J.Assoc. Publ. Analysts 1994, 30, 1-22

Determination of Chloramphenicol

Collaborative Trial

Paul Brereton, William H H Farrington, George Shearer, Hugh D Thomas and Roger Wood[1]

Ministry of Agriculture, Fisheries and Food, Food Safety Directorate, Norwich Research Park, Food Science Laboratory, Colney, Norwich, NR4 7UQ

The results of a collaborative trial to assess a method of analysis for the trace residue determination of chloramphenicol (CAP) in animal tissue are reported; 23 laboratories participated in the trial. The method tested comprised solid phase extraction, separation by reverse phase HPLC and UV detection at 285 nm.

The trial consisted of two parts. In the first part participants were asked to analyse standard solutions containing CAP at concentrations of 2.5 - 17.5 µg/L. The second part involved the analysis of samples of homogenised porcine muscle spiked with CAP. Both blind duplicate and split level samples were incorporated in the trial.

The precision for chloramphenicol in the test solutions was acceptable with Horrat values for reproducibility and for repeatability of between 1.0 and 1.3 for the range of concentrations tested (2.5 - 17.5 µg/L). The precision obtained for the analysis of the porcine muscle was only slightly worse than that for the standard solutions with only the precision for the sample containing CAP at 2.5 µg/L being unacceptable.

Introduction

Chloramphenicol (CAP) is a broad-spectrum bacteriostatic antibiotic which is administered to cattle and pigs by intramuscular injection. The Veterinary Formulary recommends that the use of this drug be restricted as chloramphenicol has been associated with human aplastic anaemia[1][2]. The Maximum Residue Limit for CAP as prescribed in both the EC Regulation 2377/90 and The Animal, Meat and Meat Products (Examination for Residues and Maximum Residue Limits) Regulations 1991, for edible tissue is 10 µg/kg[3][4]. Monitoring programs for residue levels of this drug operate within the UK and other members of the EC and are implemented under Council Directive (86/469/EEC)[5].

The MAFF Food Science Laboratory, Norwich has adopted a HPLC-UV method developed by Keukens et al.[6] for the detection of chloramphenicol in various tissues. Although the method has been collaboratively tested by Aerts *et.al.*, using incurred tissues[7], this trial

[1] to whom correspondence should be addressed

was carried out, conducted by the Food Science Laboratory, to determine whether the method was suitable for use by enforcement laboratories, who are not necessarily specialists in the analysis of veterinary drug residues. To determine the performance of the method, the analysis of spiked aqueous solutions and spiked tissue extracts was carried out and sent out to participants, who were mostly from enforcement laboratories.

The Method of Analysis being Collaboratively Tested

The method consisted of loading the standard solution or tissue extract onto an Extrelut^{R} cartridge followed by eluting the chloramphenicol with dichloromethane, evaporating off the organic phase and taking the residue up in water, washing the aqueous extract with toluene and quantifying using reverse phase HPLC with UV detection at 285 nm. (See Appendix 1 for full method)

Collaborative Trial Organisation and Sample Preparation

Participants

23 laboratories participated in the collaborative trial (21 UK Public Analyst Laboratories; Laboratory of the Government Chemist; Public Analyst's Laboratory, Galway, The Republic of Ireland.)

Trial Organisation

The collaborative trial, using spiked samples, was carried out in two stages:

Part 1: involved the analysis of seven standard solutions which consisted of a blank, two sets of blind duplicate and one set of split level samples containing known amounts of CAP.

Part 2: involved the analysis of seven spiked aqueous extracts of porcine muscle; these comprised a blank, two sets of blind duplicate and one set of split level samples.

Sample preparation

All the samples were prepared by the Food Science Laboratory, Norwich.

Part 1

Test solutions of chloramphenicol in distilled water with a range of concentrations (0-17.5 µg/L) were prepared and given code numbers from 1 to 7 on a random basis (Table I).

The solutions were dispensed into approximately 25 mL portions and stored in plastic centrifuge tubes at +4°C. A set of each of the coded samples (seven samples) was analysed on two separate occasions (Table I) to monitor stability.

Part 2

An aqueous extract was prepared by homogenising non-incurred porcine muscle with distilled water in the ratio 10 g meat to 40 mL water. This homogenate was filtered under gravity and the filtrate retained. (The aqueous extract was analysed to ensure that it was free from chloramphenicol contamination.)

Spiked extracts were prepared by adding an appropriate volume of a stock solution of chloramphenicol in distilled water to the aqueous extract (Table II). A "chloramphenicol free" aqueous extract was used as the blank.

The sample extracts were split into approximately 25 mL portions and stored in plastic centrifuge tubes and frozen at -20°C. Ten tubes containing the blank sample were analysed; no chloramphenicol was detected. Centrifuge tubes were taken at random for each sample concentration and were analysed by the coordinating laboratory to determine homogeneity. Six centrifuge tubes for samples 9 and 14, seven for 8, 10, 11 and 12 were analysed using the test procedure (by various analysts at the coordinating laboratory on different days), the results are given in Table II and are satisfactory.

Instructions for the analysis of collaborative trial samples

The participants were asked to familiarise themselves with the method in their own laboratory before analysing the trial samples.

Participants were instructed to commence the analysis of the samples from the end of the second sentence of Section **7.3** (Appendix I) of the method.

Results

These are given in tabes III-X.

Statistical analysis of the results

The trial results were examined for evidence of individual outliers ($p<0.01$) using Cochran's and Grubbs' tests progressively, by procedures described in the internationally agreed Protocol for the Design, Conduct and Interpretation of Collaborative Studies[8].

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 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[9].

The Horwitz predicted value is calculated from the Horwitz equation[9]:

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

C = measured concentration of analyte expressed as a decimal
(e.g. 1 g/100g = 0.01)

Horrat Values (Ho)

The Horrat[10] values 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 $r = 0.66R$) The Horwitz values calculated from the results of this trial are summarised in Tables XI-XII.

Repeatability and Reproducibility

Calculations for repeatability (r) and reproducibility (R) were carried out on those results remaining after removal of outliers[8]. The resulting values are given in Tables III-X and have been summarised in Tables XI-XII.

Discussion

Test solutions

The values of the repeatability (r) and reproducibility (R), for the test solutions of chloramphenicol, were of an order that would be expected for the level of analyte measured; this is demonstrated by Horrat values (Ho_r) and (Ho_R), of between 1.0 and 1.3.

The results for the test solutions, with the exception of duplicate samples 2 and 6 (the lowest concentration of chloramphenicol), were in good agreement with the results obtained by the coordinating laboratory (see Table I). The results suggest that the participants were recovering approximately 60% of CAP in the solutions. Participants reported substantially higher average values for samples 2 and 6, i.e. 2.2 and 1.4

µg/L respectively, than did the coordinating laboratory. These results suggest a recovery of CAP of 88 and 55% respectively.

Aqueous extracts of porcine muscle

The values of the repeatability (r), for the aqueous extract samples containing chloramphenicol at concentrations of 7.5 µg/L and above, were of an order that would be expected for the level of analyte measured. The samples containing 2.5 µg/L CAP (9 & 14) gave poor precision as demonstrated by the high Horrat values of Ho_r 2.9 and Ho_R 2.0. The precision obtained from the analysis of the split level samples was satisfactory and of the same order as that obtained by the blind duplicate samples. For the aqueous extracts, the average values obtained by participants for each level were considerably lower than those obtained by the coordinating laboratory (Table II). While it is possible that some loss of CAP occurred after the homogeneity testing the fact that several participants obtained similar levels to the coordinating laboratory tends to contradict this assumption. It would appear more likely that the laboratories less experienced in this type of analysis were less successful in recovering CAP.

This collaborative trial represents a validation of the cleanup, separation and quantification stages of the method but not the initial aqueous extraction. Samples were spiked after the aqueous extraction stage to ensure satisfactory homogeneity. The precision obtained from this study would be expected to be at least as good as that obtained by Aerts on incurred tissue; the fact that it was not, is a measure of the difficulties that non-specialist analysts had with this method[7]. However the precision obtained in this trial, albeit not assessing the aqueous extraction stage, was within predicted levels for a method measuring analyte at µg/L levels.

Conclusion

The precision obtained for the method tested in this collaborative trial was acceptable. Although the collaborative trial was limited in nature it suggests that the precision data obtained by Aerts for this method are not strictly applicable for analysts less experienced in this type of analysis [7].

Acknowledgements

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

Mr MJ Billington	Birmingham City Council, Birmingham;
Mr W Cassidy	Somerset County Council,Somerset;
Mr G Connelly	Kent County Laboratory, Maidstone;
Mr DS Dredge	Staffordshire County Analyst's Department, Stafford;
Dr A Ellis	Clayton Environmental, Birmingham;
Mr TD Feeley	Western Health Board, Galway;
Mr J Fulstow	Hampshire Scientific Service, Southsea;
Mr MF Godfrey	Lothian Regional Analysts Laboratory, Edinburgh;

Mr AJ Harrison	Avon County Scientific Services, Bristol;
Mr N Harrison	Leicestershire County Analyst's Laboratory, Leicester;
Mr G. Hooke	Ruddock and Sherratt, Chester;
Mr TE Johnson	Herbert J Evans and Partners, Public Analysts Department, Dyfed;
Mr G Keen	Hereford and Worcester County Council, County Laboratory, Worcester;
Mr D Lord	Lancashire County Laboratory, Preston;
Mr GS Meadows	Lincolne Sutton and Wood, Norwich;
Mr DA George	Laboratory of the Government Chemist, Teddington, Middlesex;
Mr EB Reynolds	Tickle and Reynolds, Public Analyst's Laboratory, Exeter;
Mr D Rixon	Mid Glamorgan County Public Health Laboratory, Cardiff;
Mr B Sanders	Cardiff Scientific Services, Cardiff;
Mr R Stevens	Southwark Analytical and Scientific Services, London;
Mr W Swanson	Strathclyde Regional Chemist and Public Analyst Department, Glasgow;
Mr S Thwaites	A H Allen and Partners, Sheffield;
Mr JP Wootten	Moir & Palgrave*,London;

* Incorporated into Central Scientific Laboratories, London.

References

1) "The Veterinary Formulary, Handbook of Medicines used in Veterinary Practice", Ed. Yolande Debuf, The Pharmaceutical Press, London, 1991
2) "Toxicological evaluation of certain veterinary drug residues in food", WHO Food Additives Series: 23, The 32nd Meeting of the Joint FAO/WHO Expert Committee on Food Additives, Cambridge University Press, Cambridge, 1987
3) Council Regulation (EEC) No. 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary products in foodstuffs of animal origin, OJ L 224/1, 1990
4) The Animal, Meat and Meat Products(Examination for Residues and Maximum Residue Limits)Regulations 1991, Statutory Instrument No. 2843, Food, HMSO.
5) Council Directive 86/469/EEC, of 16 September 1986, concerning the examination of animals and fresh meat for the presence of residues, OJ No L 275/36, 1986.
6) HJ Keukens, WMJ Beek and RML Aerts,"High-Performance Liquid Chromatographic screening and confirmation methods for Chloramphenicol residues in meat with off-line cartridge sample clean-up and on-line diode array UV-Vis detection", J. Chrom., 1986, **352**, 445-453
7) RML Aerts, HJ Keukens and GA Werdmuller,"Liquid Chromatographic Determination of Chloramphenicol Residues in Meat: Interlaboratory Study", JAOAC, 1989, **72**, No 4, 570-576
8) "Protocol for the Design, Conduct and Interpretation of Collaborative Studies" Ed W Horwitz, Pure and Appl. Chem., 1988, **60(6)**, 855-864

9) Horwitz W,"Evaluation of Methods Used for Regulation of Foods and Drugs", Analytical Chemistry, 1982, 57, 67A-76A

10) Peeler J T, Horwitz W and Albert R,"Precision Parameters of Standard Methods of Analysis for Dairy Products" JAOAC, 1989, 72, No 5, 784-806

TABLE I

Summary of the Analysis of Chloramphenicol Test Solutions by Coordinating Laboratory

Code Number	Spike level (ug/L)	Recovery (%)	
		Analysis1	Analysis2
7	0	-	-
2	2.5	49.2	53.4
6	2.5	62.4	56.4
5	7.5	57.9	64.2
1	8.75	57.6	67.4
3	17.5	63.2	65.9
4	17.5	73.7	59.9

TABLE II

Summary of the Homogeneity Data for Chloramphenicol in the Aqueous Extracts of Porcine Muscle

	Observed CAP concentration (ug/L)					
Code Number (CAP added)	9 (2.5)	14 (2.5)	11 (7.5)	12 (7.5)	8 (17.5)	10 (18.75)
	2.37	2.86	5.94	6.53	11.34	15.59
	2.16	2.13	6.21	5.94	15.79	15.71
	2.48	2.45	5.36	5.85	15.83	15.50
	2.15	2.14	6.09	6.15	14.85	15.53
	2.31	2.30	6.15	6.26	14.86	16.04
	2.24	2.23	5.47	5.44	10.53	15.96
			5.32	5.26	10.58	13.50
Mean	2.32		5.86		13.4	15.4
SD	0.21		0.41		2.46	0.87
RSD %	9.0		7.0		18.4	6.5
Recovery	92.8		78.1		76.6	82.1

TABLE III
Part 1 (Test Solution) Blank

Laboratory	Sample7 CAP Conc. (ug/L)
1	<0.1
2	2.6(0.8[d])
3	<1.0
4	ND (0.6[d])
5	ND
6	ND
7	0
8	2.8
9	<0.1
10	2.2
11	ND
12	ND
13	2.5
14	0.1
15	ND
16	2.3
17	ND
18	<0.5
19	1.3
20	<0.1
21	ND
22	ND
23	<2.0

For key, see Table XIII

TABLE IV
Part 1 (Test Solution) Chloramphenicol (2.5 µg/L)

Laboratory	CAP Conc. (ug/L) Sample 2	Sample 6
1	2.1	1.8
2	2.9(2.8[d])	2.3(1.7[d])
3	1.7	2.0
4	2.6(1.8[d])	2.6(1.8[d])
5	1.1	0.8
6	1.4	1.5
7	3.7	1.5
8	3.3	3.8
9	1.4	1.5
10	2.2	5.4
11	1.4	1.3
12	1.7	1.8
13	2.1	2.7
14	-[e]	-[e]
15	1.4	1.0
16	4.1	5.3
17	3.0	1.9
18	1.7	2.3
19	1.8	2.7
20	1.8	2.0
21	ND	2.2
22	1.8	1.9
23	2.1	2.7

Mean	2.2
r	2.1
S_r	0.76
RSD_r	34.9
Ho_r	1.3
R	3.0
S_R	1.06
RSD_R	48.5
Ho_R	1.2

For key, see Table XIII

TABLE V
Part 1 (Test Solution) Chloramphenicol (17.5 µg/L)

Laboratory	CAP Conc. (ug/L) Sample 3	Sample 4
1	11.0	11.1
2	13.4(3.8 [d])	5.6(13.3[d])
3	10.8	12.9
4	1.0[a](3.9[d])	18.8[a](3.7[d])
5	1.7	0.9
6	10.0	10.0
7	10.5	10.6
8	5.0	6.1
9	10.3	7.2
10	14.2	13.0
11	7.9	8.4
12	11.8	12.0
13	11.7	10.9
14	0.3	0.7
15	8.3	11.0
16	9.8	4.5
17	11.0	7.7
18	11.0	14.3
19	13.4	7.8
20	13.4	12.1
21	-[e]	6.0 [f]
22	7.5	11.2
23	15.7	15.7

Mean	9.6
r	5.9
S_r	2.09
RSD_r	21.8
Ho_r	1.0
R	11
S_R	3.94
RSD_R	41.1
Ho_R	1.3

For key, see Table XIII

TABLE VI
Part 1 (Test Solution) Split Level Samples

Laboratory	CAP ug/L Sample 5 (7.5)	Sample 1 (8.75)
1	3.5	6.8
2	5.7(5.6[d])	5.1(5.9[d])
3	5.0	6.3
4	6.4(4.3[d])	11.0 (2.3[d])
5	0.9	1.7
6	4.4	6.5
7	4.5	6.1
8	3.7	1.5
9	4.9	5.3
10	6.6	6.7
11	4.4	4.5
12	5.3[g]	6.5[f]
13	6.5	4.3
14	-[e]	-[e]
15	4.0	4.8
16	4.5	4.1
17	4.8	5.6
18	6.3	6.0
19	8.0	6.5
20	5.7	6.5
21	2.8	6.2
22	3.8	6.1
23	4.8	7.5
Mean	4.8	5.7
r	3.6	
S_r	1.27	
RSD_r	24.2	
Ho_r	1.0	
R	4.9	
S_R	1.76	
RSD_R	33.6	
Ho_R	1.0	

For key, see Table XIII

TABLE VII
Part 2 (Aqueous Extract) Blank

Laboratory	CAP Conc. (ug/L) Sample 13
1	ND
2	-[e]
3	ND
4	10.7
6	0.0
7	4.1
8	0.4
9	ND
11	ND
12	0.5
13	0.7
14	0.3
15	ND
16	1.5
17	1.0
18	ND
19	-[e]
20	0.3
21	ND
22	ND
23	0.8

For key, see Table XIII

TABLE VIII
Part 2 (Aqueous Extract) Chloramphenicol (2.5 µg/L)

Laboratory	CAP Conc. (ug/L) Sample 9	Sample 14
1	1.2	0.7
2	2.2	ND
3	0.9	1.2
4	8.9[(b)]	5.2[(b)]
6	1.5	1.2
7	1.6	4.4
8	1.6	1.1
9	0.7	0.8
11	ND	1.0
12	2.3(1.8[(d)])	2.0 (1.5[(d)])
13	2.4	1.2
14	0.3	0.2
15	0.9	0.9
16	6.0	1.5
17	1.5	1.2
18	3.5	1.7
19	1.1	0.9
20	1.5	1.4
21	4.4	ND
22	1.5	1.1
23	1.0	ND
Mean	1.5	
r	3.4	
S_r	1.23	
RSD_r	83.6	
Ho_r	3.0	
R	3.5	
S_R	1.24	
RSD_R	84.7	
Ho_R	2.0	

For key, see Table XIII

TABLE IX
Part 2 (Aqueous Extract) Chloramphenicol (7.5 µg/L)

Laboratory	CAP Conc. (ug/L) Sample 11	Sample 12
1	1.7	2.1
2	4.1	3.6
3	3.5	3.8
4	17.9[a]	7.0 [a]
6	3.0	2.9
7	3.1[a]	7.8[a]
8	5.6	5.4
9	1.7	1.7
11	3.5	2.6
12	4.7(4.2 [d])	4.6(4.1[d])
13	4.4	2.7
14	0.1	0.2
15	1.7	3.2
16	7.5	9.0
17	2.4	3.5
18	4.0	4.2
19	2.5	2.9
20	3.2	3.6
21	4.0 [a]	7.3 [a]
22	2.9	3.7
23	1.6	1.8
Mean	3.3	
r	1.5	
S_r	0.55	
RSD_r	16.7	
Ho_r	0.7	
R	5.0	
S_R	1.77	
RSD_R	53.3	
Ho_R	1.4	

For key, see Table XIII

TABLE X
Part 2 (Aqueous Extract) Split Level Samples

Laboratory	CAP (ug/ml) Sample 8	Sample 10
	18.75	17.5
1	7.8	-[e]
2	8.2	9.7
3	10.7	7.1
4	10.4	11.3
6	12.9	1.0
7	14.7	6.0
8	7.1	6.0
9	5.9	8.2
11	10.7(10.2[d])	10.9(10.4[d])
12	16.2	8.0
13	-[e]	0.5[f]
14	5.0	6.7
15	37.5[c]	22.5
16	5.1	7.5
17	9.0	9.9
18	3.9	7.6
19	5.6	10.4
20	18.9[c]	29.4[c]
21	9.2	7.7
22	4.7	3.0
23		
Mean	8.71	7.49
r	9.11	
S_r	3.25	
RSD_r	40.2	
Ho_r	1.8	
R	9.1	
S_R	3.17	
RSD_R	39.2	
Ho_R	1.2	

For key, see Table XIII

TABLE XI
Part 1 (Aqueous Solutions):
Summary of Calculated Statistical Parameters

Blind Duplicate Samples

Mean (µg/L)	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
2.2	22	2.1	0.76	34.9	1.3	3.0	1.06	48.5	1.2
9.6	21	5.9	2.09	21.6	1.0	11.0	3.94	41.1	1.3

Split Level Samples

SL Mean	Mean	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
4.8	5.7	21	3.6	1.27	24.2	1.0	4.9	1.76	33.6	1.0

For key, see Table XIII

TABLE XII
Part 2 (Aqueous Extract):
Summary of Calculated Statistical Parameters

Blind Duplicate Samples

Mean (ug/L)	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
1.5	20	3.4	1.23	83.6	3.0	3.5	1.24	84.7	2.0
3.3	18	1.5	0.55	16.7	0.7	5.0	1.77	53.3	1.4

Split Level Samples

SL Mean	Mean	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
8.7	7.5	17	9.1	3.25	40.2	1.8	9.1	3.17	39.2	1.2

For key, see Table XIII

TABLE XIII

Key to Tables III TO XII

(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 mean, repeatability or reproducibility.
(c)	An outlying result by Grubbs' Test on the cell averages of the split level test at P<0.01 level, not used in calculation of mean, repeatability or reproducibility.
(d)	A repeat analysis was carried out (value in brackets was not used in the calculation of mean, repeatability or reproducibility).
(e)	Result not reported.
(f)	A single result result reported, not used in calculation or mean, repeatability or reproducibility.
(g)	Analyst reported a gross error.
ND	Not Detected.
SL	Split level.
MEAN	The mean obtained from the collaborative trial data.
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-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 ($S_R \times 100/MEAN$).
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

Procedure for the Determination of Chloramphenicol in Animal Tissues

1. Scope and Field of Application

This method describes a procedure for the determination of trace residues of chloramphenicol in edible animal tissues. The limit of detection is 2 µg/kg. At the 10 µg/kg level average recovery falls within the range 55-60%.

2. Definition

The content of chloramphenicol: the content of *D*(-)threo-*2*-Dichloroacetamido-*1*-*p*-nitrophenylpropane-*1,3* diol, in edible animal tissues determined by the method specified.

3. Principle

A portion of homogenised meat is extracted with water. After filtration an aliquot of the filtrate is applied to an Extrelut® cartridge. Chloramphenicol is eluted with dichloromethane. The organic phase is evaporated, water is added to the residue and purification takes place by liquid-liquid extraction with toluene. The water phase is analysed with reversed phase chromatography with UV-detection.

4. Reagents

All chemicals are of analytical grade unless otherwise stated. Use water cleaned with a Milli-Q® system, or deionised and redistilled or water of similar quality.

4.1 Extrelut® Cartridges, 20 mL, Merck art. 11737

4.2 Dichloromethane

4.3 Toluene

4.4 Acetonitrile

4.5 Sodium Acetate Buffer, 0.01 mol/L: *p*H = 4.3

Dissolve 0.82 g sodium acetate (**4.5.1**) in about 700 mL water. Adjust the pH, with acetic acid (**4.5.2**), to 4.3, transfer the solution to a 1000 mL volumetric flask, make to volume and mix. Filter the solution through a 0.45 µm filter.

4.5.1 Sodium acetate, anhydrous

4.5.2 Acetic acid, 50%

4.6 HPLC Eluent

Add 750 mL acetate buffer (**4.5**) to 250 mL acetonitrile (**4.4**), mix thoroughly. Filter and degas the eluent before use.

4.7 Chloramphenicol

5. Apparatus

Normal laboratory equipment and in particular:

5.1 Meat Grinder, mincer or similar.

5.2 Homogeniser, Ultra-Turrax or similar.

5.3 Centrifuge

5.4 Waterbath, 40°C, with equipment for drying with nitrogen or rotary vacuum evaporator.

5.5 Vortex Mixer, Vibrofix or similar.

5.6 *p*H Meter

5.7 High Performance Liquid Chromatography system

5.7.1 Pump, Waters M-6000 or equivalent.

5.7.2 Injection Valve, Rheodyne 7125 or equivalent.

5.7.3 Guard Column, 1 10 mm, ID 2.1 mm (Chrompack) packed with Perisorb C 8.

5.7.4 Analytical Column, 1 200 mm, ID 3 mm (Chrompack) cartridge packed with Chromspher C-18 5 µm.

5.7.5 Variable UV/Vis Detector, Pye Unicam 4020 or equivalent.

5.7.6 Diode Array UV/Vis Detector, HP-1040A or equivalent

5.7.7 Recorder, with variable measuring range.

6. Standard

6.1 Concentrated Chloramphenicol Standard Solution, 100 µg/mL

Weigh in a 100 mL volumetric flask 10.0 mg CAP, make to volume with methanol and mix.

6.2 Diluted Chloramphenicol Standard Solution, 5 µg/mL

Pipette into a 100 mL volumetric flask 5 mL standard solution (**6.1**), make to volume with water and mix.

6.3 Working Chloramphenicol Standard Solutions

Pipette into four 10 mL volumetric flasks 200, 400, 800 and 1600 µL of standard solution (**6.2**), make to volume with water and mix. The concentrations are 0.1, 0.2, 0.4 and 0.8 µg/mL respectively (**9.1**).

7. Procedure

7.1 Pre-treatment of Sample

Excess fat is removed from the fresh meat samples (**8.3**). The meat is cut in pieces and homogenised in the meat grinder (**5.1**).

The ground meat is stored at -20°C.

7.2 Control Samples

Within each batch a reference sample to include a blank and a spike (at the 10 µg/kg level) may be included as a measure of recovery.

7.3 Sample Extraction and Clean-Up

To an accurately weighed 10.0 g of homogenised meat 40 mL of water is added. After vigorous homogenisation for 3 minutes the sample is filtered off and 20 mL of the filtrate is applied to an Extrelut® cartridge (**4.1**). After 15 minutes CAP is eluted with 100 mL dichloromethane (**4.2**). The organic phase is evaporated under a gentle stream of

nitrogen (see **8.4, 8.5**) and the residue transferred to a centrifuge tube with ca. 10 mL dichloromethane. After evaporation, 300 µL of water and 1.5 mL toluene are added to the residue. After gentle mixing (about 700 rpm) on the vortex mixer the phases are separated by centrifugation (2000 rpm). The organic phase is discarded and the partition repeated with 1 mL fresh toluene.

The aqueous phase is isolated (see **8.6**) and if necessary filtered through a Millex filter.

This solution is taken for hplc analysis.

7.4 Measurement

7.4.1 Hplc UV-Vis Detector

Wavelength	285 nm
Range detector	0.01 Aufs
Range recorder	10 mV
Paper advance	1.0 cm/min
Eluent flow	0.6 mL/min
Injection volume	0.1 mL

Wait until the system is stabilised and inject the four working standard solutions (**4.10**), the sample solutions (**6.3**) and again the working standard solutions.

Check for UV signals in the sample chromatograms with the retention time of CAP.

7.4.2 HPLC UV-Vis Diode Array Detector

Confirmatory analysis by Diode Array may be carried out using these conditions.

Wavelength	285 nm pilot signal
Band width	4 nm
Reference	550 nm
Stop time	10 min
Threshold	0.5 mAu
Spectrum at	apex, slope, base
Range	225-400 nm, step 2 nm
Eluent flow	0.6 mL/min
Injection volume	0.2 mL

8. Notes of Procedure

8.1 *The working standard solutions are stable for one week when they are stored in the dark.*

8.2 *Meat samples that have decayed cannot be analysed with this method.*

8.3 *Addition of 0.01 µg/kg CAP is carried out by pipetting 1 mL of the working standard solution of 0.1 µg/mL to the meat sample.*

8.4 *Due to a difference in the quality of the Extrelut® packing material it may, in rare occasions, happen that some water is eluted from the cartridge with dichloromethane. In that case the organic phase should be filtered through a phase separation filter (**4.11**) before evaporation.*

8.5 *It has been found that the volume of DCM may first be reduced to ca. 10 mL by rotary evaporation before transferring to a centrifuge tube.*

8.6 *When there is poor separation between the water and toluene phase the water phase becomes turbid. Toluene present in the final sample solution may cause ghost peaks in the chromatogram. This may cause problems when the chromatograms are evaluated with Diode Array UV/Vis detection.*

9. Expression of Results

9.1 Calculation

Calculate the chloramphenicol concentration using the following formula:

$$\text{Chloramphenicol Concentration (mg/Kg)} = \frac{h_m - h_{bl}}{h_{st}} \times C \times \frac{v_t . v_m}{v_e . m}$$

where:

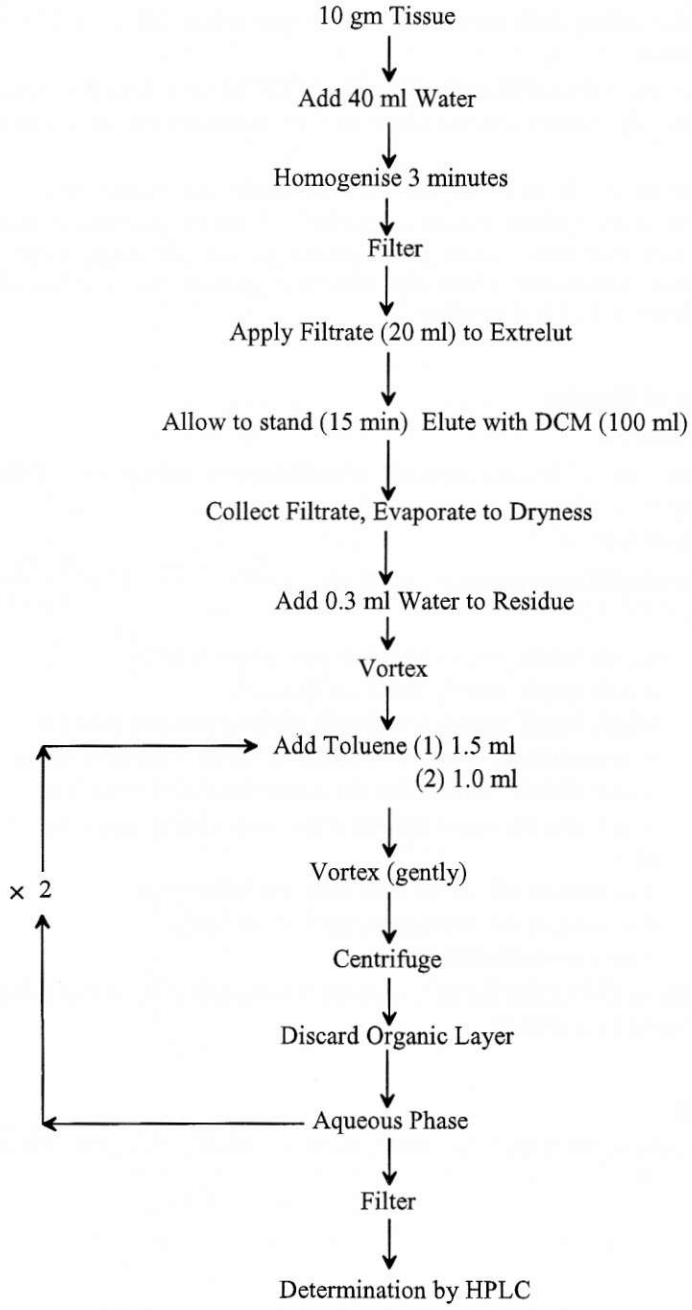
- h_m is peak height, in mm, found for the sample solution
- h_{bl} is peak height, in mm, found for the blank
- h_{st} is peak height, in mm, found for a working standard solution
- C is concentration, in µg/mL, from the working standard solution
- V_t is total volume, in mL, from the water phase after extraction 40 mL plus the water content of the meat sample (normally 7.5 mL)
- V_m is volume, in mL, of the final sample solution (0.3)
- V_e is volume, in mL, brought on the Extrelut® (20)
- m is sample weighed in g (10)

The result is corrected for recovery by multiplying by $100/r$ where r is the recovery percentage.

10. References

10.1 HJ Keukens, WMJ Beek and MML Aerts, J. Chrom., 1986, **352**, 445-453

Procedure for the Analysis of CAP in Tissue



J. Assoc. Publ. Analysts, 30, 49-54

The Determination of Theobromine in Cocoa and Chocolate Products

Collaborative Trial

Paul Brereton[(a)1], Malcolm Hague* and Roger Wood

Ministry of Agriculture, Fisheries and Food, Food Safety Directorate,
Food Science Laboratory, Colney Lane, NORWICH, UK, NR4 7UQ

* Lancashire County Laboratory, Peddars Way, Preston Riversway Dockland, Ashton-on-Ribble, Lancashire PR2 2TX

The results of a collaborative trial, involving 26 laboratories, testing a method for the determination of theobromine in chocolate and chocolate products are reported. The method tested consisted of a water extraction, addition of a clearing agent, filtration followed by separation and quantification using HPLC with UV detection.

The method demonstrated satisfactory precision for five samples containing levels of theobromine in the range 1200-5900 mg/kg. The precision of the method when determining the theobromine content of a sample of cocoa powder (approx. 20,000 mg/kg) was slightly above expected precision levels for this type of analysis. Reproducibility for six types of sample ranged from 119 mg/kg (RSD_R 3.0%) for chocolate to 4041 mg/kg (RSD_R 7.5%) for cocoa powder. The precision obtained was superior to that obtained by an AOAC HPLC method which was similar in principle.

Introduction

Theobromine and caffeine are the two most important alkaloids in cocoa and chocolate. The theobromine content of the cocoa-bean is approximately 1.8 g/100g. It has a stimulant action, a mild physiological action on the nervous system and kidneys and also considerable practical use as a diuretic. The determination of the theobromine content of a cocoa product enables an indication of the total non-fat cocoa solid content to be established: this may then be used as an indication of cocoa content.

There are no official limits for the content of theobromine in cocoa products. However levels of non-fat cocoa solids in cocoa products are prescribed in the Cocoa and Chocolate Products Regulations 1976 and Amendment Regulations 1982[(1)], which implement EC Directive No. 73/241/EEC as amended[(2)]: minimum dry non-fat cocoa solid contents are

1 (a) to whom correspondence should be addressed

prescribed for: chocolate, 14%; plain chocolates, 12% and milk chocolate, 2.5%. These levels may be estimated through the determination of theobromine.

The estimation of non-fat cocoa solids from the total theobromine and caffeine contents is calculated by the expression:[3]

$$\% \text{ dry fat free cocoa solids} = \frac{\text{theobromine concentration (mg/kg)}}{300}$$

For many years the principal method for the determination was gravimetric e.g. as described by the AOAC[4]. However methods based on this principle exhibit poor precision, are not applicable to products containing >12 % sweetening ingredients and use tetrachloroethane, a suspected carcinogen, as the extracting solvent. There are also doubts as to the accuracy of this method[5]. The method was included in the pre-trial because it is still regarded as the reference procedure by some organisations.

In view of the need for an accurate and precise validated method for theobromine, the Ministry of Agriculture Fisheries and Food (MAFF) in conjunction with the UK Association of Public Analysts (APA) agreed to validate a suitable HPLC method for the determination of theobromine in cocoa products.

The method collaboratively assessed in this trial is a variant of a HPLC technique which is the current AOAC First Action Method (1981)[5][6], it has been modified to include the use of a clearing reagent instead of centrifugation prior to chromatographic analysis.

Method of Analysis Collaboratively Tested

The following methods were used in the trial:

(1) Pre-trial only : AOAC Final Action 1975 Surplus 1981

The sample is mixed with MgO, extracted with tetrachloroethane, washed with petroleum ether and the precipitate determined gravimetrically [4].

(2) Pre-trial and trial proper: HPLC method developed by the Association of Public Analysts (APA)

The fat is removed from the weighed sample using petroleum spirit, theobromine is extracted using boiling water, solutions are cleared using Carrez reagent prior to HPLC using an ODS reverse phase column with UV detection at 273 nm. The full method is given in Appendix I

Collaborative Trial Organisation, Samples and Results

Thirty one laboratories participated in the pre-trial trial, and of these 26 participated in the trial proper (24 UK Public Analyst Laboratories, the Laboratory of the Government Chemist and Tesco Stores Ltd.)

Samples

All samples were prepared by the Lancashire County Laboratory

Sample	Sample Code No.	Sample Type
A	1-64	A retail drinking chocolate (powder)
B	129-192	A retail cocoa drink (Powder)
C	193-254	Milk Chocolate block
D	255-316	'Dark' Chocolate block
E	317-380	A malted milk drink (powder)
F	381-444	Milk Chocolate block
G	64-128	Plain Flour

Homogeneity

Eight individual analyses were carried out for each sample type, the highest variability was 1.42 % (CV) for sample C. Homogeneity data are given in Appendix II.

The chocolate blocks were shredded in a "Robot Coupe" food blender and sieved through a 2 mm sieve prior to homogeneity testing. All other samples were analysed "as received".

Trial Protocol

Pre-trial

Thirty-two laboratories participated in the pre-trial in which participants were asked to analyse two samples (C and F) in (known) duplicate by both the HPLC procedure and the AOAC gravimetric method.

Trial proper

Twenty-six laboratories participated and were asked to use the method described in Appendix I. The laboratories received fourteen samples and were asked to perform one complete analysis on each sample. The samples comprised six different chocolate products (nos. A - F) together with a blank sample (G), and were sent out as individually numbered blind duplicates.

All participants were instructed to complete the analyses within 6 weeks and to enclose their chromatograms together with their results.

Results

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

Statistical Analysis of the Results

The trial results were 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[7].

Repeatability and Reproducibility

Calculations for repeatability (r) and reproducibility (R) as defined by that Protocol[7] were carried out on those results remaining after removal of outliers. The resulting values are given in Tables I-IX and have been summarised in Tables X-XI.

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 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[8].

The Horwitz predicted value is calculated from the Horwitz equation[8]:

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

C = measured concentration of analyte expressed as a decimal.
i.e. 1 mg/L = 1×10^{-6}

Horrat Values (Ho)

The Horrat[9] values 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 greater than 2 usually indicates unsatisfactory interlaboratory 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 given in tables IX-X.

Discussion

The results of the pre-trial demonstrated that the precision of the HPLC procedure was significantly better than that of the AOAC gravimetric method. Furthermore, comments from participants indicated a reluctance to use the latter method on both analytical and safety grounds. It was therefore decided that the main trial would collaboratively test only the HPLC procedure.

Precision

Statistical analysis of the results of the collaborative trial showed that the HPLC method demonstrated satisfactory precision for all the samples except one, a cocoa powder sample (B) containing 20,000 mg/kg theobromine which had a RSD_R of 7.69%, slightly higher than would be expected for a determination at this level of analyte. The precision obtained for the method in this trial was superior to that obtained by the AOAC HPLC method in a validation study carried out by the AOAC[6].

The relationship between precision and concentration was approximately linear for the five samples in the range 1,000-6,000 mg/kg.

Recommended precision parameters for samples containing theobromine in the range 1000-6000 mg/kg are:

Repeatability, $0.06 \times C$ mg/kg;
Reproducibility, $0.13 \times C$ mg/kg;

Where C is the concentration of theobromine in mg/kg

The relatively poor precision obtained for the sample with a high theobromine content could be due to there being no defined procedure to "dilute" the theobromine content in the method when analysing samples containing very high levels of theobromine. Participants determined the theobromine content in this sample by various procedures; three, four or five fold dilution of the sample extract, comparison with 100 mg/L standard, or by taking a smaller weight. Clearly a standard procedure for the analysis of samples containing high levels of theobromine is required to be written into the method. Work carried out at Lancashire County Laboratory has shown that for samples of high theobromine content an initial weight of approximately 1g (weighed to 4 decimal places) is appropriate, the resulting sample can subsequently be diluted until a final

concentration of approximately 25 mg/L is obtained. It is therefore recommended that the method be suitably amended to include a dilution procedure for samples with a theobromine content of >6,000 mg/kg

Trueness

There has been no attempt to assess trueness in this trial. In house validation work carried out by Derbyshire Public Analyst on the HPLC method reported a mean recovery of 94.6% based on 166 determinations, the AOAC HPLC procedure (using centrifugation instead of Carrez reagents) has a mean recovery of 95.5% [5]. Two participants reported obtaining significantly higher results when not using the clearing reagents, i.e. by following the AOAC HPLC procedure [5]. One of these participants, Laboratory 18, carried out the collaborative trial without using clearing agent and obtained higher results for the five samples. Further work is required to investigate the recoveries of the two methods.

Conclusion

The results of the collaborative trial were satisfactory and demonstrate successful validation of this HPLC method for the analysis of cocoa and cocoa products. It is recommended that the method be amended slightly to include instructions on the dilution procedure used when analysing cocoa powder of high theobromine content.

The recommended precision parameters to be included in the validated method to be published by MAFF in the series of validated methods[10] are:

Repeatability, $r = 0.06 \times C$;
Reproducibility, $R = 0.13 \times C$;

where C is the concentration of theobromine in the sample in mg/kg (minimum 1000 mg/kg).

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:

R Davies(MJ Weaver)	Birmingham City Council, Birmingham;
D Dunn	A H Allen and Partners, Sheffield;
RA Ennion(K Postlethwaite/C Newton)	Ruddock and Sherratt, Chester;
MF Godfray(J Nisbet)	Lothian Regional Analyst's Laboratory, Edinburgh;
Dr W Griffiths(AR Kiddie)	Mid Glamorgan County Council, Cardiff;
PS Hall(A Cowling)	Bernard Dyer and Partners, London;
I Hampton (J Brooman)	Kent County Laboratory, Maidstone;
N Harrison(LC Graham)	Leicestershire County Analyst's Laboratory, Leicestershire;

C Heide(W Beazley)	Somerset Scientific Services, Somerset;
Dr F Hill(HE Rock/R Hayden)	Public Analyst's Laboratory, Dublin;
P Holliday(H Barker)	Official Analysts Laboratory, Jersey;
Dr F Hollywood(A Jones)	Staffordshire County Analyst's Department, Stafford;
T Ioannou(S McCudden)	Tesco Stores Ltd., Technical Services Laboratory, Herts;
TE Johnson(MB Dalling)	Herbert J Evans and Partners, Public Analyst's Department, Dyfed;
S Joyce(M Davidson)	Strathclyde Regional Chemist and Public Analyst Department, Glasgow;
Dr A Kay(D White)	Derbyshire County Council, Public Protection Department - Analyst Division, Derbyshire;
G Keen(C Lincoln)	Hereford and Worcester County Council, County Laboratory, Worcester;
J Pearse(C Evans)	The Laboratory of the Government Chemist, Teddington, Middlesex;
B Sanders(P Sweet)	City of Cardiff, City Analyst's Laboratory, Cardiff;
S Stangroom(P Osborne)	Lincolne, Sutton and Wood, Norwich;
R Stevens(M Patel/R Bottrill)	Clayton Environmental Ltd., Birmingham;
SW Swain(PJ Welsh)	Hampshire Scientific services, Southsea;
J Turnbull(A Barachizadeh)	County of Avon Scientific Services, County Laboratory, Bristol;
K Holland (S Madigan)	Isle of Man, Public Analyst's Department, Douglas;
K Wardle	Humberside County Analyst, Hull;
JP Wootten	Central Scientific Laboratories, New Cross, London;

References

(1) The Cocoa and Chocolate Products Regulations 1976 (SI 1976 No 541) and Chocolate Products(Amendment) Regulations 1982 (SI 1982 No 17)

(2) EC Directive No 73/241/EEC as amended (74/411, 74/644, 75/155, 76/628, 78/609, 80/608)

(3) Chapman W B, Fogden E and Urry S, The Determination of Total Alkaloids in Chocolate Cake and Cocoa, Journal of the Association of Public Analysts, 1963, **1**, 59-62

(4) Wadsworth R V, Analyst, 1921, **46**, 32

(5) Kreiser W R and Martin R A Jr, High Pressure Liquid Chromatographic Determination of Theobromine and Caffeine in Cocoa and Chocolate Products, JAOAC, 1978, **61(6)**, 1424-1427

(6) Kreiser W R and Martin R A Jr, High Pressure Liquid Chromatographic Determination of Theobromine and Caffeine in Cocoa and Chocolate Products: Collaborative Study, JAOAC, 1980, **63(3)**, 591-594

(7) "Protocol for the Design, Conduct and Interpretation of Collaborative Studies" Ed W Horwitz, Pure and Appl. Chem., 1988, **60(6)**, 855-864

(8) Horwitz W, Evaluation of Methods Used for Regulation of Foods and Drugs, Analytical Chemistry, 1982, **57**, 67A-76A

(9) Peeler J T, Horwitz W and Albert R, Precision Parameters of Standard Methods of Analysis for Dairy Products JAOAC, 1989, **72(5)**, 784-806

(10) MAFF Food Safety Directorate News Release, FSD 32/92, 9 June 1992

TABLE I

Theobromine Collaborative Trial : Results
Pre-Trial Samples
Sample 1 (R6/K)

Laboratory	HPLC (mg/kg)		Gravimetric (mg/kg)	
	1	2	1	2
2	1195	1179	600	3300
3	1227	1217	NR	NR
4	1156[b]	1314[b]	1410	1430
5	1203[a]	NR	1760[a]	NR
6	1325	1350	15000[a]	NR
7	1183	1157	1600	1700
8	1204	1179	1200	1400
9	1242	1255	13400[a]	NR
10	1278	1216	NR	NR
11	1261	1266	NR	NR
12	1130[a]	NR	NR	NR
14	1263	1237	6200	7200
15	1312	1226	NR	NR
16	1292	1300	NR	NR
17	1214	1277	1300	NR
18	1257	1200	600	1000
19	1202	1219	1360	600
20	1194	1206	NR	NR
21	1438[c]	1497[c]	7600	9400
22	1224	1221	NR	NR
23	1305[a]	NR	NR	NR
24	1309	1308	NR	NR
26	1205	1216	910	1400
28	1180	1219	9000	6200
29	1270	1180	820[a]	NR
30	1250	1261	44000	10000
31	1181	1181	NR	NR
32	1259	1277	NR	NR
Mean	1236		3200	
r	74		2800	
S_r	27		1000	
RSD_r	2.1		31.5	
R	130		8800	
S_R	46		3200	
RSD_R	3.7		100	

For Key, See Table XII

TABLE II

Theobromine Collaborative Trial : Results
Pre-Trial Samples
Sample 2 (M33)

Laboratory	HPLC (mg/kg)		Gravimetric (mg/kg)	
	1	2	1	2
2	1334	1350	1100	1400
3	1412	1442	NR	NR
4	1380[b]	1525[b]	1550	1550
5	1396[a]	NR	1810[a]	NR
6	1450	1500	19000[a]	NR
7	1462	1484	2200	2700
8	1361	1342	1700	2000
9	1404	1406	4400[a]	NR
10	1301	1292	NR	NR
11	1417	1410	NR	NR
12	1326[a]	NR	NR	NR
14	1385	1379	9800	10500
15	1478	1476	NR	NR
16	1402	1403	NR	NR
17	1498	1552	800	1000
18	1261[b]	1382[b]	1030	470
19	1469	1475	1240	630
20	1362	1396	NR	NR
21	1737[c]	1681[c]	6400	3500
22	1379	1391	NR	NR
23	1425[a]	NR	NR	NR
24	1430	1432	NR	NR
26	1389	1393	1640	1720
28	1365	1314	13200[a]	NR
29	1490[b]	1300[b]	1920[a]	NR
30	1424	1405	5400	9800
31	1387	1374	NR	NR
32	1413	1398	NR	NR
mean	1408		3100	
r	48		3200	
S_r	17		1200	
RSD_r	1.2		37.3	
R	157		9100	
S_R	56		3200	
RSD_R	4		105	

For Key, See Table XII

TABLE III

Theobromine Collaborative Trial : Results

Drinking Chocolate Sample (A)
(Code Numbers 1 -64)

Laboratory	Theobromine content (mg/kg)	
	1	2
1	5989	5906
2	6166	6119
3	5738	5727
4	5847	5737
5	6087	5683
6	5659	5675
7	5742	5760
8	5553	5358
11	5954	5869
14	5761	5787
15	5909	5921
17	5684	5805
18	6055[c]	6138[c]
19	6121	6445
20	5683	5519
21	5947	6178
22	5709	5692
23	5809	5819
24	5770	5873
25	5661	6044
26	5928	5852
28	5942	6023
29	6272	6888
30	5677	5939
31	6196	6165
32	5432	5305

mean	5867
r	409
S_r	146
RSD_r	2.5
R	767
S_R	274
RSD_R	4.7

For Key See Table XII

TABLE IV

Theobromine Collaborative Trial : Results
Cocoa Powder Sample (B)
(Code Numbers 129 - 192)

Laboratory	Theobromine content (mg/kg)	
	1	2
1	20580	20641
2	20464	20450
3	19475	19778
4	20079	20271
5	17907	16916
6	20237	19604
7	18691	18610
8	19070	19108
11	19151	18616
14	19660	19691
15	17707	16714
17	19815	19615
18	23003[(c)]	22086[(c)]
19	19685	21685
20	20424	20644
21	20284	19889
22	19274	19253
23	20273	19914
24	20098	19920
25	20920	21256
26	19679	19936
28	14448	15767
29	19428	20529
30	19192	17181
31	20196	19967
32	16964	17253

mean	19338
r	1509
S_r	539
RSD_r	2.8
R	4041
S_R	1443
RSD_R	7.5

For Key, See Table XII

TABLE V

Theobromine Collaborative Trial : Results

Milk Chocolate Sample (C)
(Code Numbers 193 - 254)

Laboratory	Theobromine content (mg/kg)	
	1	2
1	1861[b]	1478[b]
2	1377	1395
3	1412	1443
4	1422	1430
5	1430	1389
6	1448	1387
7	1360	1370
8	1387	1430
11	1379	1374
14	1406	1364
15	1415	1435
17	1432	1406
18	1467[c]	1542[c]
19	1456	1457
20	1348	1357
21	1488	1500
22	1380	1342
23	1385	1385
24	1367	1387
25	1386	1384
26	1419	1421
28	1532[b]	1354[b]
29	1438	1477
30	1423	1406
31	1416	1364
32	1320	1293

mean	1403
r	58
S_r	21
RSD_r	1.5
R	119
S_R	43
RSD_R	3

For Key, See Table XII

TABLE VI

Theobromine Collaborative Trial : Results
Dark Chocolate Sample (D)
(Code Numbers 255 - 316)

Laboratory	Theobromine content (mg/kg)	
	1	2
1	4279	4320
2	4479	4495
3	4214	4190
4	4079	4167
5	3610	4021
6	4155	4176
7	4115	4161
8	4099	4199
11	4167	4238
14	4022	4125
15	4114	3948
17	4060	4083
18	4538[(c)]	4510[(c)]
19	4500	4794
20	4118	4133
21	4459	4521
22	3985	4025
23	4070	4072
24	4135	4162
25	4175	4066
26	4206	4204
28	4409	4739
29	4239	4162
30	4221	4207
31	4110	4075
32	3753	3585

mean	4171
r	273
S_r	97
RSD_r	2.3
R	626
S_R	224
RSD_R	5.4

For Key, See Table XII

TABLE VII

Theobromine Collaborative Trial : Results

Malted Milk Sample (E)
(Code Numbers 317 - 380)

Laboratory	Theobromine content (mg/kg)	
	1	2
1	2024	2036
2	1808	1790
3	1904	1931
4	1952	2000
5	1764	1801
6	1905	1923
7	1918	1954
8	1886	1879
11	1921	1937
14	1929	1892
15	2007	1962
17	1948	1928
18	2127[c]	2253[c]
19	1978	1870
20	1816	1867
21	2143	2176
22	1798	1822
23	1927	1901
24	1964	1964
25	1892	1904
26	1969	1988
28	1883	1846
29	2074	2121
30	1884	1992
31	2019	1941
32	1897	1890
mean	1931	
r	88	
S_r	31	
RSD_r	1.6	
R	246	
S_R	88	
RSD_R	4.5	

For Key, See Table XII

TABLE VIII

Theobromine Collaborative Trial : Results
Milk Chocolate Sample (F)
(Code Numbers 381 - 444)

Laboratory	Theobromine content (mg/kg)	
	1	2
1	1293	1303
2	1174	1202
3	1254	1242
4	1304	1294
5	1240	1124
6	1239	1227
7	1246	1248
8	1224	1229
11	1251	1206
14	1215	1222
15	1266	1269
17	1321	1342
18	1334[c]	1282[c]
19	1317	1334
20	1231	1271
21	1398	1370
22	1190	1199
23	1224	1219
24	1239	1238
25	1209	1134
26	1264	1260
28	1265	1245
29	1374	1329
30	1288	1267
31	1207	1184
32	1151	1092

mean	1249
r	72
S_r	26
RSD_r	2
R	176
S_R	63
RSD_R	5

For Key, See Table XII

TABLE IX

Theobromine Collaborative Trial : Results
Blank Sample: Flour (G)
(Code Numbers 64 - 128)

Laboratory	Theobromine content (mg/kg)	
	1	2
1	149	ND
2	ND	ND
3	ND	ND
4	ND	ND
5	ND	ND
6	ND	ND
7	ND	ND
8	ND	ND
11	30	50.3
14	ND	ND
15	ND	ND
17	ND	ND
18	ND[(c)]	ND[(c)]
19	ND	ND
20	ND	ND
21	21	16
22	ND	ND
23	ND	ND
24	ND	ND
25	2.69	3.15
26	ND	ND
28	ND	ND
29	ND	ND
30	ND	ND
31	ND	ND
32	ND	ND
mean		
r		
S_r		
RSD_r		
R		
S_R		
RSD_R		

For Key, See Table XII

TABLE X

Summary of Calculated Statistical Parameters for Theobromine

Pre-Trial Samples : HPLC Method

Sample Number	Mean(obs) (mg/kg)	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
1	1236	23	74	27	2.1	0.6	130	46	3.7	0.7
2	1408	21	48	17	1.2	0.3	157	56	4.0	0.7

Pre-Trial Samples : Gravimetric Method

Sample Number	Mean(obs) (mg/kg)	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
1	3200	10	2800	1000	31.5	10	8800	3200	100	21
2	3100	11	3200	1200	37.3	12	9100	3200	105	22

TABLE XI

Summary of Calculated Statistical Parameters for Theobromine

Trial Samples : HPLC Method

Sample	Mean(obs) (mg/kg)	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
A	5867	25	409	146	2.5	0.9	767	274	4.7	1.1
B	19338	25	1509	539	2.8	1.2	4041	1443	7.5	2.1
C	1403	23	58	21	1.5	0.4	119	43	3.0	0.6
D	4171	25	273	97	2.3	0.8	626	224	5.4	1.2
E	1931	25	88	31	1.6	0.5	246	88	4.5	0.9
F	1249	25	72	26	2.0	0.6	176	63	5.0	0.9
G	ND	25	-	-	-	-	-	-	-	-

For Key, See Table XII

Key to Tables I to XI

(a)	A single result reported, not used in calculation of mean, repeatability or reproducibility.
(b)	An outlying result by Cochrans Test at $P<0.01$ level, not used in calculation of mean, repeatability or reproducibility
(c)	Did not use Carrez solutions, results not used in calculation of 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.
ND	Not detected; this result was not used in the calculation of the mean, repeatability or reproducibility.
NR	No result submitted.
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 ($SD_r \times 100/MEAN$).
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-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 ($S_R \times 100/MEAN$).
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

Determination of Theobromine in Cocoa and Chocolate Products by HPLC

1 Scope and Field of Application

The method is applicable to cocoa and chocolate products.

2 Principle

The sample is defatted by extracting with petroleum ether.

The sample is dispersed into distilled water by heating and stirring. The suspension/solution is cleared using two clearing agents, and then filtered through filter paper and a 0.45 µm millipore filter.

The filtered solution is analysed using reverse-phase HPLC with UV detection at 273 nm.

3 Reagents and Materials

Reagents of recognised analytical grade are to be used. Wherever the use of water is required, distilled water or water of equivalent purity is to be used.

3.1 Petroleum ether 40-60°C

3.2 Carrez solution 1

Dissolve 219 g zinc acetate (**3.2.1**),in approximately 500 mL of de-ionised water and add 30 mL acetic acid (**3.2.2**). Transfer to a 1 L volumetric flask and make up to volume with water.

3.2.1 Zinc acetate, $Zn(C_2H_3O_2).2H_2O$

3.2.2 Acetic acid, 0.1 mol/L

3.3 Carrez solution 2

Dissolve 106 g potassium ferrocyanide (**3.3.1**) in approximately 500 mL of de-ionised water and transfer quantitatively to a 1 L volumetric flask and make up to volume with water.

3.3.1 Potassium ferrocyanide, $K_4Fe(CN)_6.3H_2O$

3.4 Mobile Phase

The mobile phase contains methanol and acetic acid, 0.1 mol/L diluted 300 : 700.

3.4.1 Methanol (HPLC grade)

3.5 Theobromine Stock Solution

Dissolve 0.1000 g theobromine in water and dilute to 1 L (100 mg/L). Prepare fresh weekly.

3.5.1 Theobromine

3.6 Theobromine HPLC Standard solution

Dilute 25 mL of the theobromine stock solution (**3.5**) to 100 mL with water. Prepare immediately prior to use.

4 Apparatus

4.1 Centrifuge tube, 50 mL

4.2 Centrifuge, capable of 2000 rpm

4.3 Water bath, located in a fume cupboard
4.4 Water bath, at 100°C
4.5 Beaker, 100 mL
4.6 Volumetric flask, 100 mL
4.7 Filter paper, Whatman No. 541
4.8 Millipore filter, 0.45 μm
4.9 Stoppered tube, approximately 100 mL
4.10 HPLC System, with UV detection at 273 nm

5 Procedure

5.1 Weigh accurately to 0.1 mg an appropriate amount of sample into a weighed 50 mL centrifuge tube. (**7.1**).

5.2 To extract fat, shake the sample with 30 mL petroleum ether (**3.1**) for 2 min. centrifuge at 2000 rpm for 10 min and decant the solvent. Repeat this extraction with a further 30 mL petroleum ether.

5.3 To remove any residual petroleum ether, place the centrifuge tube in a warm water bath located in a fume cupboard.

5.4 Quantitatively transfer the residue with 50 mL of warm water to a 100 mL beaker and place on a boiling water bath for 20 minutes, stirring occasionally.

5.5 Transfer quantitatively to a 100 mL volumetric flask. Cool and add 5 mL of each clearing agent (**3.2** and **3.3**), make up to volume with water and mix.

5.6 Filter the solution through a Whatman No. 541 filter paper discarding the first 20 mL of filtrate. Pass the filtrate through a 0.45 μm millipore filter into a stoppered tube. This solution is ready for direct injection into the HPLC.

5.7 High performance liquid chromatography

5.7.1 Chromatographic conditions

Column :	Partisil 10 ODS
Flow rate :	2 mL/min.
Detector (UV) Wavelength	: 273 nm
Absorbance :	0.1
Injection volume:	25 μL
Retention time:	5 min.
Mobile phase:	See Section 3.4

6 Expression of Results

The theobromine content expressed in mg/kg of the sample is given by:

$$\text{Theobromine Content} = \frac{\text{Sample Peak Height} \times 100}{\text{Standard Peak Height} \times \text{Sample Weight}} \times 25$$

7 Notes on Procedure

7.1 Weight of sample taken for the determination of the theobromine concentration

7.1.1 Preliminary determination of the theobromine concentration

A preliminary determination of the theobromine concentration of the chocolate product sample is performed using 1 g of chocolate product sample.

7.1.2 Calculation of sample weight (g) required for the final determination

From the approximate concentration of theobromine determined by the preliminary determination, the weight of sample (g) required to produce a 25 mg/L final solution of theobromine is calculated. (i.e. The sample taken should contain approximately 2.5 mg of theobromine).

The final analysis is carried out using the calculated sample weight (g). The concentration of theobromine determined by this analysis is the value quoted.

APPENDIX II
Homogeneity Data
Drinking Chocolate (Sample 1-64)

Sample	weight(g)	Peak area	conc. mg/L	Theobromine mg/kg
(1)	0.3596	67.19	21.79	6059.5
(2)	0.3898	71.20	23.33	5985.1
(3)	0.3790	69.02	22.74	6000.0
(4)	0.4115	74.60	24.52	5958.7
(5)	0.2855	51.36	16.87	5908.9
(6)	0.2346	43.01	14.12	6018.8
(7)	0.3444	61.99	20.36	5911.7
(8)	0.4071	73.71	24.17	5937.1
	Mean			5972.5
	Standard Deviation			53.2
	CV%			0.89

Cocoa Powder (129-192)

Sample	weight(g)	Peak area	conc. mg/L	Theobromine mg/kg
(1)	0.4220	108.56	36.22	21457.3
(2)	0.4470	114.53	38.24	21387.0
(3)	0.3930	100.18	33.38	21234.1
(4)	0.5049	129.23	43.07	21326.0
(5)	0.3528	90.60	30.14	21357.7
(6)	0.4205	107.3	35.75	21254.5
(7)	0.4736	121.25	40.42	21336.6
(8)	0.3882	99.23	33.11	21322.8
	Mean			21334.5
	Standard Deviation			70.8
	CV%			0.33

Milk Chocolate Block M33 (193-254)

Sample	weight(g)	Peak area	conc. mg/L	Theobromine mg/kg
(1)	2.4603	106.49	36.09	1466.9
(2)	2.2380	96.24	32.44	1449.5
(3)	2.5515	110.5	37.25	1459.9
(4)	2.8423	122.48	41.29	1452.7
(5)	1.8454	79.06	26.72	1447.9
(6)	1.7408	74.64	24.78	1423.5
(7)	1.9710	83.87	27.91	1416.0
(8)	1.7558	74.76	24.83	1414.2
	Mean			1441.3
	Standard Deviation			20.47
	CV%			1.42

Dark Chocolate Block R5/27 (255-316)

Sample	weight(g)	Peak area	conc. mg/L	Theobromine mg/kg
(1)	0.6774	86.27	28.76	4264.5
(2)	0.7045	91.59	30.54	4334.3
(3)	0.5846	75.24	25.1	4293.5
(4)	0.7992	103.12	34.43	4308.1
(5)	0.6957	89.56	29.77	4279.1
(6)	0.7984	103.34	34.44	4313.6
(7)	0.5400	68.85	22.91	4242.6
(8)	0.8335	7.46	35.81	4296.3
	Mean			4291.5
	Standard Deviation			29.02
	CV%			0.68

Malted Milk Drink (317-380)

Sample	weight(g)	Peak area	conc. mg/L	Theobromine mg/kg
(1)	2.2340	135.49	45.61	2041.6
(2)	2.0395	122.52	40.73	1997.1
(3)	2.0009	121.12	40.15	2006.6
(4)	2.1179	127.39	42.21	1993.0
(5)	2.0979	128.23	42.56	2028.7
(6)	2.2180	133.06	44.63	2012.4
(7)	2.0734	121.75	40.92	1973.6
(8)	2.0115	188.52	39.93	1984.9
	Mean			2004.7
	Standard Deviation			22.53
	CV%			1.12

Milk Chocolate Block R6/K (381-444)

Sample	weight(g)	Peak area	conc. mg/L	Theobromine mg/kg
(1)	0.7861	30.12	9.97	1268.3
(2)	0.7033	26.75	8.89	1264.0
(3)	1.2159	46.10	15.33	1260.8
(4)	0.9395	35.74	11.87	1263.4
(5)	0.7850	30.12	10.02	1276.4
(6)	0.7650	29.42	9.78	1278.4
(7)	0.6916	26.48	8.82	1275.3
(8)	1.0144	38.72	12.91	1272.7
	Mean			1269.9
	Standard Deviation			6.7
	CV%			0.53

J. Assoc. Publ. Analysts, 30, 49-54

MAFF VALIDATED METHOD FOR THE ANALYSIS OF FOODSTUFFS

No V 34

Method for the Differentiation of Fresh and Frozen-thawed Poultry meat by the Determination of the ß-Hydroxyacyl-CoA-Dehydrogenase (HADH) Activity of Chicken Breast Press Juice

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, Food Science Laboratory, Norwich Research Park, Colney, Norwich NR4 7UQ

1. Scope and Field of Application

1.1 The freezing and thawing of meat causes damage to muscle mitochondria resulting in a partial release of certain mitochondrial enzymes into the sarcoplasm. Freeze damage to chicken breast meat muscle can be assessed via levels of the enzyme HADH.

1.2 The method describes the determination of the enzyme ß-hydroxyacyl-CoA-dehydrogenase (HADH) in chicken breast press juice by means of a photometric enzyme test using Nicotinamide-adenine dinucleotide (reduced), disodium salt, (NADH).

1.3 The method is applicable to intact chicken breast meat and can be used to differentiate between fresh meat and frozen-thawed meat which has been frozen at temperatures of -6°C or below.

1.4 The method is not applicable to minced chicken breast meat.

2. Definition

2.1 The method has no legal status.

2.2 HADH activity is expressed in the equivalent of International units per millilitre of meat press juice (U/ml) under the conditions specified.

1U represents 1 micromole of substrate converted per minute at pH 6.0 and at 37°C.

3. Principle

3.1 The press juice is expressed from the chicken breast sample and diluted with a phosphate buffer.

3.2 Determination of HADH activity is based on the following reaction:-

$$\text{Acetoacetyl-CoA} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{HADH}} \text{ß-Hydroxybutyryl-CoA} + \text{NAD}^+$$

0004-5780/94 +5 $20.00

3.3 The rate of conversion of NADH to NAD^+, which is dependent on the level of HADH activity, is measured by the decrease in absorption of the reaction solution at 340 nm.

4. Reagents

(Water should be of de-ionised, distilled or similar quality).

4.1 Phosphate buffer 0.1M (pH 6.0).

4.1.1 Potassium dihydrogen phosphate (KH_2PO_4; AR quality) 13.6g (± 0.1g) made up to one litre with water.

4.1.2 Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$; AR quality) 17.8g (± 0.1g) made up to one litre with water.

4.1.3 To one litre of KH_2PO_4 solution (**4.1.1**) add the Na_2HPO_4 solution (**4.1.2**) until a pH of 6.0 is obtained.

The solution can be stored under refrigeration (less than 5°C) for several months.

4.2 EDTA (disodium salt) solution 10mg/ml.

4.2.1 Accurately weigh 500mg (± 1mg) ethylenediamine tetra-acetic acid (disodium salt; AR quality). Transfer quantitatively to a small 50ml volumetric flask with water. Swirl to dissolve. Make up to the 50 ml mark with water, stopper and invert several times to mix thoroughly.

This solution can be stored under refrigeration (less than 5°C) for several months.

4.3 Standardised NADH solution (nominally 5mg/ml). (To be determined for each batch number of NADH).

4.3.1 Prepare stock NADH solution (10mg/ml) as follows:-

Accurately weigh 250mg (± 1mg) Nicotinamide-adenine dinucleotide (reduced), disodium salt.

($C_{21}H_{27}N_7O_{14}P_2Na_2+H_2O$; BDH Chemicals Ltd.,Poole, Dorset, England; Product 10804 ONLY; REAGENT MUST BE STORED UNDER STRICT ANHYDROUS CONDITIONS).

Transfer quantitatively to a 25ml volumetric flask with water. Swirl to dissolve. Make up to the mark with water, stopper and invert several times to mix thoroughly.

The solution can be stored under refrigeration (less than 5°C) for several days.

4.3.2 Prepare intermediate 5mg/ml NADH solution as follows:-

To 1.0ml of stock NADH solution (10mg/ml) (**4.3.1**) in a stopperable glass tube (**5.6**), add 1.0ml water and mix thoroughly.

4.3.3 Standardisation procedure:-

To a 10mm silica or glass spectrophotometer cell (**5.3**) add the following reagents:-

2.75ml phosphate buffer (**4.1**);
0.20ml (200 microlitres) EDTA disodium salt solution (**4.2**)

Place the cell in a thermostat controlled water bath (**5.2**) maintained at 37°C and allow cell contents to attain 37°C.

Add 0.05ml (50 microlitres) of intermediate 5mg/ml NADH solution (**4.3.2**). Stopper the cell, invert several times to mix and quickly place in cell holder (maintained at 37°C) of the U.V/Visible spectrophotometer (**5.1**).

Ensure the absence of air bubbles.

Measure the absorbance (extinction) of the cell contents at 37°C at 340nm against air.

The required absorbance of the cell contents under the above conditions is 0.720.

Calculation example:-

e.g. Extinction of cell contents = 0.652

∴ required concentration of NADH solution to give 0.720 is given by :-

$$\frac{0.720 \times 5}{0.652} = 5.52\text{mg/ml}$$

This concentration will be provided by mixing together:-

$$\frac{2 \times 5.52}{2 \times 5.00} = 1.104\text{ml stock NADH (4.3.1)}$$

and

$$(2 - 1.104) = 0.896\text{ml water.}$$

Stored under refrigeration (less than 5°C) this solution is stable for several days.

4.4 Acetoacetyl-CoA solution (5mg/ml).

4.4.1 Accurately weigh 5.0mg Acetoacetyl Coenzyme-A, sodium salt (Sigma Chemical Company; Product No. A-1625, stored desiccated below 0°C) to a glass tube (**5.6**)

Add 1.0ml water, swirl to dissolve, stopper and mix thoroughly. This volume will be sufficient for determinations on at least 19 samples.

Stored under refrigeration (less than 5°C) this solution is stable for several days.

(*note: On receipt of Acetoacetyl Coenzyme-A, sodium salt, it is advisable to divide the material into accurately weighed 5mg portions ready for later use*).

5. Apparatus

5.1 U.V./Visible spectrophotometer, capable of constant temperature control of the cell holder at 37°C.

5.2 Thermostatically controlled water bath suitable for use at 37°C ± 0.5°C.

5.3 Silica or glass cells, path length 10mm, e.g. from Hellma (England) Ltd.

5.4 Cast Iron Press (347195; W.H.Smith, Do-it-All by Victor Cast Ware Ltd.), with either porcelain or plastic dish and approximately 2cm thick rigid plastic disc insert; or equivalent alternative.

5.5 Pipettes or syringes capable of accurate delivery of the following volumes :-

2.60 millilitres (ml);
2.75 millilitres (ml);
200 microlitres (µl);
100 microlitres (µl);
50 microlitres (µl);

5.6 Stoppered glass tubes (capacity >2ml).

5.7 Refrigerator capable of maintaining temperature of < 5°C.

5.8 Volumetric flasks 20ml (Grade B).

5.9 Stop Watch.

5.10 Scalpel (holder and disposable blades).

6. Procedure

6.1 The chicken breast (from one side of a chicken carcass) is cut with a scalpel, transversely rather than longitudinally, to produce two halves to be labelled (a) and (b). Each of the halves are processed separately and as follows:-

6.2 Place the flesh centrally in the porcelain or plastic dish of the cast iron press (**5.4**) or equivalent alternative. Place the metal plate on top of the flesh. Lower the piston screw by rotating the handle until the latter is "hand tight". (This presses on the metal and "squashes" the sample beneath to produce "press juice").

Leave for approximately 5 minutes to allow press juice to accumulate in the base of the dish.

6.3 Transfer the press juice to an appropriately labelled stopperable glass tube (**5.6**) by means of a disposable Pasteur pipette.

A minimum volume of 0.5ml is required.

(If the volume collected is insufficient then repeat step **6.2**).

The press juice can be stored under refrigeration (less than 5°C) for a maximum of four days if necessary.

6.4 Transfer 100 microlitres (l) of the press juice into a 20 ml volumetric flask (**5.8**). Make up to the mark with phosphate buffer 0.1M (**4.1**), stopper and invert several times to mix thoroughly.

6.5 To a 10mm spectrophotometer cell (**5.3**) placed in a thermostatted water bath (**5.2**) maintained at 37°C add the following:-

2.60ml phosphate buffer 0.1M (**4.1**);
200µl (microlitres) EDTA (disodium salt) solution (**4.2**);
100µl (microlitres) diluted press juice;

Allow the cell contents to attain 37°C then add :-

50µl (microlitres) standardised NADH solution (**4.3**)

Place a stopper on the cell and invert several times to mix the contents.

Dry the cell faces quickly with a tissue.

6.6 Place the cell in the cell holder (at 37°C) of the U.V./Visible spectrophotometer (**5.1**).

6.7 Add 50µl (microlitres) Acetoacetyl-CoA solution (**4.4**) to the cell and mix to start the reaction. Ensure the absence of air bubbles.

6.8 Immediately measure the absorbance/extinction at 340nm (against air) and start the stop watch (**5.9**).

Leave the cell in the spectrophotometer.

6.9 After six minutes measure the absorbance/extinction again at 340nm.

The difference between the two readings ΔE is the decrease in absorption at 340nm, over a six minute reaction time.

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 Heatlth - Approved Code of Practice, Control of Substances Hazardous to Health Regulations, 1988) must be made before using this method.

8. Expression of Results

8.1 Formula and Method of Calculation

$$\textbf{HADH Activity (to nearest 0.1 U/ml)} = \frac{v \times \Delta E/\text{min} \times \text{dilution factor}}{-xdxa}$$

where:-

ΔE = the decrease in absorption at 340 nm, over a six minute reaction time.

v = volume of test mixture (3.0 ml)

- = extinction coefficient NADH 340 nm (**6.3**)

d = cell path length (1.0 cm)

a = volume of press juice dilution (0.1 ml)

$$\text{e.g. U/ml} = \frac{3 \times E \times 200}{6.3 \times 1 \times 0.1} = \Delta E \times 158.73$$

9. References

9.1 Billington, M. , Bowie, H., Scotter, S., Walker, H. and Wood, R. 1993. The differentiation of fresh and frozen-thawed poultry meat by the determination of the ß-hydoxyacyl-CoA-dehydrogenase (HADH) activity of chicken breast press juice:Collaborative trial. **J. Assoc. Public Anal. 1992, 28, 103-116**

APPENDIX 1

Analytical Quality Control

For summary of precision characteristics according to temperature of freezing, seeTable 1 below.

TABLE 1

Summary of precision characteristics of HADH method

Temp (°C)	Mean U/m	n	r	S_r	RSD_r %	R	S_R	RSD_R %
Fresh	5.8	8	2.6	0.93	15.9	6.6	2.36	40.6
+5	5.7	8	2.7	0.97	17.2	6.4	2.30	40.6
-6	8.2	8	4.1	1.48	18.2	8.8	3.14	38.5
-12	11.1	8	3.4	1.23	11.1	6.6	2.34	21.2
-18/20	22.7	8	13.2	4.70	20.7	13.2	4.70	20.7

Symbol	Definition
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