some of the samples to act as a competitor of *Ps. aeruginosa.*

Investigation of sample stability and homogeneity

Sample stability and homogeneity were investigated to ensure that:

i) the organisms inoculated into the samples would survive and maintain a constant level during dispatch and testing by the participants and

ii) that all samples of each batch contained, as far as was practicable, the same number/type of organisms.

The following batches of NMW were required for the trial:

A - 2 × 250ml samples containing approximately 50 cells/250ml of a typical strain of *Ps. aeruginosa*;

B - 2 × 250ml samples containing approximately 10 cells/250ml of a typical strain of *Ps. aeruginosa* and additionally 50 cells/250ml of *Ps. cepacia;*

C - 2 × 250ml samples containing approximately 20 cells/250ml of an atypical strain of *Ps. aeruginosa* and additionally 20 cells/250ml of *Ps. cepacia;*

D - 1 × 250ml sample to which no *Pseudomonas* species were added.

Sample stability

Cultures of *Ps. aeruginosa* NCDO 1369, *Ps. aeruginosa* NCIMB 10421 and *Ps. cepacia* NCTC 10743 were grown for a period of 6h in Nutrient broth (Oxoid) at 30°C. An inoculum of each organism was prepared by inoculating a 10 ml volume of Nutrient broth with 0.5 ml of the 6h culture and incubating at 30°C for 20 hours.

For each of the *Pseudomonas* species, decimal dilutions of the broth culture were prepared to 10^{-7} in Maximum Recovery Diluent (Oxoid), 1 ml volumes of the 10^{-6} dilution was added to each of four x 2 litre bottles of uncarbonated NMW.

After thorough mixing by agitation, a 150 ml sample was removed from each bottle and examined using the membrane filtration technique and a further 50 ml examined using the liquid enrichment technique. The inoculated bottles were stored at 6°C and aliquots tested as described above after storage for 1, 2, 3, 6, 7, 8, 9, 10 and 13 days.

After an initial increase in numbers during the 24h after inoculation, both the typical and atypical strains of *Ps. aeruginosa* showed a steady decline in numbers during the storage period (Fig. 1). However, as the NMW samples were to be prepared and tested within a 48h period, this decline was not considered a problem with regard to presence/absence testing. Numbers of *Ps. cepacia* remained constant

during a two-week storage period at 6°C. The technique for artificial inoculation of the samples was therefore considered satisfactory for the purposes of the trial.

Sample homogeneity

Two options were investigated for the production of homogeneous samples :-

i) inoculation of individual batches of 250 ml volumes of NMW with the relevant organisms;

ii) inoculation of a single large batches of NMW with the relevant organisms followed by thorough mixing and further sub-sampling into 250 ml volumes.

Experimental work demonstrated that the second approach resulted in less variation between samples. Pre-trial samples of 20 litres of each of the three batches (A-C) were inoculated, distributed into 250 ml sub-samples and stored at 6°C. Tests for stability and homogeneity were carried out to assess the suitability of the procedure for production of test samples. Data indicated that using this procedure homogenous and stable NMW test samples types A-C could be produced if stored at 6°C.

Appendix II

Detection and enumeration of *Pseudomonas aeruginosa* - by enrichment in a liquid medium: (from ISO Method 8360-1)

1 Scope

This procedure describes a method for the isolation of *Pseudomonas aeruginosa* and the estimation of the numbers of this organism in water samples by enrichment in a liquid medium.

The method is applicable to all types of water and associated materials.

It is recommended for use with 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 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 subculture onto plates of the same agar medium as required. Each pure culture is finally tested for certain bio-chemical characteristics (see annex A).

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 medium specified in **4.1.1** for the determination of presumptive *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:

Add the ethanol and if necessary, adjust the *p*H so that after sterilisation it is 7.2 ± 0.2. Distribute in sterile screw-capped bottles. Tighten the caps on the bottles to the point where the seal in the lid just begins to engage with the lip of the bottle. Autoclave at 121°C for 15 min. Tighten the caps on each bottle, immediately after removal from the autoclave, to prevent loss of ethanol by evaporation. Do not use polypropylene caps without seals.

Alternatively, 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 and glassware

Usual microbiological laboratory equipment, as specified below:

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**).

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 visible light.

6.3 Confirmation

6.3.1 Milk agar

Subculture a loopful of culture medium from each container showing either fluorescence or growth onto a milk agar plate (4.2.1.3). Incubate the milk agar plates at 42°C ± 0.5°C for 24 h. Examine the plates for growth, 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 42C	+	+	+
Fluorescence (under UV irradiation only)	+	+	-
Pyocyanin (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 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 Expression of results

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

8 Test report

The test report shall contain the following information:

a) all details necessary for complete identification of the sample;
b) where applicable, the confirmation methods used to identify non-pigmented strains;
c) the results obtained expressed in accordance with clause 8.
d) any particular occurrence(s) observed during the course of the analysis and any operation(s) not specified in the method or considered optional which may have influenced the results.

Annex A
(Informative)

Further information about *Pseudomonas aeruginosa*:

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.

Appendix III
Detection and enumeration of *Pseudomonas aeruginosa* by a Membrane Filtration method: (from ISO Method 8360-2)

1 Scope

This procedure describes a method for the isolation of *Pseudomonas aeruginosa* and the estimation of the numbers of this organism in water samples by the membrane filtration technique.

This method is applicable to all types of water provided that the amount of suspended particulate matter is not such that it prevents the passage of either water or nutrients through the membrane filter.

In water where the expected number of *Pseudomonas aeruginosa* is low, e.g. bottled water, or the water contains a relatively high level of residual disinfectant (e.g. swimming pools), the liquid enrichment method is recommended.

2 Definition

For the purposes of this standard the following definition applies:

Pseudomonas aeruginosa: Micro-organisms capable of growth on Drake's selective medium 19 and which produce characteristic colonies when grown on milk agar at 42°C.

3 Principle

A measured volume of the water sample is filtered through a membrane filter that has filtration characteristics equivalent to a rated pore diameter of 0.45 μm. The membrane filter is placed on the selective medium and incubated under the conditions specified for the medium.

3.1 Enumeration

The numbers of presumed *Pseudomonas aeruginosa* are obtained by counting the number of characteristic colonies on the membrane filter after incubation.

3.2 Confirmation

Subcultures are made from each membrane filter 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 either from milk agar plates or directly from the membrane filters onto solid agar media and incubated.

Pure cultures are obtained by further subculture onto plates of the same agar medium as required. Each pure culture is finally tested for certain biochemical characteristics (see annex A).

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 medium

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*:

4.2 Drake's medium 19

4.2.1 Composition

Peptone	20 g
Ethanol	25 ml
Anhydrous potassium sulphate	10 g
Anhydrous magnesium chloride	1.4 g
Hexadecyltrimethylammonium bromide (cetrimide)	0.5 g
Water	to 1,000 ml

4.2.1.1 Preparation

Dissolve all the constituents in the water and proceed in either of the procedures given below:

Add the ethanol and if necessary adjust *p*H so that after sterilisation it is 7.2 ± 0.2.

Distribute in sterile screw-capped bottles. Tighten the caps on the bottles to the point where the seal in the lid just begins to engage with the lip of the bottle.

Autoclave at 121°C for 15 min. Tighten the caps on each bottle, immediately after removal from the autoclave, to prevent loss of ethanol by evaporation. Do not use polypropylene caps without seals.

Alternatively, 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.

4.3 Confirmatory medium

4.3.1 Milk agar with cetrimide

4.3.1.1 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 1,000 ml

4.3.1.2 Preparation of medium

Prepare the yeast extract broth by dissolving all the constituents in the distilledwater 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.

4.3.1.3 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 and glassware

Usual microbiological laboratory equipment, and in particular:

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 Membrane filtration apparatus.

5.3 Membrane filters of pore size 0.45 μm and absorbent pads.

5.4 Vacuum pump.

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

5.6 Ultraviolet lamp: emitting light of wavelength 360 nm ± 20 nm used with facility capable of excluding natural light.

6 Procedure

6.1 Membrane filtration

Filter 250 ml volumes of the water sample through a sterile membrane filter with a rated pore diameter equivalent to 0.45 μm. Place each membrane on a sterile filter pad saturated with Drake's medium 19 (4.2.1.1), ensuring that no air is trapped beneath. Sterile membrane filtration apparatus should ideally be used for each sample. However, if this is not possible, the apparatus must be thoroughly rinsed by repetitive filtration of sterile distilled water between samples to prevent carry-over of bacteria from previous filtrates. Alternatively, apparatus may be dipped in boiling water for 3 min.

NOTE - Excess Drake's medium 19 should be removed from the Petri dish prior to placing the membrane on the filter pad.

6.2 Incubation of membranes

Incubate the petri dishes at 37°C ± 1°C for 48 h in containers that prevent moisture loss. Examine the membranes for blue-green or greenish-brown colonies, or colonies which exhibit fluorescence under exposure to ultraviolet light in either a darkened room or apparatus which excludes visible light.

6.3 Confirmation

6.3.1 Milk agar

Subculture the characteristic colonies from **6.2** onto the surface of milk agar plates (**4.3.1.3**). Incubate the milk agar plates at 42°C ± 0.5°C for 24 h. Examine the plates for growth, 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 42C	+	+	+
Fluorescence (under UV irradiation only)	+	+	-
Pyocyanin (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 before they are examined for pigment production.

6.3.2 Enumeration

Count as confirmed *Pseudomonas aeruginosa* all colonies which exhibit the reactions (1) and (2) (see table 1 in **6.3.1**).

Count as presumed *Pseudomonas aeruginosa* all colonies which show, after incubation, the following characteristics:

- blue-green or greenish-brown coloration or exhibit fluorescence when exposed to ultraviolet light.

*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 pick a non-pigmented colony from the membrane filter using a loop and streak on to a milk agar plate. 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 Expression of results

From the number of characteristic colonies counted on the membranes, and taking account of the confirmatory tests performed, calculate the number of confirmed *Pseudomonas aeruginosa* present in 250 ml of water sample.

8 Test report

The test report shall contain the following information:

a) all details necessary for complete identification of the sample;

b) where applicable, the confirmation methods used to identify non-pigmented strains;

c) the results obtained expressed in accordance with clause 7.

d) any particular occurrence(s) observed during the course of the analysis and any operation(s) not specified in the method or considered optional which may have influenced the results.

Note: For further description of Ps. aeruginosa see Annex A, Appendix 1

Table 2

Pseudomonas aeruginosa in Natural Mineral Water (NMW)
Collaborative Trial Results for Liquid Enrichment Examination

Sample Type	Intended Result	Laboratory Reference Number																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
C	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+
C	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+
D	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-

Table 3

Pseudomonas aeruginosa in Natural Mineral Water (NMW)
Collaborative Trial Results for Membrane Filtration Examination

Sample Type	Intended Result	Laboratory Reference Number																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A	+	+	+	+	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+
A	+	+	+	+	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+
B	+	-	-	-	-	+	-	+	-	-	+	-	-	+	+	+	+	+	+	+	+
B	+	-	+	-	-	+	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+
C	+	+	+	-	-	+	+	-	-	-	+	-	+	+	-	+	-	-	-	+	-
C	+	-	+	-	-	+	+	-	+	-	+	-	+	+	-	+	-	+	-	+	-
D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

+ = *Ps. aerugenosa* detected in 250 ml NMW; - = *Ps. aeruginosa* not detected in 250 ml NMW.

Type A - *Ps. aeruginosa* NCDO1369 (50 cell/250 ml NMW)

Type B - *Ps. aeruginosa* NCDO1369 (10 cell/250 ml NMW) + *Ps. cepacia* NCTC10743 (50 cells/250 ml NMW)

Type C - *Ps. aeruginosa* NCIMB 10421 (20 cell/250 ml NMW) + *Ps. cepacia* NCTC10743 (20 cells/250 ml NMW)

Type D - NMW with no added Pseudomonas species.

Table 4

Summary of Results for Detection of *Ps. aeruginosa* in Natural Mineral Water (NMW)

Method	Negative Controls (Sample Type D) Number of Tests	Negative Controls (Sample Type D) Positive (%)	Positive Controls (Sample Type A) Number of Tests	Positive Controls (Sample Type A) Positive (%)	Positive Controls (Sample Type B) Number of Tests	Positive Controls (Sample Type B) Positive (%)	Positive Controls (Sample Type C) Number of Tests	Positive Controls (Sample Type C) Positive (%)
Membrane Filtration	20	1 (5)	40	34 (85)	40	22 (55)	40	18 (45)
Liquid Enrichment	20	1 (5)	40	39 (98)	40	38 (95)	40	36 (90)

Type A - *Ps. aeruginosa* NCDO1369 (50 cell/250 ml NMW)

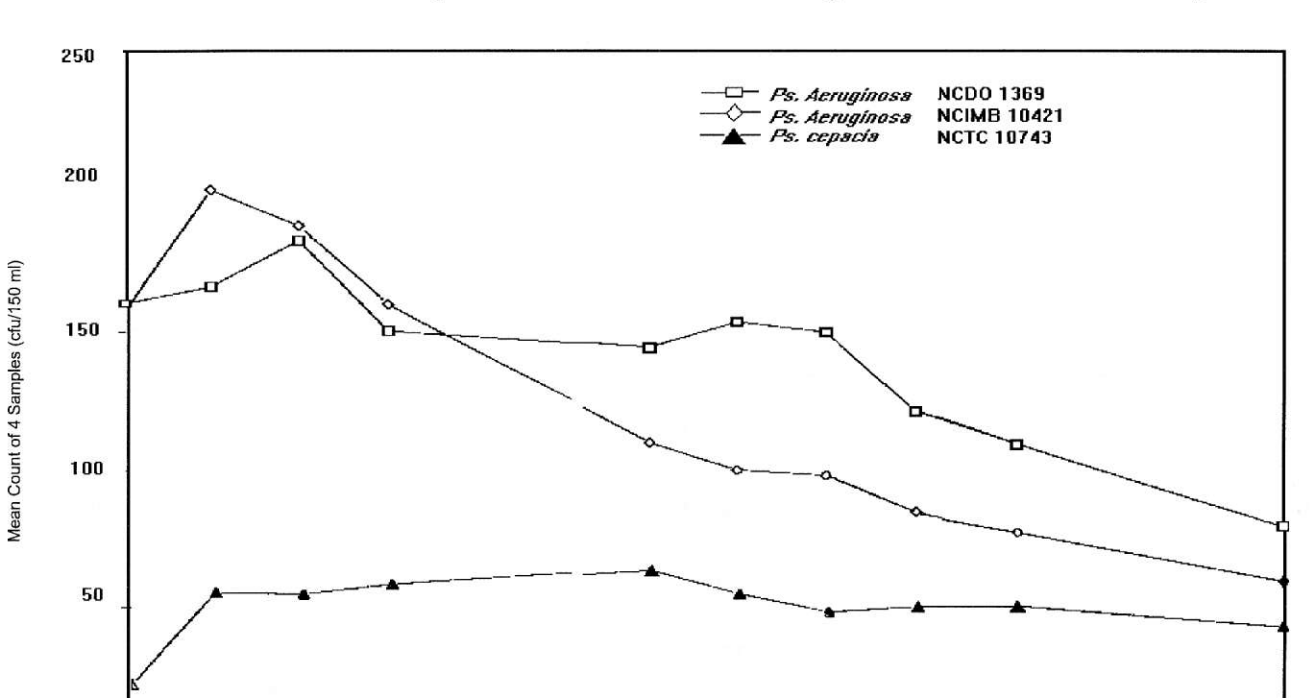
Type B - *Ps. aeruginosa* NCDO1369 (10 cell/250 ml NMW) + *Ps. cepacia* NCTC10743 (50 cells/250 ml NMW)

Type C - *Ps. aeruginosa* NCIMB 10421 (20 cell/250 ml NMW) + *Ps. cepacia* NCTC10743 (20 cells/250 ml NMW)

Type D - NMW with no added *Pseudomonas* species.

Figure 1

Survival Time of *Pseudomonas* Species in NMW determined using Membrane Filtration Technique



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MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

No. V27

Detection of Irradiated Herbs and Spices

Scottish Universities Research and Reactor Centre Procedure for Thermoluminescence Detection of Irradiated Herbs and Spices using Re-normalised Separated Minerals.

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

1. Scope and Field of Application

The method permits the detection of irradiated samples of all varieties of herbs and spices. It may be used to confirm commercial claims or to check compliance with labelling obligations under statutory instruments 1990:2489 (The Food Labelling (Amendment) (Irradiated Food) Regulations 1990 and Scottish equivalent [1990:2505 (S207)].

2. Definition

Irradiated samples comprise those which have been exposed to gamma radiation or machine sources of ionising radiation for the purpose of reducing microbial contamination. Positive results imply that a sample has been so irradiated, negative results imply that it has not. First glow refers to the thermoluminescence (TL) recorded from the prepared sample as received; second glow refers to subsequent measurement of the TL response following exposure to a fixed known dose of radiation for the purpose of normalisation. The TL glow ratio is the ratio of first to second glow TL response, evaluated over a stated temperature interval.

3. Principle

Silicate minerals adhering to the surfaces of herbs and spices store energy by charge trapping processes as a result of exposure to ionising radiation. Releasing such energy, by controlled heating of separated mineral extracts, gives rise to measurable thermoluminescence (TL) glow curves. Sample sensitivity is evaluated by re-irradiation to a standard dose followed by repeat TL analysis.

Irradiated samples are distinguished from unirradiated samples on the basis of first and second glow TL ratios, coupled to glow shape parameters. Independent checks of laboratory blanks and the concordance of duplicate aliquots are made for quality assurance purposes.

0004-5780/93 +14 $20.00

4. Reagents

Analytical grade reagents are used throughout the procedure. Deionised water is to be used. All reagents must be kept free from particulate contamination throughout the process. Stokes settling overnight and decanting into clean glassware can be used to ensure cleanliness of liquids.

4.1 Sodium polytungstate solution

Formula: $3Na_2WO_4.9WO_3.H_2O$. Available as solid or liquid from: TC Tungsten Compounds, Dr R Kamps, Sauerbruchstrasse 9, D-8630 Coburg, West Germany, or equivalent.

Stock solutions should be made up to a density of 1700-2000 kg m^3 in deionised water. Densities to be checked by weighing in small (e.g. 10 ml) volumetric flasks. The solution may be recovered and reconcentrated after use, subject to triple filtration and Stokes settling for 16 hours.

4.2 Hydrochloric acid, 1 mol/l.

4.3 Ammonium Hydroxide, 1 mol/l

4.4 Acetone

4.6 Oxygen free nitrogen gas, for TL reader

5. Apparatus

5.1 Nylon disposable sieves

5.1.1 Sieve set, GILSON Mini-Sieve-Set (51 mm in diameter) Model SV-120 supplied by GILSON COMPANY, INC, P.O. Box 677, Worthington OH 43085-0677, U.S.A. (Phone (800) 431-5935, Telex 241211 Cable: Gilscreco) or by Christison, Scientific Equipment Limited, Albany Road, East Gateshead Industrial Estate, Gateshead NE8 3AT (Phone 091-477-4261, Telex 537426) or equivalent.

5.1.2 Nylon sieve cloths, 125 and 250 μm, supplied by Plastok Associates Ltd, 79 Market St, Birkenhead, Wirral, Merseyside L41 6AN, United Kingdom, or equivalent.

5.2 Beakers, 150 ml and 500 ml.

5.3 Centrifuge tubes, about 50 ml, either glass or polypropylene.

5.4 Centrifuge, supplied with a swing-out rotor (max. centrifugal power 1000 g).

5.5 Ultrasonic bath, after filling with fresh water degas it by switching on for 15 min; ultrasonic power can be increased by adding detergent (normal cleaning solution).

5.6 Flat-bottomed settling tubes, 12 mm diameter.

5.7 Stainless steel discs, 10 mm diameter, 0.25 mm thick.

5.8 Laboratory oven, set to 50 ± 5°C

5.9 Analytical balance, 5 figure.

5.10 TL reader

5.11 Irradiation source, either beta or gamma ray source capable of irradiating the samples to a dose of 1 kGy, or suitable alternative.

6. Procedure

6.1 Sample preparation

Detailed instructions for sample preparation follow, divided into two stages. The first part (**6.2**) describes a mineral pre-concentration technique which is highly recommended for leafy samples as well as unground spices. It can be omitted for samples containing large proportions of mineral material. The second part (**6.3**) describes the density separation of minerals from organic material and preparation for presentation to the TL reader.

In either case the sample should be mixed thoroughly before starting and split into two aliquots for duplicate analysis. All laboratory surfaces and glassware should be carefully cleaned. Stainless steel discs and glassware should be checked for surface contamination (**see 7.1**) parallel process blank levels determined from the glassware. Full details on blanking are presented in section 7.1.

Sample preparation should be carried out under safelight conditions.

6.2 Preconcentration step

Preconcentration of minerals by wet sieving is recommended for most samples using the following procedure. For extremely finely ground samples it may be preferable to proceed directly to step **6.3**.

6.2.1 Place about 5 g of sample into a 150 ml glass beaker and add 50 ml deionised water.

6.2.2 Put the beaker in an ultrasonic bath for 15 min.

6.2.3 Sieve the sample in portions through a 250 μm or 125 μm nylon mesh into a 500 ml glass beaker, rinsing the minerals through with deionised water each time. Remove the residual herb/spice from the sieve and discard.

6.2.4 The minerals are now in the beaker and will settle according to Stokes Law. Leave the beaker for about 5-10 minutes to allow all but the finest grains to settle. The next sample can be prepared in parallel at this stage if desired.

6.2.5 Discard most of the water, leaving the minerals in only a few ml of water. Then transfer all material in the beaker to a 50 ml centrifuge tube. Coarse grains settle very rapidly, so keep the liquid moving during transfer, chasing any remaining grains through using a wash-bottle of deionised water.

6.2.6 Centrifuge for 1 min at 1000 g. Alternatively settle for a further 5 minutes.

6.2.7 Decant off or vacuum extract the water leaving the mineral concentrate behind.

6.3 Density separation

If the pre-concentration step is omitted then place up to 1 g of sample into a 50 ml centrifuge tube.

6.3.1 Add about 10 ml of sodium polytungstate solution (**4.1**) made up to a density of 1700-2000 kg m^{-3}. This will be used to separate the remaining organic material from the minerals.

6.3.2 Agitate the sample for 2 min in an ultrasonic bath (or longer if preconcentration steps are omitted). This is to disperse the sample and shake loose adhering mineral grains.

6.3.3 Centrifuge for 2 min at 1000 g. Silicate minerals (density 2.5-2.7 g/ml) will sink whereas organic components of herbs and spices will float.

6.3.4 It is important to now remove the upper layer and all organic material. This should be decanted off, or vacuum extracted. If necessary a small amount of water can be added to free material from the side wall, taking care to avoid the mineral grains at the tube bottom. The tube side should be cleaned by wiping with a small moist tissue or by local rinsing and vacuum extraction.

The tungstate solution can be recovered, in which case all upper layer material and subsequent washings from **6.3.5** should be filtered into a large (e.g. 1 litre) beaker. This should then be refiltered twice, covered and allowed to stand at low density overnight before decanting off the top portion for reconcentration by heating.

6.3.5 Once the organic material has been removed any remaining tungstate can be diluted in deionised water to the top of the tube, which should then be re-centrifuged for 30 sec at 1000 g, or allowed to settle for 5 minutes. Remove the water. Repeat this step twice more to recover all tungstate residues.

6.3.6 Add 2-4 ml of 1 mol/l HCl, agitate, and leave for 10 min. This is to remove any mineral salts adhering to the grains. Neutralise the acid with NH_4OH (**4.3**), fill up the tube with deionised water and centrifuge for 30 sec at 1000 g, or settle for 5 minutes.

6.3.7 Wash the sample with water twice, settling or centrifuging and removing excess water as before.

6.3.8 Fill the tube with acetone. Settle (5 mins) or centrifuge and then remove the acetone. Repeat until all water has been displaced (usually 2-3 times).

6.3.9 Prepare a set of clean flat bottomed tubes each containing a clean weighed 1 cm diameter × 0.25 mm thick stainless steel discs. Discs should be cleaned in acetone and blanks confirmed before use. The sample may either all be transferred in solution to a single tube, or can be split volumetrically into two or more portions depending on the quantity of material recovered.

6.3.10 Place the sample tube(s) upright in a laboratory oven at 50°C overnight. The acetone will dry off leaving a deposit of minerals

adhering to the discs, which should be reweighed to determine sample mass.

6.4 Thermoluminescence Measurements

All TL samples should be handled under subdued red/amber safelights inaccordance with good TL dosimetry and TL dating practice. The first glow thermoluminescence from room temperature to 400°C should be recorded from each sample, heated at 6°C s^{-1} in an oxygen-free nitrogen atmosphere. Glow curves should be kept as primary records in the laboratory, and integrated into 10°C bands from 150-350°C.

Thereafter each sample should be irradiated to a dose of 1 kGy using a ^{60}Co or ^{90}Sr source. For gamma irradiation samples should be packed individually in a manner which protects them from loss of material, exposure to light or cross contamination. Routine perspex dosimeters are used to confirm the normalisation gamma dose. After irradiation a 30 minute pre-heat at 50-60°C is recommended before recording second glow thermoluminescence under the same conditions as above.

7. Quality Assurance Considerations

Acceptance of the results of TL testing is predicated by three principle quality assurance criteria: satisfactory blank levels, satisfactory sample sensitivity, and satisfactory reproducibility. These are described more fully below.

7.1 Blank levels and Minimum detectable TL signal

Measurements of TL signal levels from blank discs, associated with glassware, and from process reagents are required both as part of the supporting quality assurance programme, and also to set acceptance levels for results.

Disc blank levels should be checked by irradiating all clean discs to a 1 kGy dose and recording TL signals. Any non-zero levels are a sign of surface contamination. Discs with levels more than 3 standard deviations above the mean blank level should be rejected, recleaned and re-tested. Only discs with a proven blank should be used.

Glassware blanks should be measured by rinsing all glassware with acetone and depositing the residues by evaporation onto clean discs. Any signals greater than 3 standard deviations above the mean clean disc blank indicate the presence of glassware derived contamination. Conversely a negative result at this stage confirms that all glassware is clean.

Finally full process blank levels should be measured in parallel with sample extractions using portions of the same stock solutions and following the procedure in all stages. Positive process blanks, again compared with mean plus 3 standard deviations of disc blanks, indicate contamination of reagents. The combined full process blank level plus three standard deviations; which should be consistent with

freedom from contamination of glassware or reagents, defines the minimum detectable TL signal level (MDL).

7.2 Sample sensitivity

Any sample with second glow TL signal level below 10 times MDL should be rejected and re-analysed. This represents the lowest sample sensitivity from which a TL glow ratio of 0.1 or less (representing an unirradiated sample) can be measured reliably.

The dynamic response of the TL reader should be characterised independently. In particular it should be noted that TL instruments based on photon counting, which are recommended for this work, will eventually experience saturation due to pulse pile up. The level at which dead time effects lead to greater than 10% reduction in apparent count rate should be defined as the upper limit of linearity (ULL). The level above which the system will not count is defined as the upper saturation limit (USL).

Any TL sample whose second glow response is measured as being at the USL should be rejected and re-analysed. Correction for dead time effects may be considered for any sample with a second glow between ULL and USL. If this is not possible then the sample should be rejected and re-analysed using either a restrictive aperture or a neutral density filter to reduce count rate.

7.3 Sample Reproducibility

The reproducibility of each result should be checked by comparing the logarithms of the TL glow ratios from each aliquot. If the difference between two results is greater than the laboratory reproducibility, r, then repeat analyses should be undertaken in accordance with the MAFF general considerations on analytical quality control.

A concordance diagram showing the paired logarithmic TL glow ratios is helpful for assessing reproducibility (see Appendix I). Any sample pair falling off the 45° line, and out with the laboratory scatter should be investigated.

In addition each individual set of glow curves should be scrutinised for ambiguities. Aberrant data whose origins have been clearly identified may be excluded.

Identification of Irradiated Samples

Having satisfied the quality assurance criteria above the following indicators are used to determine whether the sample was irradiated. These are the TL glow ratio histogram, the first-second glow plot, and the TL glow curve shape. Comparison between unknown samples and the laboratory reference set forms the basis of classification of irradiated and unirradiated samples.

TL glow ratios from irradiated samples are typically greater than unity, while those from unirradiated samples are generally below 0.1. The first-second glow plot combines this distinction with information on sample sensitivity, which may be helpful in some cases.

The TL glow curves themselves provide further discrimination between background signals due to long term exposure to low level natural radioactivity (signals in the deep traps above 300°C) and signals due to recent irradiation (in the lower temperature range from 150-250°C). Examination of the glow shape therefore can be used to support classification.

An example of the procedure to be used is given in Appendix I.

9. Precision

The procedure as described in this protocol has been subjected to an international collaborative trial organised by the SURRC under the auspices of the Community Bureau of Reference (BCR). A summary of the results obtained is given in Appendix II and Tables I and II. They indicate that clear differentiation is obtained between the unirradiated and irradiated samples used in the trial.

10. COSSH and Safety Considerations

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.

Each laboratory should follow its own safety rules and national regulations, particularly COSHH and IRR's, with respect to the sample preparations and measurement. Additional points to note are as follows.

10.1 Hydrochloric acid, HCl : corrosive. Avoid contact with skin, inhalation of fumes and ingestion.

10.2 Acetone, $(CH_3)_2CO$: highly flammable. Avoid naked flames and inhalation of fumes.

10.3 Care should be taken to avoid trip hazards when working under subdued lighting conditions.

10.4 Bottled nitrogen cylinders should be firmly supported at all times.

10.5 The electrical safety of all equipment should be ensured.

11. References

11.1 DCW Sanderson, C Slater and KL Cairns, Detection of Irradiated Food, Nature, 1989a, **340**, 23-24.

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11.3 DCW Sanderson, C Slater and KL Cairns, Luminescence Identification of Irradiated Foods, Int. J. Rad. Biol., 1989, **55**,5.

11.4 DCW Sanderson, Luminescence Detection of Irradiated Foods, in "Food Irradiation and the Chemist", ed D.E. Johnston, M.H. Stevenson, Royal Society of Chemistry, Cambridge, 1990, 25-56.

11.5 DCW Sanderson, Photostimulated Luminescence (PSL) : a new approach to identifying irradiated food, in "Potential new Methods of detection of irradiated food", ed J. Raffi, European Commission, Report EUR 13331 EN, 1991, 159-170.

11.6 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

12. Acknowledgements

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MAFF wishes to thank both Dr Sanderson and the Commission for permission to reproduce the protocol and the summary of results obtained in the trial in the MAFF Validated Methods for the Analysis of Foodstuffs Series of Bulletins.

APPENDIX I

Example of Procedure to be used to Identify Irradiated Samples

A.1 Reference Plots

The following figures illustrate reference TL glow ratio histogram and first/second glow TL plots for one laboratory, over the temperature range 220-240°C. Mean and standard deviations of the logarithmic TL glow ratios for irradiated and unirradiated samples are indicated. Irradiated and unirradiated samples are clearly distinguished.

Figure A.1 Reference Histogram

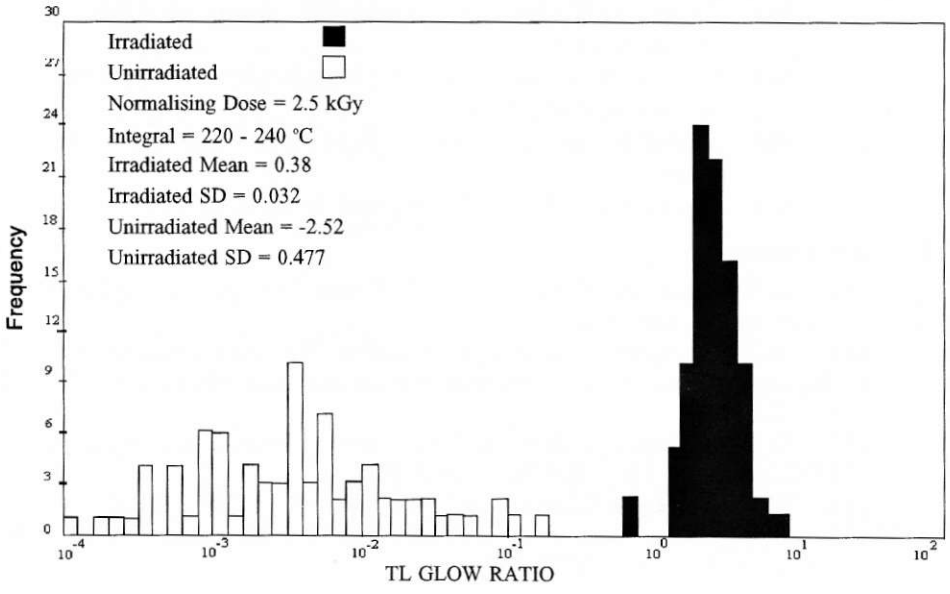
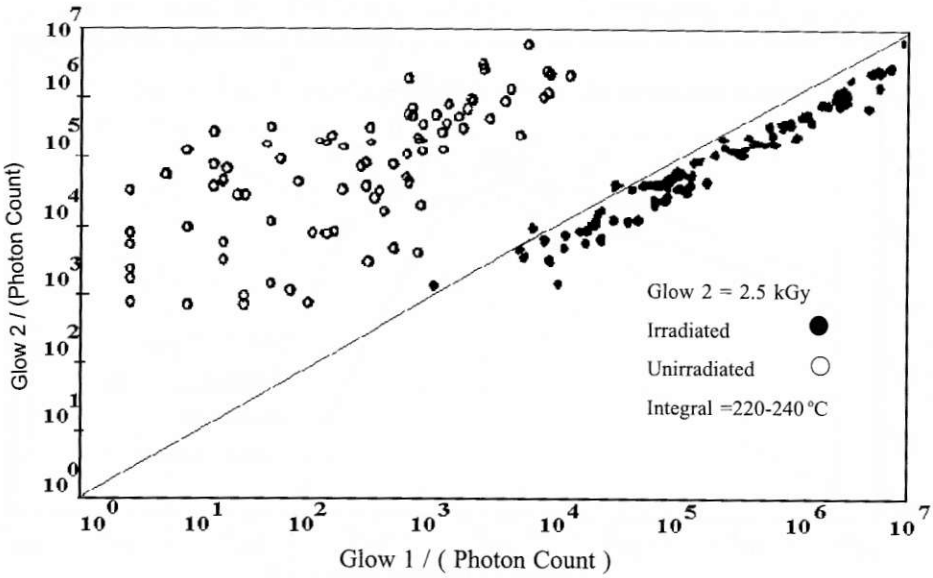


Figure A.2. First glow/second glow Reference Plot



A.2 Example of identification of an irradiated herb sample

The following sections illustrate the identification of of an irradiated herb sample, from a set of samples purchased from a retail source in Europe. All samples were subject to the standard procedure. Data were firstly compared with blank and saturation levels as described under section 7. TL glow ratios were evaluated from 220-240°C and used to construct a histogram, glow ratio plot and concordance diagram, as shown below.

Figure A.3. TL glow ratio Histogram from 17 test samples

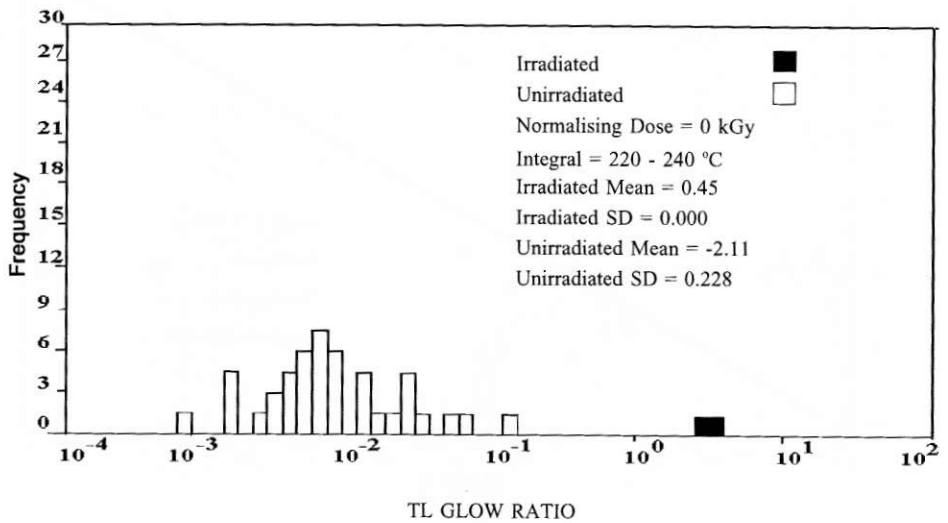
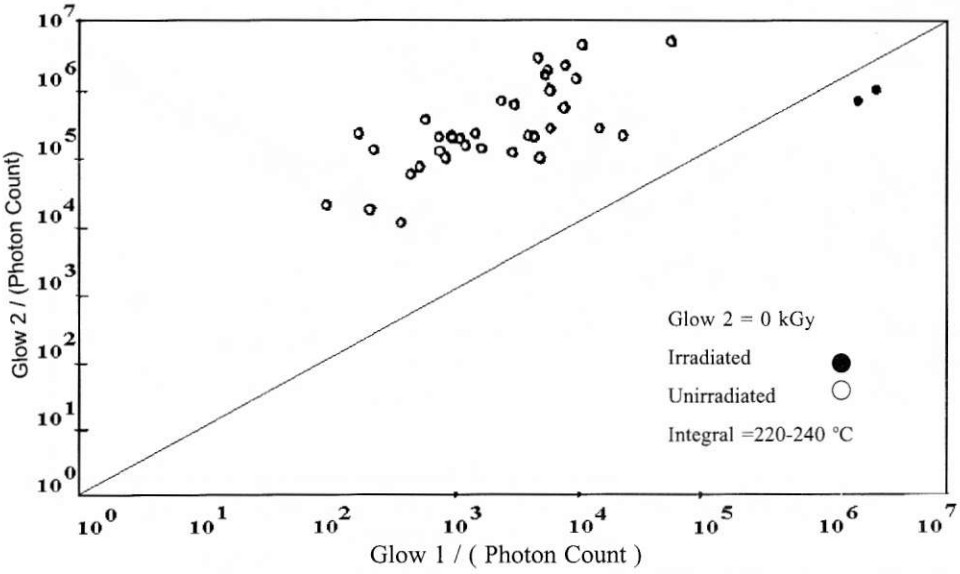
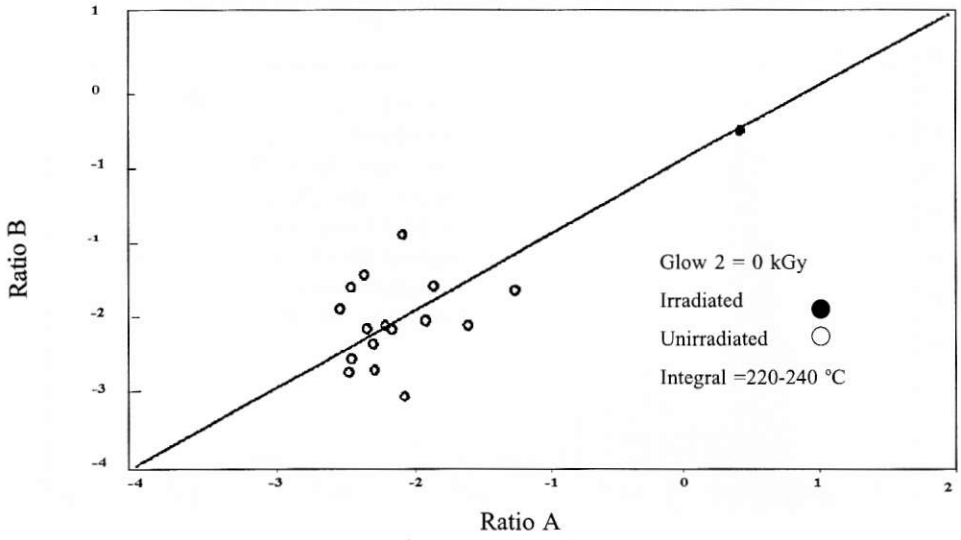


Figure A.4 First Glow / Second Glow Plot from 17 test samples



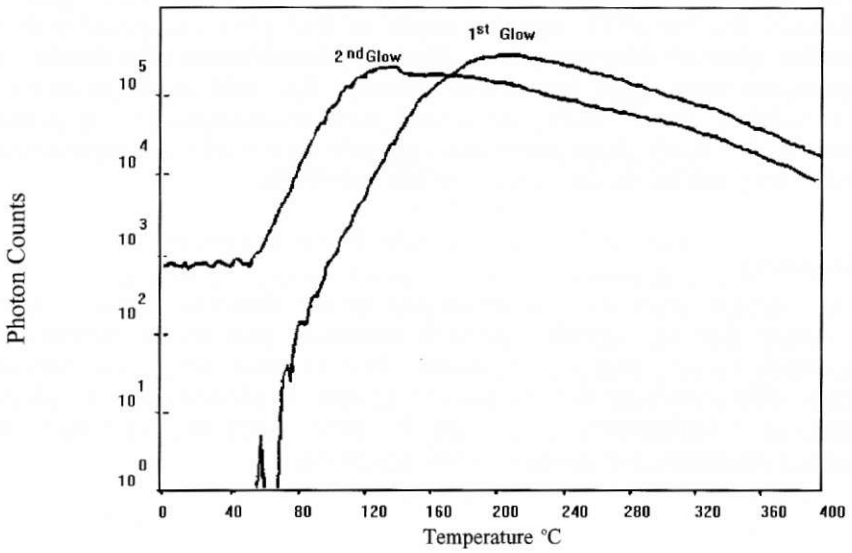
One sample pair is identified as irradiated by comparison with reference histograms. The concordance diagram, shown below indicates that this sample is highly reproducible in replicate. It also shows that the low ratios from the other, unirradiated samples, are consistent with this classification in all pairs.

Figure A.5. Concordance Diagram for Test Samples



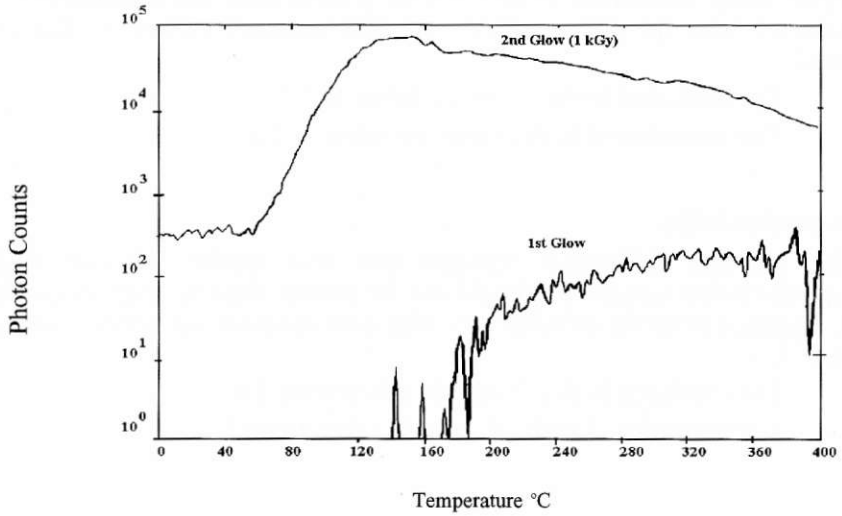
The glow curve shapes for the irradiated sample, (ref. SP303), shown and one of the typical unirradiated specimens, (ref. SP300), are shown below, to illustrate the differences between both strengths and shapes of TL signals from each category.

Figure A.6. TL glow curves from SP303 - an irradiated sample



Features to observe in figure A.6 are the rise curve of the first glow TL signal from 80°C to about 200°C followed by the broad distribution of signals up to 400°C which is characteristic of silicate polymineral samples. The second glow signal, due to the known laboratory dose of 1 kGy starts at lower temperatures, reflecting the absence of unstable short

Figure A.7 TL glow curves from SP300 - an unirradiated sample



lived components, and then follows a similar glow shape from about 160°C upwards. It may be inferred that the irradiated sample had received a dose of several kGy from the relative heights of the curves above this temperature.

The unirradiated sample illustrated in figure A.7 not only shows a dramatically lower TL signal strength in first glow compared with its second glow (1 kGy) response, the background curve also reaches its maximum intensity at higher temperatures. This reflects the increase in TL stability above 300°C, associated with archeological or geological time scales. Such shape differences provide an important complimentary indicator particularly for samples of low sensitivity.

A.3 Summary

The identification process illustrated above therefore entails: firstly ensuring that the samples are well measured and within instrumental sesitivity ranges, and are concordant. The TL glow ratio histogram and glow ratio plots may then be used to identify irradiated and unirradiated samples. Confirmatory checks on TL glow shape are advisable, and indeed necessary, for samples of low sensitivity.

APPENDIX II

Analytical Quality Control

General principles of analytical quality control are outlined in Protocol V0 of the series[6].

A1. Repeatability

The absolute difference between two test results obtained under repeatability conditions should not be greater than the repeatability, r, deduced from the collaborative trial data summarised below (Tables 1 and 2).

For irradiated herbs, r may be taken as 0.5.

For unirradiated herbs, r may be taken as 1.2.

A2. Reproducibility

The absolute difference between two test results obtained under reproducibility conditions should not be greater than the reproducibility, R, deduced from the collaborative trial data summarised below (Tables 1 and 2).

For irradiated herbs, R may be taken to be 1.0.

For unirradiated herbs, R may be taken to be 1.7.

It should be noted that these values were obtained by relatively inexperienced laboratories, and should, therefore, improve with further practice.

A3. Trueness (Bias)

Accuracy was not tested by spiking samples with known concentrations of minerals. However, there is no reason to suspect systematic bias.

A4. Statistical Data Derived from the Results of Interlaboratory Tests

Participants in the collaborative trial each analysed 12 irradiated and unirradiated samples from different countries.

Tables 1 and 2 summarises the statistical data obtained.

TABLE I

Summary of Calculated Statistical Parameters for Un-irradiated Herbs and Spices (Normalised Data)

Sample Letter	$\bar{x}$	n	r	S_r	RSD_r	R	S_R	RSD_R
C	-1.57	5	1.03	0.37	23.3	1.97	0.70	44.7
H	-1.94	5	0.94	0.34	17.4	1.96	0.70	36.1
MH	-1.73	5	1.52	0.54	31.3	1.69	0.60	34.9
S	-2.00	4	1.73	0.62	30.8	1.78	0.64	31.8
T	-2.09	4	0.56	0.20	9.6	0.81	0.29	13.9

TABLE II

Summary of Results of Statistical Parameters for Irradiated Herbs and Spices (Normalised Data)

Sample Letter	$\bar{x}$	n	r	S_r	RSD_r	R	S_R	RSD_R
C	0.61	5	0.38	0.14	22.6	0.53	0.19	31.3
CR	0.63	4	0.41	0.14	23.1	0.58	0.21	32.8
H	0.70	5	0.47	0.17	23.7	0.78	0.28	39.9
MH	0.51	5	0.48	0.17	33.8	0.98	0.35	68.9
PB	0.99	4	0.38	0.14	13.8	1.33	0.48	47.9
PFE	0.74	4	0.61	0.22	29.3	0.94	0.34	45.4
PK	0.67	4	1.20	0.43	63.6	1.74	0.62	92.2
PM	1.04	4	0.71	0.25	24.2	1.71	0.61	58.7
PR	0.66	4	0.76	0.27	41.3	0.76	0.24	35.9
S	0.50	5	0.32	0.12	23.2	0.67	0.24	48.1
SJ	0.75	5	0.45	0.16	21.2	1.05	0.38	49.9
T	0.76	5	0.33	0.12	15.4	0.54	0.19	25.4

A5. Key to Tables I AND II

A5.1 Sample Types used in Trial

Sample Letter	Description	Sample Letter	Description
C	Cumin (Turkey)	PK	Kommerz Paprika
CR	Madras Curry Powder	PM	Muntok White Pepper
H	Herbes de Provence	PR	Parsley
MH	Menthe Hartog (Mint)	S	Sage
PB	Black Pepper (Brazil)	SJ	Soupe Julienne (Mixture)
PFE	Favorit Paprika (Spain)	T	Thyme (Morocco)

A5.2 Statistical Terms

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.V28

Detection of Irradiated Bone-containing Meat

Community Bureau of Reference Draft Procedure for the Identification of All Irradiated, Bone-containing, Meats using Electron Spin Resonance Spectroscopy

Correspondence on this method may be sent to 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

The method enables the detection of all irradiated meats including poultry which contain bone fragments. It may be used to confirm commercial claims or to check compliance with labelling obligations under statutory instruments 1990:2489 (The Food Labelling (Amendment) (Irradiated Food) Regulations 1990 and Scottish equivalent [1990:2505 (S207)]).

2. Definition

2.1 Irradiated meats containing bones or bone fragments comprise those which have been exposed to gamma radiation or machine sources of ionising radiation (e.g. linear accelerator) for the purpose of reducing microbial contamination. Positive results imply that a sample has been so irradiated at a dose exceeding the minimum detectable dose.

2.2 g-value - see 'Principle' **3.2.**

2.3 Line width - see 'Principle' **3.2.**

3. Principle

3.1 Electron Spin Resonance (ESR) also known as Electron Paramagnetic Resonance (EPR) detects the unpaired electrons in paramagnetic substances (e.g. free radicals). A strong external magnetic field produces a difference in quantum energy levels between the two possible orientations of the electron spins, $M_s = +½$ and $M_s = -½$, where M_s is the spin quantum number. If electromagnetic energy of an appropriate frequency is applied the antiparallel spins absorb energy quanta and invert to the parallel state. This is known as the resonant frequency and the consequent absorption of electromagnetic energy is detected. Unpaired electrons

are produced by irradiation and these can be detected in this way in solid and dry materials such as bone where they are trapped and remain stable over a long period. In unirradiated samples there is either no signal or the signal is very weak.

3.2 For any particular absorption peak there is a constant ratio of resonant frequency to magnetic field strength called the 'g' value.

$$g = 7144.775 \cdot v/B$$

where v is the frequency in GHz and B is the magnetic field in mT. Unirradiated bones present no signal or a small symmetric signal with a g value of approx. 2.044 and a line width of 0.6 mT. Irradiated bones have an asymmetric signal defined by two g values corresponding to bone crystalline orientations parallel and perpendicular to the magnetic field. These are g 2.0030-2.0033 and g 1.9969-1.9975 with line widths of approx. 0.35 and 1.0 mT respectively (see Appendix I).

4. Apparatus

4.1 X - Band ESR-spectrometer, capable of operating at 9 GHz.

The ESR-spectrometer requires the following:

4.1.1 electromagnet having a swept field facility and field modulation,

4.1.2 microwave bridge and detector with variable output microwave power source,

4.1.3 resonant cavity,

4.1.4 signal processing units.

4.2 'Suprasil' quartz ESR cuvettes with inner diameter approximately 4.0 mm.

5. Procedure

5.1 Sample preparation

Take a fragment of bone, remove the marrow and clean the bone using distilled water if necessary and dry it on a filter paper. (*N.B.: Water absorbs microwaves and thus reduces the sensitivity of the procedure.*) Use a small piece of bone (approx 100 mg, 3.0-3.5 mm thick, 5.0-10.9 mm long) for the measurement. Put the sample into a standard ESR tube. It is possible to use a powdered sample but in either case care must be taken to completely remove the marrow.

5.2 ESR Measurements

The ESR spectrometer requires the follows:

5.2.1 Magnetic field: centre of field sweep equivalent to g=2.00 (e.g. 350 mT for a microwave frequency of 9.8 GHz).

5.2.2 Magnetic field sweep width ± 5 mT.

5.2.3 Magnetic field sweep rate 5 mT/min initially with an integrating time constant of 0.5 sec. Repeat at 2 mT/min if results are dubious with a time constant of 1-2 sec.

5.2.4 Magnetic field modulation amplitude 0.2 mT.

5.2.5 Magnetic field modulation frequency: 100 kHz.

5.2.6 Microwave power: 10 mW.

5.2.7 Temperature: room temperature.

5.3 Signal display

5.3.1 The first derivative of the spectrum should be displayed.

5.3.2 The line width is the horizontal distance in mT between the turning points on the first derivative spectrum associated with each individual ESR absorption line.

5.3.3 The g-values are the points of zero crossing or maximum gradient of the first derivative spectra corresponding to the peak maxima of each individual ESR absorption line.

6. Quality Assurance Consideration

6.1 Sample cells

All quartz cuvettes used must be cleaned and checked prior to use for no interfering background ESR spectrum. Broad lines greater in width than the overall sweep may be acceptable as subtractable background.

6.2 Sample sensitivity

Any sample with a signal level less than twice that of the noise level should be rejected and a further sample taken. If doubt remains the slower sweep rate defined above in **5.2.3** should be used with a proportionately longer time constant. However it is unlikely that such low levels of signal will be encountered since the minimum detectable irradiation dose for beef bone is much less than 0.5 kGy (see Appendix II) and commercial doses are likely to be in excess of 2 kGy.

6.3 Sample reproducibility

The reproducibility in terms of signal amplitude even for a single dose should not be expected to be good. Experiments have shown normalised standard deviations of up to ± 56% for measurements on several bone fragments given the same dose (see Appendix III). The ability to distinguish between irradiated and non-irradiated samples is not however impaired by this lack of reproducibility and trials have shown almost 100% accuracy in this task for irradiation of beef bones above 0.5 kGy (see Appendix II).

6.4 Precision

The same comments apply here as to **6.2** and **6.3** above. The procedure described has been the subject of an international collaborative trial under the aegis of the Community Bureau of

Reference (BCR) organised by CEN, Cadarache. A summary of the relevant results is given in Appendices I, II and III.

7. Identification of Irradiated Samples

The following indicators are used to determine whether the sample has been irradiated or not as already discussed in **3.1** and **3.2.**

7.1 If there is no clear ESR signal then the sample has not been irradiated.

7.2 If there is an asymmetric signal similar in shape to that shown in Fig AI.1 this indicates that the sample has been irradiated.

Detection of irradiated samples is reliable down to a minimum dose of 0.5 kGy but in many cases depending on the degree of bone calcification an identification is possible at even lower doses. Note that bones from smaller animals e.g. chicken bones have lower calcification than larger e.g. beef bones. In general the higher the degree of bone calcification the greater the signal response.

The results of this method are not significantly influenced by heating of the sample (e.g. boiling in water) if the marrow is well removed.

Detection of irradiation treatment is not significantly influenced by storage times of up to 12 months.

8. References

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9. Acknowledgements

The procedures described here were largely developed by J. Raffi at the Centre d'Etudes Nucleaire, Cadarache, France, H. Stevenson of the Department of Agriculture for Northern Ireland and the Queen's University, Belfast, UK and K-W Bögle at the Bundesgesundheitsamt (BGA), Berlin, Germany. They have been discussed and validated internationally in collaborative trials supported by the Community Bureau of Reference programme of the Commission of the European Communities.

APPENDIX I

Examples of ESR Spectra from Irradiated and Non-irradiated Bone

Fig AI.1 shows examples of spectra obtained at a frequency of 9.77 GHz from irradiated and non-irradiated beef bones. The upper curve is the unirradiated sample without marrow. The lower curve is an irradiated sample without marrow. The irradiated sample received a dose of 5 kGy. Similar spectra are found for all types of animal and poultry bones.

Fig AI.2 shows examples of spectra obtained from irradiated beef bones at different doses from 0 to 5 kGy.

APPENDIX II

Analysis of Results for BCR Collaborative Trial on Beef Bones

AII.1 Materials and Methods

Six samples of beef bones were sent to each of 21 laboratories to test blind, having been irradiated at any of six different doses (0, 0.5, 1, 2, 4 and 7 kGy). Some laboratories received two samples irradiated at the same dose but, in order to facilitate the interpretation of the results, the total number of samples for each dose was identical. All laboratories however received at least one 0 kGy and one 7 kGy sample.

The participants were instructed to follow the protocol found in this document.

Figure AI.1

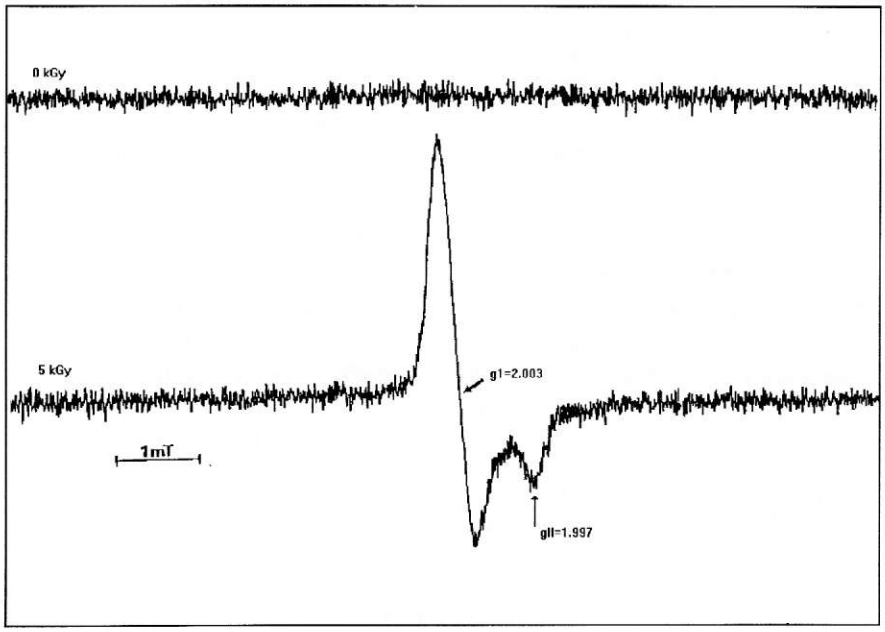
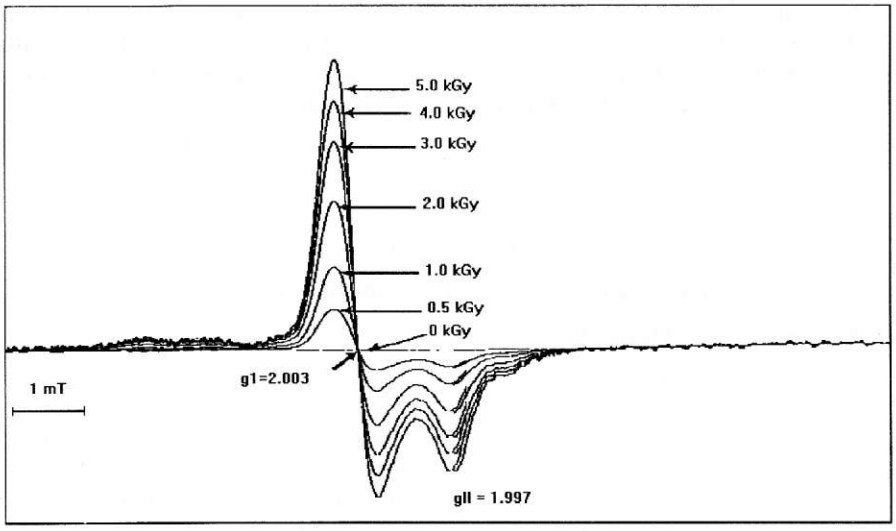


Figure AI.2



AII.2 Results and Discussion

There was in general a good agreement between laboratories. Among the 126 different samples there was only one mistake and this was later attributed to interference from a large background signal in the resonant cavity of the ESR spectrometer resonant cavity. Thus the mistake is attributable to poor technique only.

The results are presented in Table AII.1 where a 'O' indicates no irradiation detected, a '1' shows one sample found to be irradiated and a '2', shows two samples irradiated. Neglecting this one error it can be seen that down to at least 0.5 kGy there was no problem in detecting irradiated beef bone and this can be generalised readily to all other bones based on the literature.

APPENDIX III

Variation in Signal Amplitude from Single Poultry Bones

In a separate study of potential for quantitative measurement of irradiation dose carried out under the BCR programme, large within-laboratory variations were found in signal amplitudes. Eighteen fragments were taken at 3 different doses giving 54 fragments in total. The normalised standard deviations for the signal amplitudes of these 3×18 samples are shown in Table AIII.1.

These results do not affect the qualitative test for irradiation since the signals observed are unequivocally due to irradiation. Only the accuracy of amplitude measurement is affected.

The differences are at least partly related to the degree of crystallinity and the chemical composition of each bone. No link could be established between the signal intensity and the appearance of the bone.

TABLE AII.1

Qualitative test on beef bones

Laboratory No.	Value of the tested Irradiation dose					
	Ref	0.5 kGy	1 kGy	2 kGy	4 kGy	7 kGy
1	0	1	1	1	1	1
2	0	2	-	2	-	1
3	0	1	1	1	1	1
4	0	-	1	-	2	1
5	0	1	1	1	1	1
6	0	2	-	2	-	1
7	0	1	1	1	1	1
8	0	-	2	-	2	1
9	0	1	1	1	1	1
10	0	2	-	2	-	1
11	0	1	1	1	1	1
12	0	1	2	-	2	1
13	0	1	1	1	1	1
14	0	2	-	2	-	1
15	0	1	1	1	1	1
16	0	-	2	-	2	1
17	0	1	1	1	1	1
18	0	2	-	2	-	1
19	0	1	1	1	1	1
20	0	-	2	-	2	1
21	0	1	1	1	1	1
Percentage correct results		100	95.2	100	100	100

TABLE AIII.1

Normalised standard deviations of signal amplitude for chicken bone samples irradiated to different dose levels

No. of Samples	Dose (kGy)	Mean Signal Amplitude	Standard Deviation	Relative Standard Deviation (%)
18	0.3	1.084	0.553	51.0
18	3.0	2.633	0.568	21.6
18	7.0	9.03	5.06	56.0

J. Assoc. Publ. Analysts, 29, 209-220

MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

No. V29
Detection of Irradiated Poultry Meat

Method for the Detection of Irradiated Poultry Meat using the Limulus Amoebocyte Test in conjunction with a Gram Negative Bacterial Count

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

1. Scope and Field of Application

A procedure is described which will permit the presumptive identification of irradiated poultry. It comprises two methods which should be carried out simultaneously:

Method 1: Enumeration of resuscitated Gram negative bacteria in the test sample

Method 2: Determination of endotoxin concentration in the test sample using the *Limulus* amoebocyte lysate test

The procedure is generally applicable to whole or parts e.g. breast, legs, wings of fresh, chilled poultry carcasses with or without skin.

2. Interpretation of Results

Chicken carcasses which are identified as having high endotoxin levels using method 2 and similarly high Gram negative bacteria counts using method 1 show a normal microbiological profile and are not considered to have been irradiated. Thus when **$\log_{10}$ EU - $\log_{10}$ GNB = < 0 the sample is not irradiated.**

Chicken carcasses which are identified as having high endotoxin levels using method 2 but low levels or no detectable Gram negative bacteria using method 1 show an abnormal microbiological profile and **in the absence of any visible signs of processing** should be suspected of having been irradiated. Thus when **$\log_{10}$ EU - $\log_{10}$ GNB = > 0 the sample is suspected of being irradiated.**

Chicken carcasses which are identified as having endotoxin titres $< \log_{10} 2.0$ using method 2 and low levels of Gram negative bacteria using method 1 should be considered **inconclusive.**

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Method 1
Enumeration of Gram Negative Bacteria in Poultry

1. Definition

For the purposes of this method the following definition applies:

1.1 Gram negative bacteria

Organisms capable of forming colonies aerobically at 21°C in 25 h on a selective medium containing nisin, penicillin and crystal violet.

2. Principle

In general the enumeration of Gram-negative bacteria is carried out in three successive stages:

2.1 Preparation of initial test suspension in pyrogen free water.

2.2 Inoculation of non-selective solid agar medium: to allow resuscitation of stressed cells; pre-incubation at ambient temperature for 1.5 h.

2.3 Overlayering of non-selective medium: following resuscitation period with selective agar medium containing nisin, penicillin G and crystal violet; incubation at 21°C for 25 h.

3. Culture Media, Diluents And Reagents

3.1 General

For uniformity of results, it is recommended that media be prepared from dehydrated basic materials where possible. All chemicals used in the preparation of culture media shall be of analytical quality. Water shall be distilled or de-ionised and free from inhibitory substances.

3.2 Diluents

3.2.1 Pyrogen free water (Baxter Healthcare, Thetford)

3.2.2 Maximal recovery diluent

Peptone	1.0 g
Sodium chloride	8.5 g
Water	1000 ml

Preparation: Dissolve the constituents in water. Mix well and if necessary adjust the *p*H so that after autoclaving it is *p*H 7.0 ± 0.2 at 25°C. Distribute into 9 ml volumes in screw capped bottles. Sterilise by autoclaving at 121°C for 15 min.

3.3 Culture media

3.3.1 Nutrient agar

'Lab-lemco' powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar No. 1	15.0 g
Water	1000 ml

Preparation: Dissolve the constituents in water by boiling. Adjust the *p*H so that after autoclaving it is 7.4 ± 0.2 at 25°C. Sterilise by autoclaving at 121°C for 15 min. Cool to 45°C and dispense *ca* 10 ml into sterile petri dishes.

3.3.2 Gram negative selective medium

3.3.2.1 Basal medium

		IU/l
Milk agar (Oxoid CM21 or equiv.)	24.0 g	
Nisin	40.0 mg*	40,000
Water	998 ml	

** - may need to be adjusted according to commercial activity of nisin standard*

Preparation: Dissolve the constituents in water by boiling. Adjust the *p*H so that after autoclaving it is *p*H 7.1 ± 0.2 at 25°C. Distribute into final containers. Sterilise at 121°C for 15 min. Cool to 50°C.

3.3.2.2 Crystal violet solution

Crystal violet	10 mg
Water	10 ml

Preparation: Dissolve the crystal violet in the water. Filter sterilise using 0.22 µm pore size filter.

3.3.2.3 Penicillin-G solution

		IU/l
Penicillin-G	100 mg*	20,000
Water	10 ml	

** may need to be adjusted according to commercial activity of penicillin standard.*

Preparation: Dissolve the penicillin in water. Filter sterilise through 0.22 µm pore size filter.

3.3.2.4 Complete medium

		Final concentrations	
Basal medium	997 ml	milk agar	24 g/l
Crystal violet solution	2 ml	crystal violet	2 mg/l
Penicillin G solution	1.2 ml*	penicillin-G	20,000 IU/l
		nisin	40,000 IU/l

** may need to be adjusted according to commercial activity of penicillin standard.*

Preparation: Add the crystal violet and penicillin solutions aseptically to the molten basal medium tempered to 50°C. Mix well and dispense as required.

4. Apparatus and Glassware

Usual microbiological laboratory equipment and in particular:

4.1 Pyrogen free glassware

This may be achieved by heating at 180°C for 4 h. Sterile plasticware may be used in preference to glass and is normally pyrogen free.

4.2 Stomacher lab-blender and sterile stomacher bags

4.3 Sterile plastic universal bottles *ca* 30 ml (Sterilin).

4.4 Incubator : capable of being maintained at 21°C ± 1°C.

4.5 Oven: capable of being maintained at 180°C ± 2°C

5. Procedure

Samples should be analysed immediately upon receipt to reduce any further microbial proliferation. If this it is not possible, samples must be frozen at -20°C until examination which should be carried out as soon as possible.

5.1 Preparation of test sample and initial suspensions

Aseptically remove *ca* 2.5 g portions of skin from the neck, vent and under each wing of the chicken carcass to form a 10 g composite skin sample. If skinless chicken pieces are examined use 10 g surface muscle tissue excised aseptically. Place the skin or muscle portions in a stomacher bag and add 90 ml pyrogen free water (**3.2.1**). Stomach in the lab-blender (**4.2**) for 2 min. Decant *ca* 30 ml of the supernatant into a sterile universal bottle (**4.3**).

5.2 Preparation of dilutions

Ideally, plate counts should be in the range 30 to 300 colony forming units (cfu) per plate. Further serial dilutions may be prepared for plate counts by adding 1 ml of the initial suspension to 9 ml of maximal recovery diluent (**3.2.2**) to make a 10^{-2} dilution and repeating until the sample is sufficiently diluted (normally to at least 10^{-5}). No longer than 15 min should elapse between preparation of dilutions and plating out.

5.3 Plating out and enumeration

Using several dilutions (starting from 10^{-1}) prepared as in **5.1** and **5.2** above, spread 0.1 ml in duplicate over the surface of dried nutrient agar plates (**3.3.1**). Leave at room temperature (20-25°C) for 2 h. After resuscitation, overlay with *ca* 10 ml GNB selective agar (**3.3.2**). Allow to set and incubate in an inverted position at 21°C ± 1°C for 25 ± 0.5 h.

Count all the plates containing between 30 and 300 colonies and calculate the weighted mean number of cfu per g using the formula prescribed below.

$$C_s = \frac{N_i}{(n_1V_1F_1)+(n_2V_2F_2)+\ldots\ldots\ldots\ldots(n_nV_nF_n)}$$

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 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 10-fold dilution etc.)

V_s = the reference volume chosen to express the concentration of the micro-organisms in the sample.

Example:

Dilution counted	No. of colonies on duplicate plates
10^{-3}	271, 240
10^{-4}	30, 32

$$C_s = \frac{573}{(2\times 0.1\times 0.001)+(2\times 0.1\times 0.001)}$$

$$C_s = \frac{573}{2.2\times 10^{-4}}$$

$$C_s = 2.6\times 10^{6} \text{ cfu/g}$$

6. 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

7 Expression of Results

Record the number of cfu of GNB/g of skin or muscle as calculated in **5.3** If <30 colonies are present at the lowest dilution of 10^{-1} report the result as an estimated count (E). If no colonies of GNB are present from the lowest dilution of 10^{-1}, report the result as GNB not detected.

8. Quality Control Procedures

All culture media must be tested using appropriate positive and negative control reference cultures using normal in-house procedures.

9. References

9.1 Scotter, S.L., Beardwood, K. and Wood, R. 1993. Detection of irradiation of poultry meat using the *Limulus* amoebocyte lysate test in conjunction with a Gram negative bacteria count: Collaborative Trial. (In preparation)

APPENDIX I

TABLE I

Statistical data derived from inter-laboratory tests

Performance characteristics for GNB count on unirradiated samples

Sample Type	C+S*	C-S*
Irradiation Dose (kGy)	0	0
Number of Laboratories retained after eliminating outliers	17	17
Number of Laboratories eliminated as outliers	0	0
Number of results used in statistical calculations	34	32
LEVEL OF ORGANISM		
Mean observed value $\bar{x}$	5.96	5.42
REPEATABILITY		
Standard Deviation S_r	0.52	0.77
Relative Standard deviation RSD_r (%)	8.79	14.26
Repeatability r ($2.8 \times S_r$)	1.47	2.17
REPRODUCIBILITY		
Standard Deviation S_R	0.65	0.60
Relative Standard deviation RSD_R (%)	10.89	11.12
Reproducibility R ($2.8 \times S_r$)	1.82	2.17

* C+S chicken with skin; C-S skinless chicken breast fillets

Note: no statistical calculations possible for irradiated samples as GNB not detected

Method 2

Determination of Endotoxin Concentration in Poultry using the *Limulus Amoebocyte* Lysate Test

1. Definition

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

1.1 *Limulus* amoebocyte lysate (LAL) test:

A semi-quantitative assay in the form of microtitre plates containing lyophilised amoebocytes from the horseshoe crab which react to form a gel in the presence of lipopolysaccharide (also known as endotoxin/pyrogen) from the cell walls of viable and non-viable Gram-negative bacteria.

2. Principle

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

2.1 Preparation of dilutions of the initial suspension (**Method 1, 5.1**) of test sample in microtitre wells.

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

2.3 Visualising gel clots and calculation of endotoxin titres

3. Diluent and Reagents

3.1 Pyrogen free water (Baxter Healthcare, Thetford)

3.2 Lyophilised reference standard E.C 5 (*ca* 500 EU)

(Labortechnik Peter Schulz, Frankfurter Str. 61-63, D-6057 Dietzenbach, Germany)

3.3 Toluidine blue dye

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

Preparation: Dissolve the constituents in water and mix thoroughly.

4. Apparatus and Glassware

Usual microbiological laboratory equipment and in particular

4.1 All glassware coming into contact with the sample must be pyrogen free

This may be achieved by heating at 180°C for 4 h. Sterile plasticware may be used in preference to glass and is normally pyrogen free.

4.4 Non-circulating waterbath

i.e. static waterbath capable of maintaining a temperature of 37 ± 0.5°C

4.5 LAL test microtitre plates (Sensitivity 1-2 EU, 48 tests per plate, Labortechnik Peter Schulz)

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

4.6 Automatic pipettes: calibrated to a traceable source and capable of dispensing 64.8 µl and 30 µl volumes.

4.7 Parafilm

4.8 Suction pump (water or vacuum)

4.9 Vortex mixer

5. Procedure

5.1 The initial suspension prepared in Method 1 **(5.1),** is used for the measurement of endotoxin concentration

5.1.1 Dilution of sample

Using the microtitre plates, add 64.8 µl pyrogen free water to each dilution well (empty wells). Remove 30 µl from the initial test supension (Method 1, **5.1**) and add to first dilution well containing 64.8 µl pyrogen free water. Using a fresh pipette tip, mix the well contents thoroughly by filling and emptying pipette tip taking care not to introduce air bubbles into the microtitre well (Figure 1).

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

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

5.1.4 Continue with dilution of the test material in the microtitre plate as above until all eight vertical test wells have been filled for each sample. Four samples may be tested per plate.

5.2 Positive and negative controls

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

5.2.1 Positive control

Resuspend the lyophilised reference standard (**3.2**) in pyrogen free water to prepare a 50 EU/ml solution. Vortex for not less than 2 min. Test as for sample assay described above (**5.1**).

5.2.2 Negative control

Add 30 µl pyrogen free water (**3.1**) to two spare test wells in the microtitre plate.

5.3 Incubation of the LAL test

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

5.4 Visualisation of gel clots

After the incubation period, remove the plate from the water bath and dry carefully. Remove the parafilm and add 1 drop of toluidine blue dye to each test well. In order to detect gel formation, aspirate (suck out) each test well with the aid of a pasteur pipette fitted to a gentle suction pump. Gently touch the bottom of the well with the tip of the pasteur pipette with the suction on. Record a positive reaction (+) where a gel has formed in the well in which case a hole where the pasteur pipette touched will be visible. Record a negative reaction (-) when the liquid contents of the well are completely aspirated i.e. well is empty. Record a partial reaction (+/-) when there is slight gel formation.

6. Calculation of LAL Titre

Calculate the titre of the last positive well in the sequence of dilutions as shown in the examples:

Example 1:

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

Example 2:

Well No.	Gel	Dilution
1	+	$10^{-0.5}$
2	+	$10^{-1.0}$
3	+/-	$10^{-1.5}$
4	-	$10^{-2.0}$
5	-	$10^{-2.5}$
		Titre = 1.25

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

- EU/ml = $10^{(\text{titre})}$ × sensitivity of the lysate (stated on microtitre plate wrapping).
- EU/g = EU/ml × 10

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. Quality Control Procedures

General principles of analytical quality control are outlined in protocol V.0 of this series[4]

A positive (E.C.5, para.**3.2**) and negative (pyrogen-free water) endotoxin control must be included on each microtitre plate. If the negative control shows positive, all tests must be repeated with a new unopened batch of pyrogen-free water. If the positive control is > ± 0.25 titre of the expected titre, the samples must be repeated using another microtitre plate.

9. References

9.1 (See method 1)

9.2. Scotter, S.L, Wood, R. and McWeeny, D. 1990. Evaluation of the*Limulus* amoebocyte lysate test in conjunction with a Gram negative bacterial count for detecting irradiation of chicken. Radiat. Phys. and Chem., **36**, 629-638.

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9.4 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 2

TABLE II

Statistical data derived from inter-laboratory tests
Performance characteristics for LAL assay

Sample Type	C+S*	C+S	C+S	C-S	C-S
Irradiation Dose (kGy)	0	2.5	5	0	2.5
Number of Laboratories retained after eliminating outliers	17	17	17	17	17
Number of Laboratories eliminated as outliers	0	0	0	0	0
Number of accepted results after eliminating outliers	34	32	34	32	32
LAL TITRES					
Mean observed value	3.35	3.45	2.91	3.07	2.60
REPEATABILITY					
Standard Deviation S_r	0.62	0.76	0.60	0.73	0.46
Relative Standard deviation RSD_r (%)	18.59	21.88	20.67	23.90	17.86
Repeatability r (2.8 x S_r)	1.74	2.12	1.69	2.05	1.30
REPRODUCIBILITY					
Standard Deviation S_R	0.93	0.80	1.03	0.94	0.62
Relative Standard deviation RSD_R (%)	27.75	23.30	35.19	30.59	23.88
Reproducibility R (2.8 x S_r)	2.60	2.25	2.88	2.63	1.74

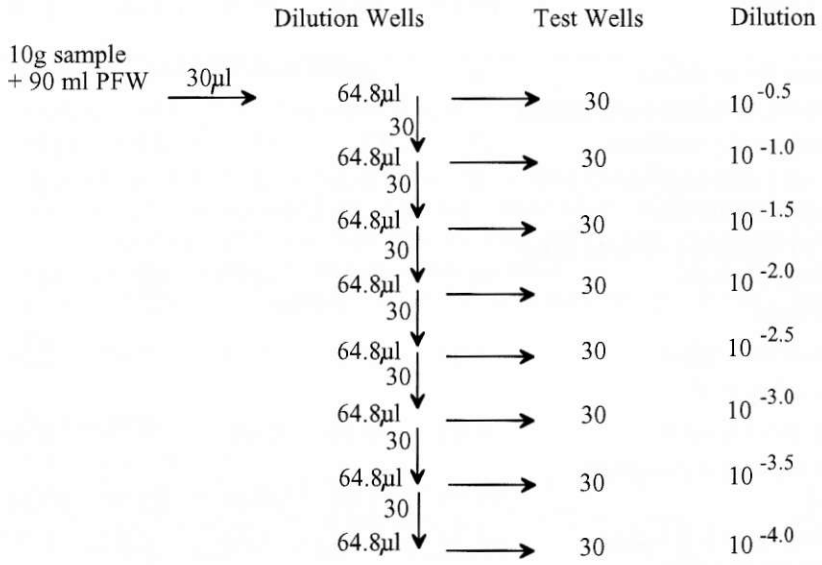
* C+S chicken with skin; C-S skinless chicken breast fillets

Key to Tables I and II

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

Figure 1

Schematic of preparation of *Limulus* amoebocyte lysate microtitre plate



***Note**: The microtitre plates are comprised of 6 segments. Each segment contains 8 vertical dilution wells and 8 vertical test wells. Schematic represents preparation of 1 segment i.e. one sample only.*