J. Assoc. Publ. Analysts 30, 101-124

The Determination of Caffeine in Roasted Coffee and Coffee Products by H.P.L.C

Collaborative Trial

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The results of a collaborative trial involving 20 participants on the determination of caffeine in instant coffee and related products are reported. The method tested comprised aqueous extraction, followed with separation by reverse phase HPLC with UV detection.

Participants were asked to analyse 7 different types of coffee and related products. The samples were sent out in the form of blind duplicate and split level samples. The method demonstrated satisfactory precision for all the samples tested except for the two decaffeinated products.

Precision for samples containing caffeine in the range of 0.66-4.06 g/100 g was acceptable as demonstrated by Horrat values of 1.8 to 1.2 respectively. The precision of the method deteriorated for lower caffeine contents. The two decaffeinated samples containing observed caffeine contents of 0.03 and 0.17 g/100 g gave RSD_R of 58 and 20 % respectively corresponding to the Horrat values of 8.9 and 3.9 respectively.

Introduction

Caffeine (1, 3, 7-trimethylxanthine) is the most abundant alkaloid present in coffee. The EC Council Directive 77/436/EEC as implemented by The Coffee and Coffee Products (Amendment) Regulations 1987 (SI No. 1986) stipulates a maximum limit for caffeine in decaffeinated coffee of 0.3 % by weight of coffee based dry matter[1][2]. Additionally the UK regulations stipulate a maximum limit of caffeine of 0.1 % by weight of coffee based dry matter in decaffeinated ground coffee or beans including coffee mixed with chicory or figs.

There is an official EC method for the determination of caffeine in decaffeinated coffee; it is described in Annex II of EC Directive 79/1066/EEC[3]. This is a "classical" spectrophotometric method involving an extensive and time consuming sample preparation stage where two chromatographic columns are used in the sample cleanup. There is a clear need for a suitable validated method that can be used on a routine basis for the determination of caffeine levels in coffee and, in particular, decaffeinated coffee.

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The Ministry of Agriculture, Fisheries and Food collaborated with the Association of Public Analysts to collaboratively test a HPLC method developed within the latter Association, for the determination of caffeine in coffee and decaffeinated coffee.

The Method of Analysis being Collaboratively Tested

The method requires hot water extraction, filtration, followed by separation using reverse phase HPLC with UV detection. A full description of the method is given in Appendix I. It was developed by the Association of Public Analysts as part of its Validated Enforcement Methods Service (VEMS) series of methods.

Collaborative Trial Organisation, Samples and Results

Twenty (UK Public Analysts) laboratories participated in the collaborative trial.

Samples

All the samples were prepared by Ruddock and Sherratt, Public Analyst's Laboratory, Chester.

Fourteen individual samples, comprising of six sets of blind duplicates and two split level samples, all of instant coffee products, were used in the trial.

Sample Preparation

A range of retail products were purchased. Each material was mixed and reduced to a fine powder by means of high speed blending in a food processor. Samples were used either directly in the form as purchased or were prepared by blending to obtain suitable concentrations of caffeine.

Sample scheme and caffeine content (g/100 g) obtained during homogeneity testing

Sample	A	B	C	D	E	F	G	G
(Numbers)	(2,7)	(5,11)	(4,12)	(3,8)	(9,14)	(6,13)	(10)	(1)
Caffeine	4.06	3.85	3.17	0.66	0.29	0.12	1.8	1.56

A (2 & 7) A "mild" instant coffee

B (5 & 11) A branded instant coffee

C (4 & 12) A branded freeze dried coffee

D (3 & 8) A blend of instant coffee and a chicory and coffee product**

E (9 & 14) (29, 124*) A leading brand decaffeinated instant coffee

F (6 & 13) (62, 213*) A generic (own brand) decaffeinated instant coffee

G (10) A leading brand "light" blend of decaffeinated instant coffee and instant coffee.

G (1) A generic (own brand) coffee and chicory product** blended with an instant coffee

*Samples of decaffeinated coffee were sent out in a follow up exercise to those participants who originally had taken 0.5 g weight for decaffeinated samples. These samples 6, 13, 9 & 14 were renumbered 62, 213, 29 & 124 respectively and reissued to participants.

**The caffeine content of these samples was adjusted by blending with other materials (see below).

The following blends were prepared in the laboratory for the purposes of this trial.

Sample D (0.66 g/100 g)

Chicory/coffee product containing 0.17 g/100 g caffeine was blended with an instant coffee containing 3.7 g/100 g caffeine, in the ratio of 88:12 (by weight) respectively. This product when analysed had an average caffeine content of 0.66 g/100 g (see homogeneity data, Appendix II)

Sample G1 (1.56 g/100 g)

In order to obtain a suitable split level to compare with sample G10 a coffee/chicory mix was blended with instant coffee in the ratio of 84.6:15.4 (by weight). This split level sample when analysed had an average caffeine content of 1.56 g/100 g (Sample G (1)) (see homogeneity data, Appendix II)

Homogeneity Testing

The homogeneity testing consisted of analysing 6 samples drawn randomly from each packing run, 5 samples each in the case of the split level pair (10 & 1). Results of the homogeneity testing are given in Appendix II.

Packing

The materials were packed in suitable plastic containers. A number of containers were withdrawn at this stage and used for homogeneity testing. The other containers were stored under desiccated conditions prior to dispatch.

Results

The results obtained in the trial are reported in Tables I-VII.

TABLE I

Caffeine Content g/100g

Sample A (4.06 g/100 g)

Laboratory	Sample Number	
	2	7
1	3.93	4.19
2	4.01	3.79
3	3.95	4.02
4	3.77	3.79
5	3.83	3.84
6	3.94	3.84
7	3.81	3.81
8	4.06	4.12
9	3.91	3.86
10	3.78	3.86
11	4.01 [(a)]	3.15 [(a)]
12	4.00	4.05
13	3.61	3.67
14	3.80	3.76
15	3.88	3.78
16	4.04	4.23
17	3.97	3.70
18	3.90	3.67
19	3.83	4.09
20	3.70	3.67
Mean	3.88	
r	0.28	
S_r	0.1	
RSD_r	2.6	
R	0.42	
S_R	0.15	
RSD_R	3.9	

For key, see Table IX

TABLE II

Caffeine Content g/100g
Sample B (3.85 g/100 g)

Laboratory	Sample Number	
	5	11
1	3.93	3.70
2	3.68	3.80
3	3.75	3.78
4	3.57	3.57
5	3.58	3.59
6	3.68	3.75
7	3.50	3.50
8	3.86	3.60
9	3.64	3.62
10	3.47	3.57
11	3.71	3.69
12	3.73	3.81
13	3.40	3.36
14	3.51	3.59
15	3.49	3.54
16	3.72	3.78
17	3.83	3.53
18	3.59	3.48
19	3.65	3.60
20	3.43	3.46
Mean	3.63	
r	0.23	
S_r	0.08	
RSD_r	2.3	
R	0.38	
S_R	0.14	
RSD_R	3.7	

For key, see Table IX

TABLE III

Caffeine Content g/100g

Sample C (3.17 g/100g)

Laboratory	Sample Number	
	4	12
1	3.08	3.32
2	3.13	2.99
3	3.18	3.22
4	2.97	2.98
5	3.03	3.05
6	3.16	3.25
7	2.97	2.81
8	3.21	3.36
9	3.08	3.08
10	2.92	3.02
11	2.89	3.21
12	3.16	3.22
13	2.90	2.78
14	3.31 [(a)]	4.12 [(a)]
15	2.91	2.84
16	3.22	3.20
17	3.21	3.00
18	3.10	3.00
19	3.05	3.38
20	2.81	2.91

Mean	3.07
r	0.3
S_r	0.11
RSD_r	3.5
R	0.44
S_R	0.16
RSD_R	5.1

For key, see Table IX

TABLE IV

Caffeine Content g/100g
Sample D (0.66 g/100 g)

Laboratory	Sample Number 3	8
1	0.69	0.73
2	0.68	0.71
3	0.66	0.67
4	0.62	0.62
5	0.64	0.63
6	0.62	0.68
7	0.54	0.56
8	0.69	0.81
9	0.61	0.61
10	0.61	0.64
11	0.62	0.59
12	0.67	0.64
13	0.59	0.59
14	0.62	0.63
15	0.61	0.61
16	0.64	0.61
17	0.63	0.57
18	0.63	0.63
19	0.60	0.69
20	0.64	0.62

Mean	0.64
r	0.09
S_r	0.03
RSD_r	4.8
R	0.14
S_R	0.05
RSD_R	7.7

For key, see Table IX

TABLE V

Caffeine Content g/100g

Sample E (0.29 g/100 g)

Laboratory	Sample Number 9		Sample Number 14	
1	0.15	(0.25)	0.15	(0.22)
2	0.19	(0.25)	0.18	(0.25)
3	0.17	(0.20)	0.18	(0.22)
4	0.17		0.17	
5	0.15	(0.19)	0.15	(0.20)
6	0.16		0.18	
7	0.08		0.08	
8	0.00[b]	(0.58)	0.00[b]	(0.50)
9	0.17		0.17	
10	0.20		0.20	
11	0.47[b]		0.44[b]	
12	0.22	(0.17)	0.24	(0.17)
13	0.23	(0.18)	0.20	(0.15)
14	0.15	(0.18)	0.15	(0.25)
15	-	(0.18)	-	(0.16)
16	0.20		0.18	
17	0.15	(0.19)	0.15	(0.19)
18	0.14	(0.19)	0.16	(0.16)
19	0.33[b]		0.32[b]	
20	0.19	(0.15)	0.19	(0.14)

Mean	0.17
r	0.03
S_r	0.01
RSD_r	5.39
R	0.1
S_R	0.04
RSD_R	20.5

For key, see Table IX

TABLE VI

Caffeine Content g/100g

Sample F (0.12 g/100 g)

Laboratory	Sample Number			
	6		13	
1	0.02	(0.07)	0.02	(0.07)
2	0.06	(0.00)	0.06	(0.00)
3	0.03	(0.06)	0.03	(0.06)
4	0.05		0.05	
5	0.04	(0.03)	0.04	(0.03)
6	0.03	(0.05)	0.03	(0.04)
7	0.00		0.00	
8	0.00	(0.46)	0.00	(0.29)
9	0.04		0.04	
10	0.06		0.06	
11	0.27[(b)]		0.28[(b)]	
12	0.08	(0.03)	0.07	(0.03)
13	0.01	(0.00)	0.01	(0.00)
14	0.03	(0.01)	0.03	(0.03)
15	-	(0.00)	-	(0.00)
16	0.06		0.06	
17	0.03	(0.00)	0.03	(0.00)
18	0.04	(0.03)	0.04	(0.03)
19	0.11[(a)]		0.02[(a)]	
20	0.04	(0.02)	0.05	(0.02)

Mean	0.04
r	0.01
S_r	0.0024
RSD_r	6.65
R	0.06
S_R	0.0213
RSD_R	58.51

For key, see Table IX

TABLE VII

Caffeine Content g/100g

SAMPLE G: Split Level Sample 10: 1.80 g/100 g & Sample 1: 1.56g/100 g

Laboratory	Sample Number 10		Sample Number 1
1	1.68		1.74
2	1.96		1.57
3	1.73		1.53
4	1.58		1.49
5	1.65		1.49
6	1.86		1.49
7	1.62		1.46
8	1.70		1.59
9	1.71		1.46
10	1.61		1.44
11	1.92		1.34
12	1.79		1.53
13	1.59		1.44
14	1.66		1.50
15	1.54		1.44
16	1.72		1.51
17	1.52		1.49
18	1.62		1.48
19	1.73		1.56
20	1.54		1.43
Mean	1.69		1.50
r		0.27	
S_r		0.10	
RSD_r		6.3	
R		0.29	
S_R		0.10	
RSD_R		6.3	

For key, see Table IX

Statistical analysis of the results

The collaborative trial results were examined for evidence of individual aberrant systematic error ($p<0.01$) using Cochran's and Grubb's tests progressively, by procedures described in the internationally agreed Protocol for the Design, Conduct and Interpretation of Collaborative Studies[4].

Horwitz Predicted Precision Parameters

There is often no validated reference/statutory method with which to compare precision criteria when assessing a method. In such cases it is useful to compare the precision data obtained from a collaborative trial with predicted acceptable levels of precision. These levels, predicted by the Horwitz equation, give an indication as to whether the method is sufficiently precise for the level of analyte being measured[5].

The Horwitz predicted value is calculated from the Horwitz equation[5]:

$$RSD_R = 2^{(1-0.5\log C)}$$

C = measured concentration of analyte expressed as a decimal

e.g. 1 g/100 g = 0.01

Horrat Values (Ho)

The Horrat[6] values quoted in Tables VIII give a comparison of the actual precision measured with the precision predicted by the Horwitz equation for a method measuring at that particular level of analyte. It is calculated as follows:

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

A Ho_R value of 1 usually indicates satisfactory interlaboratory precision, while a value of >2 indicates unsatisfactory precision i.e. one that is too variable for most analytical purposes or where the variation obtained is greater than that expected for the type of method employed. Similarly Ho_r is calculated, and used to assess intralaboratory precision, using the approximation $RSD_r(\text{Horwitz}) = 0.66RSD_R(\text{Horwitz})$. (This assumes the approximation $r = 0.66R$).

Repeatability and reproducibility

Calculations for repeatability (r) and reproducibility (R) were carried out on those results remaining after removal of outliers. The resulting values are given in Tables I-VII and have been summarised in Table VIII.

TABLE VIII

Summary of Calculated Statistical Parameters
Blind Duplicates

Sample Letter	Hom. test	(g/100g) obs.	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
A	4.06	3.88	19	0.28	0.10	2.6	1.2	0.42	0.15	3.9	1.2
B	3.85	3.63	20	0.23	0.084	2.3	1.1	0.38	0.14	3.7	1.1
C	3.17	3.07	19	0.30	0.11	3.5	1.6	0.44	0.16	5.1	1.5
D	0.66	0.64	20	0.085	0.030	4.8	1.7	0.14	0.049	7.7	1.8
E	0.29	0.17	16	0.026	0.009	5.4	1.6	0.10	0.035	20.5	3.9
F	0.12	0.04	17	0.028	0.024	6.7	1.5	0.06	0.021	58.5	8.9

Split Level Samples (G)

Sample numbers	Hom. test	(g/100g) obs.	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
10&1	1.80 (1.69)	1.61 (1.50)	20	0.27	0.10	6.1	2.5	0.29	0.10	6.5	1.7

For key, see Table IX

TABLE IX

Key to Tables I to VIII

(a)	An outlying result by Cochran's Test at P<0.01 level, not used in calculation of mean, repeatability or reproducibility.
(b)	An outlying result by Grubbs' Test at P<0.01 level, not used in calculation of the mean, repeatability or reproducibility.
(c)	Result recorded as "less than" or "not detected" and not used in the calculation of the mean, repeatability or reproducibility.
( )	Result for decaffeinated samples using 0.5 g sample weight, not used in the calculation of mean, repeatability or reproducibility.
Hom. test	The mean obtained from homogeneity data.
obs.	The observed mean, the mean obtained from the collaborative trial data.
n	Number of laboratories whose data were used in the statistical calculation, excluding outliers.
r	Repeatability (within laboratory variation). The value below which the absolute difference between two single test results obtained with the same method on identical test material under the **same conditions** may be expected to lie with 95 % probability.
S_r	The standard deviation of the repeatability.
RSD_r	The relative standard deviation of the repeatability (S_r h 100/MEAN).
Ho_r	The HORRAT value for repeatability is the observed RSD_r divided by the RSD_r value estimated from the Horwitz equation using the assumption r = 0.66R.
R	Reproducibility (between-lab variation). The value below which the absolute difference between two single test results obtained with the same method on the identical test material under **different conditions** may be expected to lie with 95 % probability.
S_R	The standard deviation of the reproducibility.
RSD_R	The relative standard deviation of the reproducibility (S_R h 100/MEAN).
Ho_R	The HORRAT value for reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

The results demonstrate satisfactory precision for the five samples containing caffeine at concentrations of $\geq$ 0.66 g/100 g. The reproducibility for all these samples was acceptable as demonstrated by Horrat (Ho_R) values of 1.1-1.8. The intralaboratory precision (repeatability) was acceptable for four of these five samples with only the split level samples G & H being outside the acceptable predicted levels (Ho_r 2.5).

The precision obtained from the analysis of the two decaffeinated samples was unsatisfactory both in terms of repeatability and reproducibility. There was some confusion amongst participants about the sample weight to be taken. Seven participants followed the intended directions of reanalysing the decaffeinated samples using a larger sample weight of 4 g once a decaffeinated sample had been identified. The other 13 laboratories used a sample weight of 0.5 g, these results are given in brackets in Tables V and VI. These thirteen laboratories were asked if they could repeat their analysis on the decaffeinated samples using the procedure identified in the method, i.e. **8.2.2.**, of the thirteen laboratories, twelve submitted results using the full procedure and their results were therefore included in Tables V and VI.

Discussion

The results indicate that the precision of the method is satisfactory for the determination of caffeine in coffee products in the range 0.6-4 g/100 g caffeine, but is less satisfactory when determining lower levels of caffeine. The method has been used routinely by the laboratory involved in the sample preparation, for the determination of caffeine in decaffeinated coffee with satisfactory within-laboratory precision. However, it is clear from the results of this trial that the method as written is not sufficiently robust when determining low levels of caffeine, i.e. when the method is used by other laboratories.

The method specified the content of the mobile phase and flow rate, these specifications can only ever be a guide and only then apply to the column specified in the method (25 cm × 4.9 mm Partisil ODS). Participants used a variety of columns (see Appendix III), some optimised the system to accommodate the different column. Other laboratories followed the protocol exactly, the result of which was that these laboratories often had too short a retention time and could not successfully resolve the caffeine peak from the shoulder of that of the co-extractives. Some participants, even when using the same HPLC conditions as specified in the method (including the suggested column), obtained significantly shorter retention times than those quoted. Several participants experienced problems in obtaining stable retention times when analysing the samples, this problem was particularly apparent when analysing the decaffeinated samples using a 4 g initial sample weight. This could suggest that in these cases the

sample being injected into the HPLC was not clean enough and particulate matter was affecting the integrity of the system.

Most participants obtained satisfactory peak shape although slight tailing was common.

Despite the problems identified above, the precision obtained for the caffeinated samples i.e. caffeine contents 0.64-3.88 g/100 g, was satisfactory.

Virtually all the laboratories reported difficulties in analysing the decaffeinated coffee samples. Participants reported problems in successfully separating the caffeine peak from the co-extracted material. The caffeine peak was usually quantified on the shoulder of the peaks of the co-extracted material causing problems in quantification, and is probably the main reason for the poor precision obtained for the decaffeinated samples. It should be noted that while this method has been shown to be far from ideal at measuring low levels of caffeine, there are very few suitable validated methods for the determination of caffeine in decaffeinated coffee products.

Recommended precision parameters to be included in the validated method are:

Caffeine content (g/100g)	r (g/100g)	R(g/100 g)
3-4	0.27	0.40
0.66	0.09	0.14
0.17	0.03	0.10

It would appear that although this method has been used routinely by the laboratory which developed it and which prepared the samples used in the trial for the determination of low levels of caffeine in coffee products, the results at low levels are not easily reproducible. An improvement in the sample clean-up may improve the robustness of this method.

Conclusion

The results obtained from this collaborative trial show that the method is suitable for the determination of coffee/coffee products containing caffeine at concentrations of 0.6-4 g/100 g. While the precision of the method when analysing samples containing caffeine at concentrations of less than 0.3 g/100 g is poor, the method can be used for routine analysis in the absence of a superior alternative method. Further work is required to improve the sample cleanup for the determination of low levels of caffeine before the method is suitable for the determination of caffeine in decaffeinated coffee samples.

Acknowledgements

The authors wish to thank the following analysts and their staff members (named in parentheses) who participated at the time of the collaborative trial:

Mr D Dunn (HJ Hanslip)	A.H. Allen and Partners, Sheffield;
Mr N K Payne (D Gibbons)	Central Scientific Laboratories, New Cross, London;
Mr R Davies (MJ Weaver)	City of Birmingham Public Analyst's Laboratory, Birmingham;
Mr P Sweet	City of Cardiff, City Analyst's Laboratory, Cardiff;
Mr R Stevens (Mr M O'Meara)	Clayton Environmental Consultants Ltd, Birmingham;
Mr J S Turnbull (M Somers)	County of Avon Scientific Services, County Laboratory, Bristol;
Dr A C Smith (A Chapman)	Greater Manchester Scientific Services, Manchester;
Mr E E J King (P J Welsh)	Hampshire Scientific Services, Hampshire;
Mr T E Johnson	Herbert J Evans and Partners, Public Analysts Department, Dyfed;
Mr B McLean (Mr L A Yeates)	Hereford and Worcester County Council, County Laboratory, Worcester;
Mr K Wardle (Dr M Thomas)	Humberside County Scientific Service, Kingston-Upon-Hull;
Mr I Hampton (J Griffin)	Kent County Laboratory, Maidstone;
Laboratory of the Government Chemist,	Teddington, Middlesex;
Miss L C Graham	Leicestershire County Analyst's Laboratory, Leicestershire;
Mr S Stangroom (A Alexander)	Lincolne, Sutton and Wood, Norwich;
Mr F Gorton	Melling and Arden, Salford;
Dr W Griffiths (A R Kiddie)	Mid Glamorgan County Council, County Scientific Laboratory, Cardiff;
F Hollywood (A Jones/H Sellman)	Staffordshire County Analyst's Department, Stafford;
Mr C J Heide (A R Janes)	Somerset County Council, Somerset;
Mr S Joyce (W Swanson)	Strathclyde Regional Chemist and Public Analyst Department, Glasgow;
Mr E B Reynolds (T Williams)	Tickle and Reynolds, Public Analyst's Laboratory, Exeter;

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APPENDIX I

The Determination of Caffeine in Roasted Coffee and Coffee Products by H.P.L.C.

1. Scope and Field of Application

The method describes the determination of the caffeine content of roasted coffee, decaffeinated roasted coffee, instant coffee and decaffeinated instant coffee.

2. Definition

The caffeine content means the caffeine content extracted and determined by the method as described.

3. Principle

Caffeine is extracted from the sample with hot water and is determined by H.P.L.C.

4. Health and Safety

4.1 *Care should be taken when handling hot objects. Use tongs.*

4.2 *Methanol is highly falmmable and toxic by inhalation or if swallowed. Avoid contact with skin. Keep away from sources of ignition.*

4.3 *Caffeine is toxic if swallowed avoid contact with skin and eyes. If unwell, seek medical advice.*

4.4 *Perchloric acid may explode if heated Contact with combustible material may cause fire. Causes severe burns. Do not breathe vapour. In case of contact with eyes rinse immediately with copious amounts of water and seek medical advice. Wear suitable protective clothing*

4.5 *Acetic acid is flammable and causes severe burns. Do not breathe fumes. In cases of contact with eyes rinse immediately with copious amounts of water and seek medical advice.*

5. Pre-Training Requirements

5.1 Use of analytical balance.

5.2 Use of H.P.L.C. System.

5.3 Use of volumetric glassware.

6. Reagents

6.1 GPR and AR grade reagents are suitable unless otherwise stated. Water should be de-ionised, distilled or of similar quality.

6.2 Caffeine, anhydrous - Prepare a stock solution containing 500 mg/L Caffeine in water.

6.3 H.P.L.C. Mobile phase.

Methanol (H.P.L.C. grade) 450 volumes : Water (H.P.L.C. grade) 1,050 volumes : Perchloric acid (A.R. grade) 3 volumes. Filter and degas before use.

7. Apparatus

7.1 Normal laboratory glassware and apparatus.

7.2 Coffee grinder.

7.3 H.P.L.C. system capable of delivering the mobile phase @ 2.0 mL min^{-1}, fitted with a 20μl injection loop. UV detector monitoring at 272 nm, electronic integration and/or chart recorder.

7.4 25 cm × 4.9 mm I.D. Partisil 10 ODS H.P.L.C. Column. The retention time of caffeine on this column under the conditions specified is about 9 minutes. Other columns with similar resolving power may be suitable.

7.5 Whatman 54 & GF/C filter circles.

7.6 Membrane filters. Gelman Acro LC3A filters are suitable.

8. Procedure

8.1 Whole coffee beans must be ground before commencing the analysis.

8.2 Preparation of sample extracts.

8.2.1 Coffee and decaffeinated coffee

Extract about 5 g of ground sample (10 g for decaffeinated coffee), weighed to the nearest 0.001 g with approximately 100 mL of boiling water by refluxing for one hour. Filter the extract (Whatman 54) into a 500 mL volumetric flask (250 mL flask for decaffeinated coffee). Boil the insoluble residue with a further 100 mL of water, under reflux, for 15 minutes and filter into the same flask. Cool to 20°C, make up to volume with water, mix and filter (GF/C).

8.2.2 Instant Coffee and Decaffeinated Instant Coffee

Dissolve about 0.5 g of instant coffee (4 g for decaffeinated instant coffee) weighed to the nearest 0.0001 g, in hot water and transfer to a 100 mL volumetric flask. Dilute with water to about 75 mL. Cool to 20°C, make up to volume with water, mix and filter (GF/C).

8.3 Extracts from **8.2** must be passed through membrane filters before analysis by H.P.L.C.

8.4 Prepare a range of working standards by pipetting 5, 10, 20 and 50 mL of stock standard solution (**6.2**) into separate 100 mL volumetric flasks. Dilute to volume with water and mix. These working standards contain 25, 50, 100 and 250 mg/L caffeine respectively. Each standard must be passed through a membrane filter before analysis by H.P.L.C.

8.5 Inject 20 μl aliquots of each working standard into the chromatograph.

8.6 Determine peak height or areas (by electronic integration) and plot a calibration graph. This should be rectilinear over the required concentration range.

8.7 Inject 20 μl aliquots of sample extract into the chromatograph. Identify the peak due to caffeine by comparison of retention time with the working standard chromatograms. From the peak height or area (electronic integration), use the calibration graph to determine the concentration C of caffeine in the sample extract.

9. Calculation

9.1 Since the volume of sample and standard solutions injected into the chromatograph were the same (20 μl), the concentration C of the sample extract, in mg/L may be determined directly from the calibration graph.

9.2 The concentration of anhydrous caffeine in the sample, expressed as a percentage of the sample by weight is given by:-

$$\text{Concentration of anhydrous caffeine (\%)} = \frac{V}{10^4} \times \frac{C}{M}$$

where V is the volume of volumetric flask for sample extract (**8.2**) mL

V = 500 for Coffee

V = 250 for decaffeinated coffee

V = 100 for instant coffee and decaffeinated instant coffee

C = Concentration of caffeine in the sample extract, mg/L

M = Weight in grams, of the test portion

10. Expression of Results

Record the result to the nearest 0.01%.

11. Interpretation

The Coffee and Coffee Products Regulations, 1978 (as amended) set limits for residual anhydrous caffeine in decaffeinated designated products. Samples which exceed these limits when analysed by this routine method must be re-analysed using the designated reference method for enforcement purposes (**12.2**).

12. References

12.1 The Coffee and Coffee Products Regulations S1 1978/1420 (as amended).

12.2 Commission Directive 79/1066/EEC laying down Community methods of analysis for testing coffee extracts and chicory extracts, OJ No L327, 17-28, 24.12.79.

APPENDIX II

Homogeneity Data

For blind duplicate samples, six extra containers were packed, spread throughout the the filling run, these were then withdrawn and checked for uniformity of caffeine content. For the split level samples the same procedure was followed except only five extra containers for each material were packed and subsequently analysed.

	Sample A (Sample No 2 & 7)	Sample B (Sample No 5 & 11)	Sample C (Sample No 4 & 12)
	Caffeine g/100g	Caffeine g/100g	Caffeine g/100g
1	4.05	3.83	3.19
2	4.07	3.87	3.18
3	4.07	3.84	3.17
4	4.06	3.86	3.17
5	4.04	3.84	3.15
6	4.07	3.87	3.15
Mean	4.06	3.65	3.17
s	0.0126	0.0172	0.016
CV(%)	0.31	0.45	0.51

	Sample D (Sample No 3 & 8)	Sample E (Sample No 9 & 14) (Decaffeinated)	Sample F (Sample No 6 & 13) (Decaffeinated)
	Caffeine g/100g	Caffeine g/100g	Caffeine g/100g
1	0.65	0.29	0.13
2	0.65	0.29	0.12
3	0.65	0.29	0.11
4	0.66	0.28	0.11
5	0.66	0.29	0.13
6	0.66	0.29	0.11
Mean	0.66	0.29	0.12
s	0.005	0.004	0.010
CV(%)	0.83	1.41	8.31

	Sample G (Sample No 10) Caffeine g/100g	Sample G (Sample No 1) Caffeine g/100g
1	1.80	1.57
2	1.78	1.58
3	1.81	1.55
4	1.79	1.54
5	1.80	1.57
Mean	1.80	1.56
s	0.011	0.016
CV(%)	0.63	1.05

APPENDIX III

Comments from participants

Laboratory 1

Hypersil 5 ODS rt 4.6 min. The calibration graph using peak heights was not linear. Calibration using peak areas was linear throughout concentration range. Peak heights were used to quantify very low concentrations.

Laboratory 3

Partisil 10 ODS 2 used. Retention time 14 min.

Laboratory 4

Column, 25 cm Hypersil ODS. At a flow rate of 2 ml/min this gave a retention time of 5.1 min but caffeine peak unresolved from small coeluting peak. Flow rate was changed to 1.0 ml/min giving rt 10.1 min. Samples 6 & 13 gave slightly unusual peak shape.

Laboratory 6

25 x 4.9 mm Spherisorb 5 ODS-2. 1 ml/min.

Laboratory 7

Column ODS-3 10 m 25 x 0.46 cm. Samples 3, 6, 8, 9, 13 & 14 all repeated using 4 g.

Laboratory 9

Column 10 cm RP 18 10 m 3 mm id, flow rate 0.4 mL/min (0.3 mL/min for decaffeinated) Injection vol. 10 L. Calibration graph found to be linear results calculated vs 100 mg/L standard for caffeinated samples, 25 mg/L for decaffeinated samples (peak height). Strongly recommend using acetic acid (0.1M) in place of perchloric acid/water used in this trial. The pH of the eluent used in this trial (pH 1) will cause unnecessary degradation of the silica based column and will be detrimental to the pump and tubing in the long term.

Laboratory 10

Erratic pressure fluctuations were obtained using HPLC system described in method. Retention times varied at start of batch becoming more constant towards the end. Retention times of caffeine using conditions and column specified were 6.5 min compared to 9 mins recorded in method. Could not obtain satisfactory separation of caffeine from remaining sample constituents. This led to a slope in the

base of the caffeine peak making determination of the caffeine peak more difficult.

Laboratory 11

Took 0.5 g for samples 9 & 14 and 4 g for 6 & 13.

Laboratory 12

Column used 16 cm 5 m ODS. Mobile phase 80/20/3 H_2O/methanol/perchloric acid. Flow 1.2 ml/min. Rt caffeine = 6.9 min.

Laboratory 14

Column used Spherisorb ODS 5 m. Large variations in retention time 8.7-9.6 min.

Laboratory 17

8.2.2. "hot water" needs more precise definition.

Laboratory 18

Method does not state temperature of the hot water used for extraction. Used 5 g for samples 6, 9, 13 & 14. A 10 mg/L standard was more appropriate for samples 6 & 13. The absorbance for high standards was greater than we would normally consider good practice. Higher sample dilutions/lower standard concentrations would be preferable.

J. Assoc. Publ. Analysts 30, 125-152

Determination of Sulphadimidine Residues in Porcine Muscle

Collaborative Trial

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The results of a collaborative trial to assess a method for the trace residue determination of sulphadimidine in incurred animal tissue are reported. Fourteen laboratories participated in the trial. The method tested comprised solvent extraction, cleanup using anion/cation solid phase extraction and separation by HPLC with fluorescence detection.

Participants analysed 12 test materials comprising six different concentrations of incurred sulphadimidine in the range 30 - 570 µg/kg. The precision of the method was satisfactory for all the concentrations analysed except for the lowest concentration of sulphadimidine (30 µg/kg). The precision for the two concentrations near the MRL of 100 µg/kg was well within theoretical predicted limits.

Introduction

Sulphadimidine (sulphamethazine), (*N*'-4,6-dimethylpyrimidin-2-yl sulphanilamide), is a sulphonamide antibiotic drug which is used for the prevention and control of disease in animals and humans. The application of sulphonamides extends to therapeutic, prophylactic, and also growth promotion uses in cattle, pig, sheep, poultry, and fish.[1] The widespread use of these agents in food producing animals raises the possibility of residues remaining in edible tissue after slaughter.

MAFF routinely monitors the incidence of sulphonamides present in the UK food supply through statutory surveillance as prescribed under EC Directive 86/469 and also at the request of the MAFF Steering Group on Food Surveillance.[2][3] This collaborative trial is testing a method for the determination of sulphadimidine, one of the most widely used sulphonamides. Incidence of residues of sulphadimidine have decreased in recent years following a publicity campaign by MAFF and the introduction of Maximum Residue Limits (MRLs).[4][5]

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A Maximum Residue Limit of ***100 µg/kg*** for total sulphonamide residues in edible tissue has been prescribed by European Community Regulation (EEC) No. 2377/90 and also by The Animals, Meat and Meat Products (Examination for Residues and Maximum Residue Limits) (Amended) Regulation, 1991.[4][5]

There are over 20 sulphonamide class antibiotics with veterinary applications. Methods of analysis for residues of these compounds must therefore be capable of determining individual residues at concentrations well below the MRL, in case a mixture of sulphonamides is present. A wide variety of food types can potentially contain residues of sulphonamides; these include liver, kidney and muscle of all farmed animals, and also milk, eggs and processed food products made with these ingredients.

The Food Science Laboratory, Norwich, has developed an HPLC - Fluorescence detection method for the determination of sulphadimidine in meat. The extraction and clean up are based on the method of Haagsma[6], but includes an additional anion exchange (NH_2) solid phase extraction column to act as a filter for some co-extractives present in some samples.

The determination of veterinary drug residues has always been a problematical area, and has in the past been performed only by specialist laboratories. However there is a clear need for an accurate, precise and robust method to determine sulphadimidine in meat and that can be used by non-specialist food analysts. The Food Science Laboratory has therefore collaboratively tested such a method; if successful the method will be published in the series of MAFF Validated Non-Statutory methods.[7]

Method of Analysis being Collaboratively Tested

The method comprises extraction into 5% acetic acid in ethyl acetate, cleanup by anion and cation exchange (SAX & SCX) solid phase extraction cartridges, derivitisation and separation by HPLC with fluorescence detection. (See Appendix I for full description of method)

Collaborative Trial, Organisation, Samples and Results

Participants

14 laboratories participated in the collaborative trial (14 UK Public Analyst Laboratories).

Trial Organisation

The participants were asked to familiarise themselves with the method in their laboratory prior to analysing the collaborative trial samples. The collaborative trial used samples of incurred tissue and was carried out in two stages:

Pre-trial

A test material containing approximately 700 μg/kg of sulphadimidine in incurred pig muscle tissue was sent to each participant to be analysed in duplicate (See Table I). As a result of the pre-trial, slight alterations to the method were made for the purposes of the trial proper. Comments received from participants are given in Appendix II.

Trial proper

The 6 samples comprising 6 different concentrations of sulphadimidine were sent to participants in the form of blind duplicates i.e. each participant received 12 test materials to be analysed by the prescribed method. (See Table II)

Sample preparation and homogeneity

Sample Preparation

All the trial test materials were incurred porcine muscle tissue and were prepared by Dr R Patterson, Department of Animal Husbandry, University of Bristol. The base incurred porcine tissue was originally analysed to determine the approximate concentration of sulphadimidine. The base tissue was then diluted with blank porcine tissue, i.e. porcine tissue containing no sulphadimidine, to achieve a suitable sample range. The samples were finally tested at the Food Science Laboratory for homogeneity. (See Table III)

Homogeneity Testing

For each of the six concentrations (except S7) five test materials were taken and analysed in duplicate. Each batch of samples comprised these ten samples plus two samples of blank tissue (S7), one spiked at 100 μg/kg sulphadimidine.

The results of the homogeneity testing were subjected to an analysis of variance test described in the International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories.[8] All the results were found to be satisfactory.

Results

The results received are given in Table IV. The results from two laboratories (7) & (10) were eliminated from the trial during the initial screen of the results. They both failed to get the satisfactory recoveries of sulphadimidine required for quantitative analysis (Table V). In the case of Laboratory 7, the reported results were clearly gross outliers, the reasons for such atypical results have not been ascertained.

Statistical analysis of the results

Due to the unfamiliarity of most of the participants with the analysis for veterinary drug residues and the variable results of the pre-trial, the data were submitted to Youdens ranking test to detect laboratories that were performing atypically across the sample range, prior to analysing the data for individual outliers using Cochrans and Grubbs tests[9][10].

The results of Youdens ranking test identified laboratory 14 as an outlier laboratory, their results, which were consistently low, were therefore not included in the further statistical analysis (Table VI).

The trial results were then examined for evidence of individual aberrant systematic error ($p<0.01$) using Cochran's and Grubbs tests progressively, by procedures described in the internationally agreed Protocol for the Design, Conduct and Interpretation of Collaborative Studies[10]. Only one result, Lab 1 S3 was found to be an outlier (Table VII).

Horwitz Predicted Precision Parameters

There is often no validated reference or statutory method with which to compare precision criteria when assessing a new method. In such cases it is useful to compare the precision data obtained from the collaborative trials with predicted acceptable levels of precision. These levels, predicted by the Horwitz equation, give an indication as to whether the method is sufficiently precise for the level of analyte being measured.[11]

The Horwitz predicted value is calculated from the Horwitz equation[11]:

$$RSD_R = 2^{(1-0.5\log C)}$$

C = measured concentration of analyte expressed as a decimal

i.e. 1 g/100g = 0.01

Horrat Values (*Ho*)

The Horrat values give a comparison of the actual precision measured with the precision predicted by the Horwitz equation for a method determining that particular level of analyte.[12] It is calculated as follows:

A Ho_R value of 1 usually indicates satisfactory interlaboratory precision, while a value of >2 indicates unsatisfactory precision i.e. one that is too variable for most analytical purposes or where the variation obtained is

greater than that expected for the type of method employed. Similarly Ho_r is calculated, and used to assess intralaboratory precision, using the approximation $RSD_r(Horwitz) = 0.66RSD_R(Horwitz)$ (this assumes the approximation $r = 0.66R$). The Horwitz values calculated from the results of this trial are included in Table VIII.

Repeatability and Reproducibility

Calculations for repeatability (r) and reproducibility (R) were carried out on those results remaining after removal of outliers.[9] The resulting values are given in Table VII and have been summarised in Table VIII.

Discussion

Accuracy and precision

The results of the statistical analysis show that the performance of the method was satisfactory. The intralaboratory precision, the repeatability, was acceptable for all the samples analysed. This can be seen by the corresponding Horrat values (Ho_r) which ranged from 0.6 (S2 & S3) to 1.9 (S5). The interlaboratory precision, the reproducibility, was acceptable for all the samples bar the "blank" (S7) with the corresponding Horrat values (Ho_R) ranging from 1.2 (S2 & S3) to 2.2 (S7). The relationship between concentration and precision was not linear. The precision was optimum for S2 and S3 i.e. the samples containing analyte in the middle of the concentration range of sulphadimidine. It would normally be expected that the relative precision (RSD) would decrease with increasing analyte concentration, the fact that the RSD increased for the samples containing the highest concentrations of sulphadimidine (S4 & S5) demonstrates that the method performed less well for these levels containing higher amounts of analyte. Although there was no attempt to estimate trueness in this collaborative trial, in general the results from participants agreed well with the results obtained by the co-ordinating laboratory during homogeneity testing. This is demonstrated when comparing the mean concentration obtained for a particular level by participants, with the corresponding mean value (Target Value (TV)) obtained by the co-ordinating laboratory. While any such comparison is essentially a measure of the reproducibility of the method, the co-ordinating laboratory has considerable expertise in the determination of veterinary drug residues and is one of two reference laboratories for this type of work in the UK, the Target Values therefore are of importance.

Chromatography

Most laboratories did not use the HPLC column prescribed in the trial method (See Table IX). The recommended column was the Waters Radial-Pak column with a Nova-Pak C18 cartridge which was chosen for its ability to resolve a large number of different sulphonamide type

antibiotics. The use of other columns did not pose a significant problem for the purpose of this exercise (providing that the chromatography was acceptable) as sulphadimidine was the only sulphonamide present in the trial samples. Some participants using different columns to that recommended, could only analyse very small batches due to the longer retention times produced by their columns making the overall analysis of the samples more time consuming. All the columns used in the collaborative trial gave satisfactory performance.

Recovery

Most operators experienced problems with low recovery until they became familiar with the method. Two laboratories, 7 & 10, could not resolve these problems (Table IX). Reasons for low recovery may include:

(i) leaving dry extracts on blow-down apparatus.

(ii) using weak ammonia (< 35% or old stock) to elute the SCX cartridge.

(iii) allowing Solid Phase Extraction (SPE) cartridges to dry out before final elution stage.

(iv) loading SPE cartridges with a higher than specified flow rate.

(v) non-quantitative transfer between dry extract and sample vial.

(vi) unstable derivatised extracts. The derivatives take a short time to form and are unstable. Analysis should therefore be carried out at about 20 minutes after adding the derivatising agent and certainly within 4 hours.

Blanks

The blank tissue (S7) provided was found to contain residues of sulphadimidine. This may have been caused by contamination of the blank sample during preparation or by contamination or carry over during analysis. Sulphadimidine can adhere to glassware under some conditions. Meticulous attention to cleaning equipment between samples and separating glassware associated with different samples is necessary to avoid carry over or cross contamination of extracts. It is possible to contaminate subsequent extracts on the HPLC system. To completely avoid this problem it may be necessary to intersperse samples with blank or solvent only extracts until no peaks are seen for blank extracts and it may be necessary to introduce further measures such as cleaning glassware with chromic acid. When the method is used for surveillance purposes, the vast majority of extracts are negative and carry-over is not a substantial problem. The fact that residues were also found by the co-ordinating laboratory during homogeneity testing suggests that contamination may have occurred during initial sample preparation or that the "blank tissue" (obtained from a pig fed on a sulphadimidine free diet) initially contained sulphadimidine.

The method is designed for surveillance purposes to determine sulphadimidine concentrations near the MRL of 100 μg/kg and below.

The results of this collaborative trial would appear to demonstrate this, the method performed at its best for the samples S1, S2 & S3, i.e. <350 μg/kg. The precision for S4 & S5 deteriorated but was still typical for a method measuring analyte at sub mg/kg levels. Clearly if the method was to be used routinely to determine sulphadimidine at very high concentrations then the calibration curve and instrument sensitivity would need to be adjusted and a defined procedure included to deal with samples that are out of the calibration range.

Future work

The inclusion of a suitable internal standard would improve precision of the method by correcting for recovery in individual extracts, and may also correct for problems with derivative instability. Sulphamerazine differs from sulphadimidine by the loss of one methyl group, and is not a widely used sulphonamide. This would therefore be a good candidate for use as an internal standard.

The Waters Radial-Pak column with a Nova-Pak C18 cartridge is capable of resolving most of the different sulphonamide drugs. The extraction and clean-up procedure is also suitable for most of these compounds. Extensive validation could indicate the suitability of the method as a multi-residue procedure for sulphonamides. A wide variety of tissue types could also be investigated for analysis by this method.

Conclusion

The precision obtained for the method tested in this collaborative trial was satisfactory and was well within theoretical predicted range for the samples containing sulphadimidine at concentrations close to the MRL. The method is recommended to be published as a MAFF validated method in the "Validated Non-Statutory Methods for the Analysis of Foods" series.

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Acknowledgments

The authors wish to thank the following analysts and their staff members (named in parentheses) who participated at the time of the collaborative trial.

D. Dunn (S. Thwaites)	A H Allen & Partners, Sheffield
E.W. Langridge (S.Johnson)	Aynsome Laboratories Ltd., Cumbria
J.Turnbull (P.Stanley)	Avon Scientific Services, Bristol
S. Davis (M.Weaver)	City of Birmingham Laboratory, Birmingham
B.A. Evans (P.A.Sweet)	Cardiff Scientific Services, Cardiff
A. Smith (A.J. Chapman)	Greater Manchester Scientific Services, Manchester
B Mclean (J. Flude)	Hereford & Worcester County Laboratory, Worcester
I. Hampton (P.Maynard)	Kent County Laboratory, Kent
M.F. Godfray (M.Cavens)	Lothian Regional Laboratory, Edinburgh
C. Boynes (C.C. Maitland)	Lyne Martin & Radford, Reading
P. Lenartowicz (R.Kiddie)	Mid Glamorgan Council, Cardiff
E.C. Conchie (J.D. Watkins)	Ruddock & Sherratt, Chester
B.S. Dredge (M.Bentley)	Staffordshire County Laboratory, Stafford
E.B. Reynolds (T.Williams)	Tickle & Reynolds, Exeter

Table I
Pre-trial results corrected for recovery

Laboratory	Sulphadimidine (=570 μg/kg)	
	(1)	(2)
1	266*	247*
2	276	257
3	595	487
4	687	691
5	396	431
6	644	630
7	490	4460#
8	165	76*
9	334	220*
10	111	132*
11	640	580
12	157	198
13	743	725
14	NR	NR

* not corrected for recovery due to inconsistent recovery data

gross error

Table II
Test material identification codes

Sample No. / Laboratory	S1		S2		S3		S4		S5		S7	
	Test Material (No.)											
1	637	102	573	539	497	312	412	505	105	229	130	604
2	530	640	280	805	277	917	310	297	519	454	381	329
3	833	918	788	757	636	216	758	714	266	624	466	510
4	350	712	932	622	404	686	286	230	795	841	594	819
5	924	489	548	245	413	614	610	672	615	509	200	646
6	273	880	159	820	532	515	496	319	907	609	431	435
7	900	311	748	947	851	388	780	547	210	182	938	687
8	440	508	393	133	921	755	555	638	703	882	773	175
9	692	158	101	419	110	768	840	420	719	374	739	926
10	465	800	452	902	766	166	908	878	950	953	831	108
11	565	378	814	304	231	844	895	836	460	303	209	495
12	729	675	468	272	171	912	593	564	859	113	264	797
13	155	822	890	154	743	149	700	114	191	711	553	715
14	246	581	682	601	891	590	228	898	423	331	661	235

Table III
Homogeneity data

Sample	S1	S2	S3	S4	S5	
Replicate No.			μg/kg			
IA	119	227	363	630	656	
lB	111	131	460	534	438	
2A	99	194	410	596	569	
2B	92	145	352	548	486	
3A	124	202	315	614	590	
3B	81	71	356	614	552	
4A	126	173	321	646	583	
4B	126	178	362	544	644	
5A	112	122	285	452	NR	
5B	116	104	275	534	409	
mean	111	154	350	571	547	$\bar{x}$
S7.	29	18	17	56	29	30
Rec. (%)	74	51	52	50	52	

Table IV
Trial proper results, corrected for recovery

Sample	S1		S2		S3		S4		S5		S7	
	μg/kg		μg/kg		μg/kg		μg/kg		μg/kg		μg/kg	
1	170	172	126	117	231	561	504	754	760	465	19	21
2	95	48	144	130	286	240	336	348	377	518	25	27
3	88	83	191	223	486	439	735	954	827	1340	16	15
4	88	87	129	137	266	264	477	428	531	453	27	28
5	128	120	185	152	270	307	520	426	555	504	31	56
6	126	124	210	206	425	403	629	665	732	766	31	38
*7**	*9*	*162*	*356*	*679*	*1297*	*536*	*1742*	*2372*	*2571*	*1173*	*30*	*44*
8	159	116	237	194	333	396	576	>620	766	678	19	21
9	110	95	174	156	257	261	514	455	589	587	17	15
*10**	*21*	*32*	*92*	*88*	*316*	*271*	*256*	*245*	*440*	*267*	*33*	*19*
11	222	120	200	210	350	350	560	600	760	940	19	25
12	125	222	154	131	244	269	387	306	369	362	86	62
13	146	147	249	248	35	360	609	493	677	730	39	37
14	44	41	19	59	123	110	188	176	262	152	15	19

* Results not included in subsequent statistical analyses due to low recoveries.

Table V
Recoveries obtained in trial proper

Laboratory	Recovery % (fortification μg/kg)			
1	58 (100)	51 (100)	52 (100)	56 (100)
2	75 (400)	60 (200)	55 (100)	
3	57 (50)	56 (100)	37 (200)	
4	77 (200)	77 (200)		
5	88 (200)	87 (100)		
6	68 (107)	68 (107)		
7	23 (230)	17 (230)		
8	55 (100)	53 (100)	51 (100)	48 (100)
9	65 (200)	61 (200)	55 (200)	62 (200)
10	14 (100)			
11	62 (210)	16* (106)		
12	84 (100)	77 (50)		
13	75 (101)	81 (99)		
14	76 (100)	70 (100)		

* not used to correct for recovery

Table VI
Youdens Ranking Test

Sample	S1		S2		S3		S4		S5		S7		SUM
Lab	$\bar{x}$	Φ	$\bar{x}$	Φ	$\bar{x}$	Φ	$\bar{x}$	Φ	$\bar{x}$	Φ	$\bar{x}$	Φ	
1	342	2.5	243	11	792	3	1258	3	1225	6	40	8.5	34
2	143	11	274	9	526	9	684	11	895	10	52	6	56
3	171	9	414	4	925	1	1689	1	2167	1	31	12	28
4	175	8	266	10	530	8	905	9	984	9	55	5	49
5	248	6	337	6	577	7	946	8	1059	8	87	2	37
6	150	10	416	3	828	2	1294	2	1498	3	69	4	24
8	275	5	431	2	729	4	>1196	4	1444	4	40	8.5	27.5
9	205	7	330	7	518	10	969	7	1176	7	32	11	56
11	342	2.5	410	5	700	6	1160	5	1700	2	44	7	27.5
12	347	1	285	8	513	11	693	10	731	11	148	1	42
13	293	4	497	1	705	5	1102	6	1407	5	76	3	24
14	85	12	78	12	233	12	364	12	414	12	34	10	70*

$\bar{x}$: mean of duplicate results for level Φ : ranking score, highest mean result designated 1, lowest mean result designated 12.

* the score is significantly high ($p<0.05$); acceptable score limits for 12 laboratories and 6 levels are 15 (lowest acceptable score) and 63 (highest acceptable score). The results for laboratory 14 were not included in any further statistical analysis.

See Table X for full description of symbols

Table VII

Statistical analysis of results

Sample	S1 µg/kg		S2 µg/kg		S3 µg/kg		S4 µg/kg		S5 µg/kg		S7 µg/kg	
1	170	172	126	117	231[c]	561[c]	504	754	760	465	19	21
2	95	48	144	130	286	240	336	348	377	518	25	27
3	88	83	191	223	86	439	735	954	827	1340	16	15
4	88	87	129	137	266	264	77	428	531	453	27	28
5	128	120	185	152	270	307	520	426	555	504	31	56
6	126	124	210	206	425	403	629	665	732	766	31	38
8	159	116	237	194	333	396	576	>620[(1)]	766	678	19	21
9	110	95	174	156	257	261	514	455	589	587	17	15
11	222	120	200	210	350	350	560	600	760	940	19	25
12	125	222	154	131	244	269	387	306	369	362	86	62
13	146	147	249	248	345	360	609	493	677	730	39	37
$\bar{x}$	126.9		177.4		327.6		535		649.4		30.64	
s_r	33.16		15.48		23.47		86.09		138.6		7.71	
RSD_r	26.14		8.73		7.17		16.09		21.4		25.17	
s_R	43.59		42.96		74.54		162.9		225.5		17.85	
RSD_R	34.36		24.22		22.76		30.45		34.73		58.28	
r	92.9		43.35		65.7		241		388		21.6	
R	122		120.3		209		456		631		50	

C = Cochrans outlier

(1) = result out of range of sensitivity of detector, neither result included in statistical analysis.

See Table X for full definition of terms

Table VIII
Summary of results

Sample	S1	S2	S3	S4	S5	S7
(TVμg/kg)	(110)	(155)	(350)	(571)	(547)	(30)
n	11	11	10	10	11	11
$\bar{x}$	126.9	177.4	327.6	535	649.4	30.6
RSD_r	26.1	8.7	7.2	16.1	21.4	25.2
RSD_R	34.4	24.2	22.8	30.5	34.7	58.3
r	92.9	43.4	65.7	241	388	21.6
R	122	120.3	209	456	631	50
Ho_r	1.8	0.6	0.6	1.4	1.9	1.4
Ho_R	1.6	1.2	1.2	1.7	2.0	2.2

See Table X for full description of symbols

Table IX
Type of column used by participants

Laboratory	Type of Column Used
1	Waters Nova-Pak C18 100 × 5 mm
2	5μ 4.6 mm × 100 mm ODS2
3	Waters Nova-Pak C 18
4	Waters Radial-Pak 100 × 5 mm; Nova-Pak C 18 cartridge
5	Lichrospher KP 18 125 × 4.0 mm.
6	Hypersil ODS
7	Radial Pak Cartridge 100 × 5 mm, Nova Pak C18 4μ
8	Hypersil 5 ODS
9	Chrompack ODS2 150 × 4.6 mm
10	Partisil ODS II 10μ
11	Apex ODS 3μ
12	Partisil 10 ODSI
13	Spherisorb ODS 2 250 mm
14	Radial Pak Cartridge 100 × 5 mm, Nova Pak C18 4μ.

Table X

Key	Definition of term
NR	Result not reported.
(c)	An outlying result by Cochran's Test at P<0.01 level, not used in calculation of mean, repeatability or reproducibility.
1	Result above maximum range of detector. Value was not used in the calculation of mean, repeatability or reproducibility.
x	Mean
n	Number of laboratories used in the calculation of the statistical parameters after the elimination of outliers.
r	Repeatability (within laboratory variation). The value below which the absolute difference between two single test results obtained with the same method on identical test material under the **same conditions** may be expected to lie with 95% probability.
S_r	The standard deviation of the repeatability.
RSD_r	The relative standard deviation of the repeatability ($S_r \times 100$/Mean)
Ho_r	The HORRAT value for repeatability is the observed RSD_r divided by the RSD_r value estimated from the Horwitz equation using the assumption r = 0.66R.
R	Reproducibility (between-laboratory variation). The value below which the absolute difference between two single test results obtained with the same method on the identical test material under **different conditions** may be expected to lie with 95% probability.
S_R	The standard deviation of the reproducibility.
RSD_R	The relative standard deviation of the reproducibility ($S_R \times 100$/MEAN).
Ho_R	The HORRAT value for reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

APPENDIX I

Notes on Method

Additional instructions were given to participants for the analysis of frozen samples used in the collaborative trial and to accomodate the large range in analyte concentration.

Section 6 " For the purposes of this collaborative trial standard solutions should be prepared in the range equivalent to 50-350 µg/kg of sulphadimidine in the sample."

Section 7.2.1. - for the purposes of the collaborative trial amend to "Weigh 5 g of frozen sample, allow to thaw and add 25 mL.....".

Determination of Sulphadimidine at Residue Levels in Cattle kidney, Pig Kidney, Liver and Muscle

Warnings and Safety Precautions

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.

1. Scope

This procedure may be used to determine sulphadimidine in animal tissues. This includes kidney, liver and muscle from pigs and cattle.

The method may also be suitable for other tissues and species. e.g. sheep kidney, milk, eggs, pate, liver sausage and salami.

2. Principle

Tissue is extracted into 5% acetic acid in ethyl acetate and the extract is applied to pre-packed anion and cation exchange columns arranged in tandem, the former placed on top of the latter. Sulphadimidine is eluted using 1:1 methanol:ammonia, derivatised using fluorescamine, and quantified using HPLC with fluorescence detection with the excitation wavelength set at 405 nm and emission at 495 nm.

3. Sampling

For analysis of tissue sub samples are taken from thin slices through the frozen sample to gain as representative a sample as possible. Homogenisation of whole sample is best avoided as loss of analyte through increased enzymic activity may result.

4. Reagents

Chemicals and solvents are analytical grade reagents except where stated. Deionised water processed through an Elga UHP (or equivalent) is used throughout.

4.1 Chemicals

4.1.1 Acetone

4.1.2 Acetonitrile

4.1.3 Ammonia solution, 35 %(*w/v*)

4.1.4 Ammonium acetate

4.1.5 Ethyl acetate
4.1.6 Glacial acetic acid
4.1.7 Methanol
4.1.8 Sulphadimidine (Sulphamethazine)
4.1.9 Hexane
4.1.10 Sodium sulphate, anhydrous
4.1.11 Fluorescamine

4.2 Solutions

4.2.1 5% acetic acid in ethyl acetate

Glacial acetic acid (**4.1.6.**) is diluted 5 mL 100 mL with ethyl acetate (**4.1.5.**).

4.2.2 1:1 methanol:ammonia

Ammonia solution (**4.1.3.**) is diluted 50 mL 100 mL with methanol (**4.1.7.**). Prepare fresh daily.

4.2.3 HPLC mobile phase

Dissolve 0.77 g ammonium acetate (**4.1.4.**) in 800 mL water, then make up to 1 L with acetonitrile (**4.12**).

4.2.4 2% acetic acid solution

Acetic acid (**4.1.6**) is diluted 2 mL 100 mL with water.

4.2.5 Fluorescamine solution

Dissolve 6 mg fluorescamine (**4.1.11**) in 3 mL acetonitrile (**4.1.2**).

4.3 Column Preparations

4.3.1 SAX and SCX Solid Phase Extraction Columns

Condition the Bond-Elut SAX and SCX columns (**5.3.2**) with 6 mL hexane (**4.1.9**) followed by 6 mL 5% acetic acid (**4.2.1**). Connect the SCX column to the top of the Vac-Elut chamber and connect the SAX column to the top of the SCX column using an adapter (**5.3.1**). Connect a 75 mL reservoir (**5.3.3**) to the top of the SAX column.

5. Apparatus

5.1 Glassware

5.1.1 Centrifuge tubes, 100 mL capacity.
5.1.3 Glass filter funnels 75 mm diameter
5.1.3 Conical flasks, 150 mL
5.1.4 HPLC vial inserts, 0.3 mL capacity.
5.1.5 All glass filter holder, 47 mm - Millipore.
5.1.6 Test tubes. 15 mL capacity, to fit blow down apparatus

5.2 Equipment

5.2.1 Homogeniser, Ultra-Turrax or equivalent.
5.2.2 Ultrasonic bath, L&R 140S or equivalent.

5.2.3 Centrifuge, IEC Centra-7R or equivalent.

5.2.4 Filter paper, 15 cm, type 1PS - Whatman.

5.2.5 Nitrogen blow-down apparatus and hot block

5.2.6 Vortex mixer, Whirlimixer (Fisons) or equivalent.

5.2.7 Disposable syringes, 1 and 10 mL capacity.

5.2.8 Disc filter, 0.45 μm, HVLP, 13 mm - Millipore U.K. Ltd.

5.2.9 Disposable filter units, 0.2m Anotop or equivalent

5.2.10 Membrane filter, 0.45 μm "Durapore", 47 mm - Millipore U.K. Ltd.

5.2.11 Safety pipette, 5.0 mL - Gilson Pipetman.

5.2.12 Positive displacement pipette, 25 μL - Gilson

5.2.12 Rotary evaporator, Buchi

5.3. Solid phase extraction

5.3.1. Bond-Elut adapters

5.3.2 Bond-Elut SAX and SCX cartridges, 500 mg/2.8 mL (Jones Chromatography Ltd).

5.3.3. Bond-Elut reservoirs, 75 mL capacity.

5.3.4 Vac-Elut vacuum manifold, (Jones Chromatography Ltd.).

5.4. High Performance Liquid Chromatography

5.4.1 Pump, LKB 2150 or equivalent.

5.4.2 Column, Waters Radial - Pak 100 mm × 5 mm Nova-Pak C_{18}, 4m particle size, and RCM 8 × 10 cartridge holder. Flow rate 1.0 mL/min.

5.4.3 Detection, Fluorescence detection. Excitation wavelength 405 nm, emission wavelength 495 nm.

5.4.4 Injection, Gilson 231 autosampler, with rack for 300 μl vial inserts (20 μl injections).

6. Standards

6.1 Sulphadimidine (sulphamethazine) standards:

Solutions of sulphadimidine should be stored in a refrigerator. Stock solution should be made fresh monthly, intermediate and working standards weekly.

6.2. Stock standard (100 μg/mL)

Dissolve 10.0 mg standard (**4.1.8**) in 100 mL methanol (**4.1.7**).

6.3 Working standard (10 μg/mL)

The stock standard (**6.2**) is diluted 1.0 mL to 10 mL with methanol (**4.1.7**).

7. Procedure

7.1 The extraction should be performed on batches of 10 samples per day.

7.2. Extraction

7.2.1 Weigh 5 g of finely sliced tissue and add 25 mL 5% acetic acid solution (**4.2.1**) into a centrifuge tube (**5.1.1**). Homogenise (**5.2.1**) for approximately. 1 min. Centrifuge (**5.2.3**) for approximately 5 min. at approximately 2500 rpm.

7.2.2 Filter the supernatant through a Whatman 1PS paper (**5.2.4**), containing sodium sulphate, (**4.1.10**) into a 150 mL conical flask (**5.1.3**).

7.2.3 Re-extract the remaining residue from **7.2.1** (as in **7.2.1** and **7.2.2**) using a further 25 mL 5% acetic acid solution (**4.2.1**). Homogenise (**5.2.1**) for approximately 30s.

7.3. Clean-Up

7.3.1 Apply the pooled filtrates through the prepared Bond-Elut SAX and SCX columns (**5.3.2, 4.3.1**) at a flow-rate of 5 - 10 mL/min (**5.3.1, 5.3.3, 5.3.4**), by adding the extract to the columns and reducing the pressure in the Vac-Elut chamber.

*THE COLUMN SHOULD NOT BE ALLOWED TO RUN DRY AT ANY STAGE DURING THE LOADING, WASHING AND ELUTION STEPS AS THIS MAY RESULT IN LOW OR INCONSISTENT RECOVERIES. THE RECOVERY REFERENCE SPIKE (**10.2**) INCLUDED WITH EACH BATCH WILL INDICATE WHETHER THE BATCH WILL NEED TO BE REPEATED.*

7.3.2 Discard the SAX column.

7.3.3. Wash the SCX column with 5 mL water followed by 10 mL acetone (**4.1.1**) and 10 mL acetonitrile (**4.1.2**).

7.3.4 Elute the sulphadimidine, with 10 mL methanol:ammonia solution, (**4.2.2**) into a 15 mL test tube (**5.1.6**) using a 10 mL syringe (**5.2.7**).

7.3.5 Place the tubes into the blow-down apparatus (**5.2.5**) and evaporate using a gentle stream of nitrogen at 95°C until the samples just reach dryness.

Do not leave dry extracts on the blow down apparatus.

7.3.6 Add 200 μL 2% acetic acid solution (**4.2.4**). Vortex mix and add 100 μL fluorescamine solution (**4.2.5**) and vortex mix again.

7.3.7 Use a 1 mL syringe to withdraw the extract and pass it through a 0.2 mμ filter (**5.2.9**) into a 0.3 mL vial insert (**5.1.4**) .

7.3.8 HPLC is performed on 20 μL of extract.

FLUORESCAMINE DERIVATIVES ARE UNSTABLE. HPLC MUST BE PERFORMED BETWEEN 20 MINUTES AND 4 HOURS AFTER SAMPLE PREPARATION.

8. Interpretation of Chromatographic Data

8.1 Identification of analyte

Identification of suspect peaks is made by comparison of the retention times of analytes in spiked samples, or in standard solution, with those of suspect peaks in the sample.

8.2 Standard Curve

A minimum of 2 injections of standard sulphadimidine solutions are carried out to determine average peak height. Solutions are prepared by adding an appropriate volume (25 μL gives a standard of concentration equivalent to 50 ppb sulphadimidine in a sample, assuming 100 % recovery) of working standard solution to a 0.3 mL vial insert, making the volume up to 200 μL using 2 % acetic acid solution (**4.2.4**), and adding 100 μL flurescamine solution. The vials must be capped and mixed well before analysis.

8.3 Correction for recovery.

Recovery of analyte is determined by including with each batch, two blank samples spiked with sulphadimidine at 100 ppb. A blank is also included with each batch. The recovery should be between 50 and 110%.

8.4 Calculation of results

The concentration of sulphadimidine in the meat sample is calculated from the standard curve, using a least squares technique, and the result corrected for recovery.

9. Quality Assurance Procedures

9.1 Validation of method prior to use

When this method is to be used by an operator for the first time it should be validated for in house repeatability and reproducibility. The full procedure should be carried out on one batch of samples on each of three separate days. Each batch will consist of up to eight samples of which six are spiked at the 0.05 or 0.1 mg/kg level (25 or 50 μL of working standard (**6.3**)). Batches should include at least one blank sample. All tissue should be from the same source.

When returning to the method after a break, a validation analysis should be carried out. This should consist of at least one batch of samples.

Recovery of analyte should fall within the range 50 - 110%. Relative standard deviation values should be less than 15% for inter and intra batch precision.

APPENDIX II
Comments from Participants

Pre-Trial

Laboratory 1

Obtained erratic and low recoveries. Diluted sample.

Laboratory 2

Initially obtained low recoveries.

Laboratory 3

Novopak C18 3.9 × 150. Recovery 41 %

Laboratory 4

Centrifugation not necessary.

Laboratory 5

Used Lichrospher RP18 100 mm × 4.6 mm × 5 μm with 20 mm × 2 mm pre-column packed with Co-pell ODS C18 30-38 μm. Extracts were filtered directly into Bond Elut reservoir rather than being pooled. This served to wash the retained remnants through the filter. Used 500 ppb std for quantification.

Laboratory 6

Used a Hypersil ODS column. Used a strong standard for quantification.

Laboratory 8

Hypersil 5ODS. Problems encountered with dilution. Instruction required in method.

Laboratory 9

Chrompak ODS 2 150mm × 4.6mm. Variable recoveries, problem not resolved.

Laboratory 10

Partisil ODS II 10 μm × 25 cm gave long retention times. Suction apparatus used instead of Vac-Elut system. Water bath at 95°C with nitrogen stream instead of blow down apparatus. Problems maintaining a head of liquid over the SCX material. Darkening of

ammonia/methanol extract on evaporation. Problems obtaining suitable blank samples.

Laboratory 11

APEX ODS 5 μ 250 × 40 mm with 28 % CH_3CN. Liquid resisted evaporation. Brown material resisted cleanup. Minor changes made to the method.

Laboratory 13

Spherisorb ODS2 25cm. Dilution or smaller sample weight was used.

Trial Proper

Laboratory 2

Deviations from the specified procedure:

7.2.1 the minimum volume of extraction solution was 50 mL for a Silverson top-drive. This resulted in a total volume of 125 mL

7.2.2 5g of sodium sulphate was used.

7.3.1 a 2.p.s. 24 vacuum station was utilised for the column chromatography.

7.3.5 evaporation was afforded at 65°C.

3.6 residue was re-dissolved in 0.5 mL of 2 % aqueous acetic acid.

Laboratory 3

Samples were stored in a refrigerator in methanol: ammonia solution (**4.2.2**) prior to analysis.

Steps **7.3.5,7.3.8** were always carried out on the same day. HPLC mobile phase 21% CH_3CN 79 % ammonium acetate buffer using low pressure mixing. Flow rate 2 mL/min.

Laboratory 4

The centrifuge step was omitted from the extraction. About 2 mL of 5 % acetic acid solution (**4.2.1**) was added to both NH_2 and SCX columns before loading the sample extract. Peak areas were used for quantification. The derivitisation did not appear to work properly for standard solutions where the volume of 2 % acetic acid used in the make up fell below approximately 100 μL. In this case the standard solution was evaporated to dryness and dissolved in 200 μL of 2 % acetic acid.

Laboratory 5

Standards made up as recommended gave a curved calibration. A straight line calibration was obtained by diluting the appropriate

volume of 10 μg/mL sulphadimidine (e.g. 25 μL for a 50 ppb standard) to 400 μL with 2% acetic acid and treated with 200 μL of fluorescamine. This also eliminated problems due to the small final sample volume. Other modifications as noted for pre-trial. Where dilutions of the final analysis solution were required (despite note to **8.2**) these were made as follows: 60 μL solution, 40μL 2 % acetic acid, 20 μL fluorescamine. Final analysis solutions were allowed to stand at room temperature for 20 minutes after mixing, then transferred to a refrigerator and only removed at the time of injection.

Laboratory 6

Sample blanks yielded small peaks (24 μg/kg) after applying 1.47 recovery factor. despite stringent efforts not to contaminate these samples with traces of sulphadimidine. Hence greatest error in results will be for lower level sulphadimidine samples, i.e. samples 431 and 435, where their levels are very close to the 'lowest detectable level'. To avoid declaring a false positive result, it may be safer to assume a higher detection limit, e.g. none detected/ less than 50 μg/kg.

Laboratory 7

Sample solutions made up to 0.9 mL (not 0.3) before filtering and HPLC.

Laboratory 8

The detector used for this trial was a Perkin-Elmer fluorescence spectrometer LS-38. As noted at the end of the pre-trial this detector seems to be more sensitive than the written method allows for (the maximum output of the detector is 1 volt). This is equivalent to 10 μL of 2 μg/mL sulphadimidine standard solution. Solutions were therefore made more dilute by adding more than the stated amount of 2 % acetic acid and the fluorescamine solutions at the step **7.3.6.** Additionally to reduce sensitivity, 10 μL sample loop was used in place of the 20 μL loop stated in the method.

Laboratory 9

Standard solutions prepared in the range equivalent to 50-350 μg/kg in the sample, according to method. **8.2** (standard curve) gives an unusable calibration graph. In order to obtain results for this trial suitable aliquots of working standard sulphadimidine solutions were evaporated to dryness as at **7.3.5** and prepared for HPLC as in **7.3.6** and **7.3.7**. A four point calibration graph gave a linear response. The HPLC flow rate was increased to 2.5 mL/min to give a sulphadimidine retention time of 8 minutes.

Laboratory 10

During the trial a recovery of 14.4% was recorded for spiked sample material, as opposed to 18-25% at the time of the pre-trial. No reason for this has been identified, but the recovery was found to be repeatable, therefore the results have been corrected appropriately. The method as amended specified standard solutions in the range equivalent to 50-350 μg/kg of sulphadimidine in the sample. Assuming 100% recovery, this corresponds to 0.83 -5.83 μg/mL in the solution as injected into the HPLC. We found the linear range of calibration to extend only up to a maximum of 4 g/mL as injected (though given the poor recovery of 14.4% this corresponds to some 1660 μg/kg in the sample.

Laboratory 11

Due to an instrument failure, the analysis of the spiked extract could not be repeated. In view of past experience 62% has been taken as typical recovery. HPLC was satisfactory using a column of Apex ODS, 3 m, 250 × 4 mm, with 28 % acetonitrile in the mobile phase giving a retention time of 7 min. Injections were made manually by syringe. Macerator head is not specified : we used T25-18G at 20,500 rpm which was the highest speed not to cause excessive heat when the head had been rinsed by running in cold water before each use. We interpreted **7.2** to mean that the second extract follows the first through the same filter, but doubt whether there is sufficient rinsing to recover sulphadimidine trapped in the partly hydrated sodium sulphate. The extract was dried down on a waterbath; it is difficult to see how the moment of dryness can be observed when using a hot-block. A deposit of brown solid from drying down the extracts is not re-dissolved in the acetic acid solution; it may have trapped some sulphadimidine.

Laboratory 13

The blank levels were (i) laboratory blank 5.5 μg/kg; FSL blank 27.0 μg/kg; these were taken into account when calculating recovery. HPLC calibration: to avoid potential errors in the dispensing of very small volumes, a working standard containing 1 μg/kg was used. The larger volumes required were evaporated and treated as in steps **7.3.5** and **7.3.6**. Linearity was checked up to the equivalent of 460 μg/kg of sulphadimidine in the sample (assuming 100 % recovery). The higher levels found in a few of the samples were determined on a test portion of less than 5 g.

Laboratory 14

Since the pre-trial the retention time of the sulphadimidine had almost doubled. The samples were run with a flow rate of 1.5 mL/min. The blanks provided gave results of 15 μg/kg.

ERRATA

Due to an omission in volume 30 part II, the final paragraphs of the report of the Hon. Secretary of the Association were not published. Please read these paragraphs in conjunction with the Annual report of the Honarary Secretary of the Association, Dr. Peter Clare, J.A.P.A. 30(2) 89-95

European Issues

Late in the year Council Directive 93/99/EEC on the Subject of Additional Measures concerning the Official Control of Foodstuffs was published. This directive complements Council Directive 89/397/EEC the Food Control Directive concerned with the inspection, sampling and analysis, staff hygiene, examination of records and verification systems for the control of foods. The "Additional Measures" directive sets out the requirements for accreditation and performance criteria for official laboratories and qualification requirements for food control officials. The implementation of these criteria in the United Kingdom is expected to be considered in the form of a series of discussion papers with the Ministry of Agriculture, Fisheries and Food.

The forum of the Food Law Enforcement Practitioners (FLEP) met in June in Mastrict. 33 delegates from EC countries were present, the Association being represented by the President. Topics raised at this meeting included food frauds, the meaning of basic terms such as meat and milk, co-ordinated sampling and analysis programme, standards for the training of food law practitioners and the quality.
The Journal of the Association is also finding invaluable contacts within the community. Support from the Food Science Laboratories of MAFF in the form of a series of validated methods for the analysis of foods and of collaborative trials of analytical methods to which Public Analysts Laboratories have contributed form the backbone to the published papers. The Journal is now widely available throughout the community and it is anticipated to be in increasing demand with the introduction of requirements for accreditation in official laboratories.

Conclusion

Public Analysts are analytical scientists whose functions are specifically written into statute with corresponding prescribed responsibilities, who must be formally appointed by the Enforcing Authority and report to that authority, and who are charged with

providing expert opinion on matters referred to them. These terms of reference are therefore very wide, encapsulated in law and rest on the results of analysis and testing, and on expert opinion based on these results and related experience.

These responsibilities are reflected in the annual statistics of samples analysed and other work undertaken and which is presented and discussed in the Annual Statistics for the Association which are produced by Mr. P. Lenartowicz.

Local government reorganisation and developments in food legislation brought about as a consequence of community membership will together provide demanding challenges to members of the Association- It is therefore fitting to conclude this annual report with the final comment of 25 years ago contained in the Annual Report of Council for 1968 presented by Mr.F.A.Lyne when the Report of the Royal Commission on Local Government was about to be published. This subsequently lead to the corresponding reorganisation of local government of 1973 in Scotland and 1974 in the rest of the United Kingdom . "It remains to be seen how the future pattern will emerge but the pioneers who laid the foundations of the Public Analysts service a century ago , were men of enthusiasm, integrity and dedication and to paraphrase Isaac Newton ' if we now see a little further than we did, it is because we stand on the shoulders of giants'"-