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DETERMINATION OF SYNTHETIC COAL-TAR DYES IN SOFT DRINKS, SKIMMED MILKS AND CAKES: COLLABORATIVE TRIAL

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The results of a collaborative trial, involving 9 laboratories testing a method for the determination of synthetic coal tar dyes in soft drinks, skimmed milks and cakes are reported. The method tested consisted of a quantitative extraction, as ion pairs with cetylpyridinium chloride, from aqueous solutions into butan-1-ol. The colouring materials were analysed using reversed phase, ion pair gradient elution HPLC with diode array detection.

The method was tested on Carmoisine (70.2, 93.8 mg/kg) Tartrazine (93.7,120.0 mg/kg) and Erythrosine (91.4, 118.8 mg/kg) in cake crumb; Carmoisine (97.4, 80.9 mg/kg) Tartrazine (91.7, 79.6 mg/kg) and Amaranth (80.5, 69.3 mg/kg) in skimmed milk; Tartrazine (85.8, 65.5 mg/kg), Amaranth (57.6, 64.1 mg/kg) and Red 2 G (49.4, 61.4 mg/kg) in lemonade. Analytical precision appeared unaffected by the chemical nature of the dye but was considerably influenced by the nature of the food matrix. Reproducibility for the lemonade sample was good with RSD_R values ranging from 5-9 %. For cake samples, the RSD_R values of 12-15% were considered satisfactory but for skimmed milk samples the RSD_R values of 17-25% were considered higher than desirable and resulted in Horrat values of 2-3.

INTRODUCTION

In most countries of the world the coloration of food is regulated by Government statute. This is usually achieved by the publication of permitted lists and purity specifications for each colorant and sometimes by the limitation of permitted colorant levels in certain food products.

Safety in use of food additives is constantly under review, with colouring matter particularly, the subject of close scrutiny. In the United Kingdom, the process of harmonisation of the relevant food colouring legislation with that of the European Economic Community was completed with the implementation of the latest amendment (94/36/EC) to Council Directive (89/107/EEC) concerning food additives authorised for use in foodstuffs intended for human consumption.

The latest amendment[1] lists 43 E numbers which cover permitted food colours. About one quarter of the colours are permitted for use in defined foods only, with the permitted maximum level specified for each colour. The colours include Amaranth, Erythrosine and Red 2 G.

Other colouring matters, including Tartrazine, are permitted to be used singly or in combination up to a maximum level of 100 mg/kg or mg/L in specified food items. However, for non alcoholic drinks, edible ices, desserts and fine bakery wares (confectionery) certain colours including Carmoisine and Sunset Yellow may not exceed 50 mg/kg or 50 mg/L.

Methods of analysis of synthetic food colours invariably involve aqueous ion pair extraction and then back extraction into organic solvent followed by clean up using column chromatography. Determination may be qualitative (TLC; colorimetry) or quantitative (TLC with densitometric detection; HPLC). Most HPLC methods employ reversed phase ion pair chromatography using gradient elution to effect a separation of the different colouring materials[2-8].

Many of these methods are concerned with the identification of individual dyes in mixtures of food dyes. In view of the desire to restrict the usage of synthetic food dyes there is still a shortage of published, official analytical methodology for the analysis of foods, particularly within the EC.

There is a clear need for a validated method to detect synthetic 'coal tar' dyes in foods. The Food Science Laboratory has therefore collaboratively tested a method as part of the MAFF collaborative trial programme. The method tested here identifies and quantifies a range of synthetic 'coal tar' dyes when present in soft drinks, skimmed milk and cakes. Fat is removed from cake samples and enzyme digestion is used to release any colours bound during processing. This facilitates the formation of an aqueous extract of the cake sample which can then be treated in the same way as the soft drink and skimmed milk samples. The colouring materials are then extracted from aqueous solution, as ion pairs, into butan-1-ol. They

can then be determined using reversed phase, ion pair HPLC with diode array detection.

METHOD OF ANALYSIS COLLABORATIVELY TESTED

The method tested in this trial is a quantitative extraction from aqueous solutions into butan-1-ol as ion pairs with cetylpyridinium chloride. The colouring materials are analysed using reversed phase, ion pair gradient elution HPLC with diode array detection. The method was developed by Avon County Scientific Services and is detailed in Appendix I; it was amended as described in Appendix II following the pre-trial.

COLLABORATIVE TRIAL ORGANISATION AND SAMPLES

Ten laboratories participated in the pre-trial, and of these, nine participated in the trial proper; all were UK Public Analyst Laboratories. In the pre-trial, participants were asked to analyse two samples (PTi and PTii) using the method described in Appendix I. For the main trial, the laboratories received three pairs of samples containing various synthetic colouring materials. One of the pairs was a lemonade sample (Q and R); one pair was a skimmed milk (J and X) and the other pair, a dried sieved cake crumb sample (A and D). Each pair contained the same dyes at different concentrations (i.e. split levels). Comments received from participants at the pre-trial stage were taken into account and revisions to the method for use in the main trial are detailed in Appendix II. Comments from participants on both the pre-trial and the main trial are detailed in Appendix III.

Samples

All samples were prepared by Avon County (now Bristol City Council) Scientific Services and are described in the table below:

Composition of samples used in the collaborative trial

SAMPLE TYPE	SAMPLE CODE	ANALYTE ADDED mg/kg					
		SUNSET YELLOW	TARTRAZINE	CARMOISINE	RED 2 G	AMARANTH	ERYTHROSINE
LEMONADE	PTi	25.0	25.0	25.0	25.2	25.0	
LEMONADE	PTii	35.0	35.2	35.1	35.1	35.0	
LEMONADE	Q		85.8		49.4	53.6	
LEMONADE	R		65.5		61.4	64.1	
SKIMMED MILK	J		91.7	97.4		80.5	
SKIMMED MILK	X		79.6	50.9		69.3	
CAKE CRUMB	A		93.7	70.2			91.4
CAKE CRUMB	D		120.0	93.8			118.8

Their homogeneity was checked by performing up to six replicate analyses and the results are tabulated in Appendix IV. The CVs measured for the pre-trial were rather variable ranging from 4.6-13.1%. The CV's for the main trial were considerably better ranging from 1-5.6%. These data were considered to demonstrate sufficient homogeneity for the trial to be undertaken.

THE TRIAL RESULTS AND THEIR STATISTICAL ANALYSIS

Tables I - XIV give the results for specific dyes in the matrices used in both the pre-trial and main trial. The results were examined for evidence of individual systematic error using single Grubbs and double Grubbs test progressively. These outliers are identified in Tables I-XIV by "(G)". Tables XV-XVIII summarise the findings for each food matrix; lemonade pre-trial, main trial lemonade, skimmed milk and cake crumb respectively.

Repeatability and Reproducibility

Calculations for repeatability (r) and reproducibility (R) as defined in the internationally agreed Protocol for the Design, Conduct and Interpretation of Collaborative Studies[9], were carried out on those results remaining after removal of outliers. The resulting values are included in Tables I - XVIII.

Horwitz Predicted Precision Parameters

There is often no validated reference or statutory method with which to compare precision criteria when assessing a new method. It is therefore useful to compare the precision data obtained from a collaborative trial with acceptable levels of precision predicted by the Horwitz equation. These give an indication as to whether the method is sufficiently precise taking into account the concentration of analyte being measured.

The Horwitz predicted value is calculated from the Horwitz equation[10]:

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

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

Horrat Values (Ho)

The Horrat values[11] 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:

$Ho_R = RSD_R$ (measured)/RSD_R (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) = 0.66 RSD_R (Horwitz). This assumes the approximation r = 0.66R. The Horwitz values calculated from the results of this trial are given in Tables I - XIV and have been summarised in Tables XV - XVIII.

DISCUSSION

Precision

A summary of the pre-trial results, obtained using a lemonade matrix, is shown in Table XV. Ho_R values ranged between 0.48 and 1.3 for the five colours analysed and demonstrated that the method was performing satisfactorily for all analytes. Nevertheless, some laboratories reported having difficulties with the HPLC analysis. It was not considered appropriate to modify this aspect of the method since it was anticipated that analysts would benefit from the experience gained in the pre-trial. However other comments were taken into account and the method modified as detailed in Appendix II before analysis of the trial samples proper.

The main trial again demonstrated the applicability of the method for analysis of colours in lemonade (summary Table XVI). The maximum Horrat value observed was 1.07 (Ho_R Amaranth) thus demonstrating that the precision was within acceptable limits for this type of analysis. The repeatability, reproducibility and associated Horrat values showed good agreement with those obtained in the pre-trial.

The analysis of the skimmed milk samples is summarised in Table XVII. The method used was identical to that adopted for the lemonade sample and two of the same colours, Amaranth and Tartrazine, were analysed. However the precision proved considerably poorer for this more complex matrix. All the Horrat values were above 2 thus indicating that the variability of results was greater than would be expected for a method measuring analyte at ppm concentrations.

Data for the cake crumb mixture are summarised in Table XVIII. Their analysis used a rather more thorough extraction technique than that adopted for the skimmed milk samples. Of the dyes investigated, Carmoisine had also been measured previously in the lemonade pre-trial and Tartrazine was measured in the skimmed milk samples but Erythrosine was only analysed in the cake matrix. The precision (RSD_R 15.1%) for Tartrazine was considerably worse than that for the lemonade sample (RSD_R 5.4%) but slightly better than that of the skimmed milk samples (RSD_R 17.3%). This trend was also evident for Carmoisine where the pre-trial lemonade sample (RSD_R 6.9%) showed better precision than the cake crumb (RSD_R 12.4%) but the precision found for the skimmed milk sample was poorer (RSD_R 24.6%).

Although the precision found for all the dyes in the cake sample was worse than for those in the lemonade, the Ho_R values found for all three dyes in the cake sample were below 2 and hence the method can be considered satisfactory for a cake matrix. These values proved remarkably similar, ranging from 1.44 for Carmoisine to 1.82 for Tartrazine. Indeed it was generally the case in this trial that the precision obtained with the method, for the many dyes studied, seems to depend almost completely on the food matrix from which they were analysed with the chemical structure of the analyte evidently having little influence.

Evaluation of the trial

The precision characteristics have demonstrated that the method tested is acceptable for lemonade and cake crumb matrices. Nevertheless, comments from participants indicate that the method, as specified, is not particularly easy to follow. Some themes recur in the comments and it may be advantageous to take these comments into account in future use of the method. A difficulty was identified by laboratories 4, and 7 when butan-1-ol extracts were diluted with mobile phase prior to injection. This may also have contributed to the split peaks reported by laboratories 5 and 10. The problem was overcome by laboratory 4 by a further dilution of the butan-1-ol with mobile phase to make 25% butan-1-ol at injection.

Similarly, a number of laboratories expressed dissatisfaction with the chromatographic conditions employed or reported problems which might have resulted from unsatisfactory chromatography. These included laboratories 3,4,7,8,9 and 10. Ion pairing HPLC does not provide the most straightforward of chromatographic methods. The presence of the ion

pairing reagent can prevent a convenient degassing of the mobile phase because of froth formation - particularly in commonly used helium sparging systems. Considerable difficulties can also be encountered in column re-equilibration at the start of a chromatographic run. The interaction between ion pair reagent and column stationary phase is much slower than for typical solvent mixtures and may require an hour where 10 minutes would be sufficient for a normal or reverse phase separation[12] These equilibria are also temperature sensitive so that column temperature control may also aid stability. It is also considered advisable to dedicate a column to ion pair work because of the difficulty of achieving complete removal of the ion pair reagent by solvent rinse. Since ion pairing is essentially a dynamic ion exchange system, it may be that the use of an ion exchange solid phase extraction followed by ion exchange HPLC might provide a more robust chromatographic system.

Although the majority of comments referred to chromatographic problems, it seems unlikely that these would have led to the poor repeatability encountered for the skimmed milk samples. The statistical evaluation indicated that the sample matrix was the major contributing factor to the variability of results. Lemonade samples which were relatively easy to extract showed good repeatability so that sound chromatography for these samples may be assumed. Cake samples had extra steps added to facilitate extraction of the colours and the results from this matrix were satisfactory. The major difficulty arose from the skimmed milk samples. These did not have any special treatment and were treated like liquid samples. Only one comment from participants referred to difficulties with extraction of the skimmed milks and this was as a result of emulsion formation (laboratory 9). It may be that adopting the full extraction procedure applied to cakes would provide a sufficiently precise method for skimmed milks. Certainly there are practical difficulties in following the current procedure.

Trueness

The trial was not set up to evaluate the trueness of the method. However some comments suggest that residual colour was left after extraction of the cake samples. Further evaluation of whether this truly represented a failure to extract the dye or whether there was some other cause for this observation may be appropriate. Efforts to confirm the accuracy of the method may be beneficial.

CONCLUSION

The results of the trial demonstrated successful validation of this HPLC method for the analysis of soft drinks and cakes but further method development is appropriate for the analysis of skimmed milk.

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TABLE I

AMARANTH CONTENT mg/kg

PRE TRIAL SAMPLES

(PTi, 25.0 mg/kg and PTii, 35.0 mg/kg)

	SAMPLE	
LABORATORY	PTi	PTii
1	26	36
2	28[G]	18 [G]
3	21	32
4	24	34
5	24	34
6	25	39
7	24	34
8	26	37
9	26	38
10	24	34
MEAN	24.4	35.3
r	2.70	
S_r	0.97	
RSD_r	3.23	
Ho_r	0.51	
R	5.52	
S_R	1.97	
RSD_R	6.6	
Ho_R	0.69	

TABLE II
CARMOISINE CONTENT
PRE TRIAL SAMPLES
(PTi, 25.0 mg/kg and PTii, 35.1 mg/kg)

	SAMPLE	
LABORATORY	PTi	PTii
1	24	34
2	20	33
3	24	34
4	25	35
5	24	35
6	27	35
7	24	33
8	27	37
9	24	40
10	26	35
MEAN	24.5	35.1
r	4.59	
S_r	1.64	
RSD_r	5.50	
Ho_r	0.87	
R	5.73	
S_R	2.05	
RSD_R	6.87	
Ho_R	0.72	

TABLE III

RED 2 G CONTENT mg/kg

PRE TRIAL SAMPLES

(PTi, 25.2 mg/kg and PTii, 35.1 mg/kg)

	SAMPLE	
LABORATORY	PTi	PTii
1	23	33
2	16	29
3	22	32
4	23	32
5	22	31
6	22	36
7	23	32
8	26	35
9	24	35
10	26	30
MEAN	22.7	32.5
r	5.35	
S_r	1.91	
RSD_r	6.92	
Ho_r	1.08	
R	7.13	
S_R	2.55	
RSD_R	9.22	
Ho_R	0.95	

TABLE IV
SUNSET YELLOW CONTENT mg/kg
PRE TRIAL SAMPLES
(PTi, 25.0 mg/kg and PTii, 35.0 mg/kg)

	SAMPLE	
LABORATORY	PTi	PTii
1	21	31
2	18	33
3	23	33
4	21	31
5	25	35
6	33 (G)	39 (G)
7	24	33
8	28	39
9	23	36
10	23	34
MEAN	23.9	34.4
r	4.69	
S_r	1.67	
RSD_r	5.7	
Ho_r	0.9	
R	10.0	
S_R	3.57	
RSD_R	12.2	
Ho_R	1.3	

TABLE V
TARTRAZINE CONTENT mg/kg
PRE TRIAL SAMPLES
(PTi, 25.0 mg/kg and PTii, 35.2 mg/kg)

	SAMPLE	
LABORATORY	PTi	PTii
1	24	34
2	26	35
3	23	33
4	25	35
5	24	35
6	$32^{(G)}$	$49^{(G)}$
7	23	34
8	$31^{(G)}$	$44^{(G)}$
9	27	37
10	24	33
MEAN	24.5	34.5
r	1.5	
S_r	0.54	
RSD_r	1.81	
Ho_r	0.29	
R	3.82	
S_R	1.36	
RSD_R	9.61	
Ho_R	0.48	

TABLE VI
CARMOISINE CONTENT mg/kg
CAKE CRUMB
(SAMPLE A, 70.2 mg/kg and SAMPLE D, 93.8 mg/kg)

	SAMPLE	
LABORATORY	A	D
1	51.9	75.6
2	59.6	87.8
3	53.5	80.0
4	46.8	60.2
5	55.0	76.6
6	50.0	64.0
7	57.4	75.1
8	39.0	62.5
9	50.3	73.3
MEAN	51.5	72.8
r	10.31	
S_r	3.68	
RSD_r	5.92	
Ho_r	1.04	
R	21.53	
S_R	7.69	
RSD_R	12.37	
Ho_R	1.44	

TABLE VII

TARTRAZINE CONTENT mg/kg

CAKE CRUMB

(SAMPLE A, 93.7 mg/kg and SAMPLE D, 120.0 mg/kg)

	CAKE CRUMB	
LABORATORY	A	D
1	77.7	92.8
2	88.0	113.2
3	59.9	77.3
4	56.0	67.4
5	72.9	93.7
6	84.0	99.0
7	67.8	90.8
8	65.6	87.6
9	62.2	83.5
MEAN	70.5	89.5
r	8.95	
S_r	3.2	
RSD_r	4	
Ho_r	0.73	
R	33.77	
S_R	12.06	
RSD_R	15.08	
Ho_R	1.82	

TABLE VIII
ERYTHROSINE CONTENT mg/kg
CAKE CRUMB
(SAMPLE A, 91.4 mg/kg and SAMPLE D, 118.8 mg/kg)

	CAKE CRUMB	
LABORATORY	A	D
1	75.4	112.0
2	76.8	117.0
3	67.6	93.8
4	56.2[(G)]	72.1[(G)]
5	74.7	101.1
6	63.0	85.0
7	70.4	97.4
8	0.0[(G)]	40.6[(G)]
9	62.7	91.5
MEAN	68.4	96.2
r	15.20	
S_r	5.43	
RSD_r	6.60	
Ho_r	1.21	
R	31.89	
S_R	11.39	
RSD_R	13.84	
Ho_R	1.68	

TABLE IX
CARMOISINE CONTENT mg/kg
SKIMMED MILK

(SAMPLE J, 97.4 mg/kg and SAMPLE X, 80.9 mg/kg)

	SKIMMED MILK	
LABORATORY	J	X
1	97.5	82.9
2	93.6	82.6
3	85.1	61.9
4	45.6	70.0
5	86.4	74.7
6	98.0	136.0
7	100.5	83.6
8	62.8	64.2
9	90.3	73.8
MEAN	84.4	81.1
r	41.46	
S_r	14.81	
RSD_r	17.89	
Ho_r	3.29	
R	56.91	
S_R	20.32	
RSD_R	24.56	
Ho_R	2.98	

TABLE X
TARTRAZINE CONTENT mg/kg
SKIMMED MILK
(SAMPLE J, 91.7 mg/kg and SAMPLE X, 79.6 mg/kg)

	SKIMMED MILK	
LABORATORY	J	X
1	81.2	71.4
2	78.6	77.4
3	85.1	61.9
4	65.5	67.5
5	79.2	71.9
6	76.0	32.0
7	88.5	78.6
8	57.2	60.7
9	86.6	74.1
MEAN	77.5	66.2
r	29.12	
S_r	10.4	
RSD_r	14.47	
Ho_r	2.61	
R	34.71	
S_R	12.4	
RSD_R	17.25	
Ho_R	2.05	

TABLE XI

AMARANTH CONTENT mg/kg

SKIMMED MILK

(SAMPLE J, 80.5 mg/kg and SAMPLE X, 69.3 mg/kg)

	SKIMMED MILK	
LABORATORY	J	X
1	82.2	68.9
2	69.8	67.6
3	61.0	46.5
4	34.5	57.3
5	71.8	64.6
6	76.0	93.0
7	81.4	70.4
8	50.6	51.7
9	76.9	63.7
MEAN	67.1	64.9
r	27.13	
S_r	9.69	
RSD_r	14.68	
Ho_r	2.61	
R	40.96	
S_R	14.63	
RSD_R	22.17	
Ho_R	2.6	

TABLE XII
TARTRAZINE CONTENT mg/kg
LEMONADE
(SAMPLE Q, 85.8 mg/kg and SAMPLE R, 65.5 mg/kg)

	LEMONADE	
LABORATORY	Q	R
1	78.4	59.6
2	83.1	65.2
3	85.5	66.5
4	72.9	55.9
5	85.2	65.5
6	83.0	63.0
7	83.7	63.1
8	85.1	63.1
9	88.8[(G)]	60.5 [(G)]
MEAN	82.1	62.7
r	3.1	
S_r	1.11	
RSD_r	1.53	
Ho_r	0.28	
R	11.04	
S_R	3.94	
RSD_R	5.44	
Ho_R	0.65	

TABLE XIII

AMARANTH CONTENT mg/kg

LEMONADE

(SAMPLE Q, 53.6 mg/kg and SAMPLE R, 64.1 mg/kg)

	LEMONADE	
LABORATORY	Q	R
1	49.5	62.1
2	52.3	69.7
3	53.6	65.9
4	43.2	56.3
5	52.9	65.5
6	41.0	51.0
7	53.4	64.5
8	49.1	59.4
9	49.3	57.6
MEAN	49.4	61.3
r	5.08	
S_r	1.81	
RSD_r	3.27	
Ho_r	0.57	
R	14.55	
S_R	5.2	
RSD_R	9.39	
Ho_R	1.07	

TABLE XIV
RED 2 G CONTENT mg/kg
LEMONADE

(SAMPLE Q, 49.4 mg/kg and SAMPLE R, 61.4 mg/kg)

	LEMONADE	
LABORATORY	Q	R
1	43.9	55.9
2	44.6[(G)]	61.1[(G)]
3	48.6	60.8
4	39.9	53.2
5	46.8	59.7
6	46.0	57.0
7	48.7	60.3
8	49.5	61.1
9	45.1[(G)]	50.4[(G)]
MEAN	46.2	58.3
r	1.58	
S_r	0.56	
RSD_r	1.08	
Ho_r	0.19	
R	8.92	
S_R	3.19	
RSD_R	6.10	
Ho_R	0.69	

TABLE XV

SUMMARY OF CALCULATED STATISTICAL PARAMETERS FOR LEMONADE SAMPLES

PRE-TRIAL SAMPLES

	ANALYTE									
	AMARANTH		CARMOISINE		RED 2 G		SUNSET YELLOW		TARTRAZINE	
	PTi	PTii	PTi	PTii	PTi	PTii	PTi	PTii	PTi	PTii
MEAN (mg/kg)	24.4	35.3	24.5	35.1	22.7	32.5	23.9	34.4	24.5	34.5
n	9		10		10		10		8	
r	2.70		4.59		5.35		4.69		1.5	
S_r	0.97		1.64		1.91		1.67		0.54	
RSD_r	3.23		5.50		6.92		5.7		1.81	
Ho_r	0.51		0.87		1.08		0.9		0.29	
R	5.52		5.73		7.13		10.0		3.82	
S_R	1.97		2.05		2.55		3.57		1.36	
RSD_R	6.6		6.87		9.22		12.2		9.61	
Ho_R	0.69		0.72		0.95		1.3		0.48	

TABLE XVI
SUMMARY OF CALCULATED STATISTICAL PARAMETERS FOR LEMONADE SAMPLES
MAIN TRIAL

	ANALYTE					
	TARTRAZINE		AMARANTH		RED 2 G	
	Q	R	Q	R	Q	R
MEAN (mg/kg)	82.1	62.7	49.4	61.3	46.2	58.3
n	8		9		9	
r	3.1		5.08		1.58	
S_r	1.11		1.81		0.56	
RSD_r	1.53		3.27		1.08	
Ho_r	0.28		0.57		0.19	
R	11.04		14.55		8.92	
S_R	3.94		5.2		3.19	
RSD_R	5.44		9.39		6.10	
Ho_R	0.65		1.07		0.69	

TABLE XVII
SUMMARY OF CALCULATED STATISTICAL PARAMETERS FOR SKIMMED MILK SAMPLES
MAIN TRIAL

	ANALYTE					
	CARMOISINE		TARTRAZINE		AMARANTH	
	J	X	J	X	J	X
MEAN (mg/kg)	84.4	81.1	77.5	66.2	67.1	64.9
n	9		9		9	
r	41.46		29.12		27.13	
S_r	14.81		10.4		9.69	
RSD_r	17.89		14.47		14.68	
Ho_r	3.29		2.61		2.61	
R	56.91		34.71		40.96	
S_R	20.32		12.4		14.63	
RSD_R	24.56		17.25		22.17	
Ho_R	2.98		2.05		2.6	

TABLE XVIII
SUMMARY OF CALCULATED STATISTICAL PARAMETERS FOR CAKE CRUMB SAMPLES
MAIN TRIAL

	ANALYTE					
	CARMOISINE		TARTRAZINE		ERYTHROSINE	
	A	D	A	D	A	D
MEAN (mg/kg)	51.5	72.8	70.5	89.5	68.4	96.2
n	9		9		8	
r	10.31		8.95		15.2	
S_r	3.68		3.2		5.43	
RSD_r	5.92		4		6.6	
Ho_r	1.04		0.73		1.21	
R	21.53		33.77		31.89	
S_R	7.69		12.06		11.39	
RSD_R	12.37		15.08		13.84	
Ho_R	1.44		1.82		1.68	

KEY TO TABLES I TO XVIII

(G)	Outlier identified using Grubbs test.
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 ($S_r \times 100$/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

METHOD FOR THE DETERMINATION OF SYNTHETIC COAL-TAR DYES IN SOFT DRINKS, SKIMMED MILKS AND CAKES

WARNING 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 AND FIELD OF APPLICATION

The method identifies and quantifies the following range of synthetic colouring materials when present in soft drinks, skimmed milks and cakes:

Amaranth

Carmoisine

Erythrosine

Red 2 G

Sunset Yellow

Tartrazine

2. DEFINITION

Synthetic colouring materials: any substance which is capable of and is used for colouring food, other than those substances occurring naturally.

3. PRINCIPLE

The colouring materials are quantitatively extracted from aqueous solutions into butan-1-ol as ion-pairs with cetylpyridinium chloride. The colouring material is analysed using reverse-phase, ion-pair, HPLC with UV detection.

4. REAGENTS

All reagents should be of recognised analytical grade unless otherwise specified. Distilled water or water of an equivalent purity is to be used.

4.1 Butan-1-ol, water-saturated.

Shake 500 mL of butan-1-ol (**4.1.1**.) with an excess of water and separate the water saturated butan-1-ol from the water.

4.1.1 ***Butan-1-ol***

4.2 Cetylpyridinium chloride(CPC), 0.1 mol/L in water.

Weigh 35.80g of cetylpyridinium chloride (**4.2.1**) and dissolve in 500 mL of water in a 1 L beaker, warm slightly to dissolve if necessary. Transfer to a 1 L volumetric flask and make up to volume with water.

4.2.1 ***Cetylpyridinium chloride monohydrate****, 98% purity*

4.3 Phosphate buffer, 0.05 mol/L, pH 7.0

Dissolve 6.805 g potassium *di*-hydrogen *ortho*-phosphate in 500 mL water and adjust to pH 7.0 with 1.0 mol/L potassium hydroxide solution (**4.5**). (Approximately 30 mL of 1 mol/L potassium hydroxide solution is required.) Make to 1 L in a volumetric flask with water.

4.3.1 ***Potassium di-hydrogen ortho-phosphate***

4.4 Phosphate buffer, 0.1 mol/L, pH 7.5

Dissolve 13.610 g potassium *di*-hydrogen *ortho*-phosphate in 500 mL water and adjust to pH 7.5 with 1.0 mol/L potassium hydroxide solution (**4.5**). (Approximately 80 ml of 1 mol/L potassium hydroxide solution is required.) Make to 1 L in a volumetric flask with water.

4.5 Potassium hydroxide, 1.0 mol/L

Weigh accurately 56.1 g of potassium hydroxide and dissolve in 100 mL of water and cool. Then make to 1 L in a volumetric flask.

4.5.1 ***Potassium hydroxide****,* pellets

4.6 Sodium hydroxide, 0.1 mol/L

Weigh accurately 4.00 g of sodium hydroxide and dissolve in 100 mL of water and cool. Then make to 1 L in a volumetric flask.

4.6.1 ***Sodium hydroxide****,* pellets

4.7 Mobile phase

4.7.1 ***Solution A***

Phosphate buffer (**4.4**) and water are diluted 50 mL + 850 mL, and this solution is de-gassed. To the de-gassed solution, 50 mL of cetylpyridinium chloride solution (**4.2**) is added and the final solution made to 1 L in a volumetric flask. The solution is de-gassed before the addition of cetylpyridinium chloride solution (**4.2**) to avoid frothing.

4.7.2 ***Solution B***

Cetylpyridinium chloride solution (**4.2**) is diluted 50 ml → 1 L with a 1 L + 1 L dilution of acetonitrile and methanol (**4.10**).

4.8 Standard colour solutions

Dissolve 50 mg of dye in 100 mL water. Dilute 5 mL of the aqueous standard and 2 ml of 0.1 mol/L cetylpyridinium chloride in water (**4.2**) to 50 mL with butan-1-ol (**4.1**). Shake vigorously, then dilute with a solution of Solution A (**4.7.1**) and Solution B (**4.7.2**) diluted 1 L + 1 L. Mixed standards can be prepared when the component peaks of each individual dye have been identified.

4.9 α-Amylase, eg BDH, bacterial source, about 40 Eu per mg, contains about 50% reducing sugars.

4.10 Acetonitrile-methanol solution

Acetonitrile and methanol are diluted 500 mL + 500 mL.

4.10.1 *Acetonitrile*

4.10.2 *Methanol*

4.11 Petroleum spirit, 40-60

5. APPARATUS

5.1 Laboratory Glassware

5.1.1 ***Beakers***, *25 mL*

5.1.2 ***Centrifuge***

5.1.3 ***Centrifuge tubes***, glass, screw-cap, 55 mL

5.1.4 ***Filters***, glass, microfibre, Whatman GF/A 70 mm

5.1.5 Flasks, glass, volumetric, 25 mL

5.1.6 Pipettes, glass, pasteur, disposable

5.1.7 ***pH meter***

5.1.8 ***Shaker mechanical***

5.1.9 ***Water bath***

5.2 HPLC Equipment

5.2.1 ***Pumps***, capable of delivering solvent at 1.5 mL per minute.

5.2.2 ***Binary Gradient Controller***, capable of linear gradient.

5.2.3 ***Injection valve***, fitted with 20 µL loop.

5.2.4 Column, 250 × 4.6 mm Spherisorb C8 5 µm.

5.2.5 ***Guard column***, packed with 40 µm reverse phase material (eg Perisorb RP8 30-40 µm).

5.2.6 *Detector*

Diode-array detector, wavelength range 500 nm, bandwidth 200 nm or variable wavelength detector:

430 nm for yellow dyes

480 nm for orange dyes

520 nm for red dyes

5.2.7 *Mobile Phase Composition*

The sample is separated using a linear gradient. The composition of the mobile phase starts at 60% (*V/V*) Solution B (**4.7.2**) and 40% (*V/V*) Solution A (**4.7.1**) and ends at 80% (*V/V*) Solution B (4.7.2) and 20% (*V/V*) Solution A (**4.7.1**) after 20 min. The flow rate of the mobile phase is 1.5 mL/min. The gradient should be adjusted to produce optimum resolution of peaks of interest depending upon the age and condition of the column. (Allow 10 min at final conditions followed by a 10 min reverse and 10 min at initial conditions before injecting next sample).

6. PROCEDURE

6.1 Analysis of Soft Drinks and Skimmed milk

6.1.1 *Sample Preparation*

Accurately weigh 10 g of sample into a 25 mL beaker and adjust to pH 7.0 with 0.1 mol/L sodium hydroxide (**4.6**).

6.1.2 *Extraction*

Transfer neutralised sample to centrifuge tube. Rinse beaker and pH electrode with 2 × 5 mL portions of water and transfer washings to centrifuge tube (**5.1.3**). Add 5 mL 0.1 mol/L cetylpyridinium chloride in water (**4.2**), mix and add 10 mL of water-saturated butan-1-ol (**4.1**). Shake vigorously for 10 min on mechanical shaker. Centrifuge at 1000 g for 5 min and transfer the upper organic layer to a 25 mL volumetric flask using a Pasteur pipette. Repeat the procedure with three further 5 mL portions of water-saturated butan-1-ol (**4.1**).

Make the combined butan-1-ol extracts up to 25 mL with water-saturated butan-1-ol (**4.1**). Mix and filter a portion through a microfibre filter (**5.1.4**). Accurately dilute an aliquot of the filtrate with an equal volume of mobile phase (1 L + 1 L dilution of mobile phase Solution A (**4.7.1**) and Solution B (**4.7.2**)).

6.2 Analysis of Flour Based Products

6.2.1 *Sample Preparation*

Weigh accurately 5 g of sample into a 50 mL beaker. Defat the sample by stirring and decanting with 3 × 50 mL portions of petroleum spirit 40-60 (**4.11**) at no greater than 40°C. Discard petroleum spirit and air-dry the sample at ambient temperature under fume hood with occasional stirring.

6.2.2. *Extraction*

Transfer the air-dried, defatted sample to a centrifuge tube. Add 10 mL 0.05 mol/L phosphate buffer pH 7.0 (**4.3**). Add 100 mg α-amylase to the tube, shake vigorously to disperse the amylase and incubate at 40°C for 2 hr in a shaking water bath or by regular manual shaking. Add 5 mL cetylpyridinium chloride in water (**4.2**), mix and add 10 mL of water-saturated butan-1-ol (**4.1**). Shake vigorously on the mechanical shaker for 10 min followed by centrifugation at 1000 g for 10 min. If a gel forms in the upper organic layer, add 2 mL water-saturated butan-1-ol (**4.1**) and gently stir into upper layer, with glass rod, until emulsion breaks. Re-centrifuge. Transfer the upper organic phase by pasteur pipette to a 25 mL volumetric flask. Repeat the extraction procedure with three further 5 mL portions of water-saturated butan-1-ol (**4.1**). make the combined butan-1-ol extracts up to 25 mL with water-saturated butan-1-ol (**4.1**). Mix and filter a portion through a microfibre filter (**5.1.4**.). Accurately dilute an aliquot of the filtrate with an equal volume of mobile phase (1 L + 1 L dilution of mobile phase Solution A (**4.7.1**) and Solution B (**4.7.2**)).

6.3 Quantitative Determination

6.3.1 *HPLC*

Load 20 μL of standard/sample extract onto the column. Use gradient (linear) elution (**5.2.7**) to achieve optimum separation of the peaks of interest. Monitor the peak elution by selecting the appropriate wavelength (**5.2.6**).

7. EXPRESSION OF RESULTS

Calculate the concentration of colouring matter present in the extract by comparison of peak areas with standard dye solutions of known concentrations. The amount of colouring matter present in the sample can be calculated by applying the appropriate dilution factor.

Results should be expressed as mg of colouring material per kg.

APPENDIX II

AMENDMENTS TO METHOD MADE AFTER PRE-TRIAL SAMPLES ANALYSED

4.8 Standard colour solutions

Dissolve 50mg of the supplied dye(s) in 100mL of water. Take 5mL of this solution and 2mL of cetylpyridinium chloride solution (**4.2**) and make to volume in a 50mL volumetric flask with butan- 1- o1(**4.1.1**) Shake vigorously. To 10mL of this solution add 5mL of each of solutions A and B (**4.7.1** and **4.7.2**). Mix and filter through a filter (**5.1.4**).

Mixed standards can be prepared when the component peaks of each individual dye have been identified.

5.1.4 Filters, glass, microfibre, Whatman GF / A 70mm or a 0.45 μm eg Millipore Millex- HV.

6.1.2 Transfer neutralised sample to centrifuge tube. Rinse beaker and pH electrode with 2 × 5 mL portions of water and transfer washings to centrifuge tube (**5.1.3**). Add 5 mL 0.1 mol/L cetylpyridinium chloride in water (**4.2**), mix and add 10 mL of water- saturated butan- 1- ol (**4.1**).

Shake vigorously for 10 min on mechanical shaker. Centrifuge at 1000g for 5 min and transfer the upper organic layer to a 25 mL volumetric flask using a Pasteur pipette. Repeat the procedure with three further 5 ml portions of water- saturated butan- 1- ol (**4.1**).

Make the combined butan- 1- ol extracts up to 25 mL with water-saturated butan- 1- ol (**4.1**). Accurately dilute an aliquot of the filtrate with an equal volume of mobile phase (1 L + 1 L dilution of mobile phase solution A (**4.7.1**) and solution B (**4.7.2**)). Mix and filter a portion through a filter (**5.1.4**).

APPENDIX III

COMMENTS RECEIVED FROM PARTICIPANTS

Lab No 1

Pre-trial:

1. The use of water saturated butan-1-ol in the preparation of standards resulted in a two phase solution.
2. Standard and sample preparation differ in that samples are diluted 1 : 1 with mixed mobile phase but standards are not. Dilution of standard and sample in the same way would be recommended.
3. The method for soft drinks involves a dilution of effectively five fold. Given the absorbances of the final solutions it would seem advantageous to actually concentrate the colour from the sample rather than dilute thus achieving a final solution of more reasonable absorbance.

Lab No 3

Main Trial:

1. Colour was not completely extracted from cake samples (A & D).
2. Samples were chromatographed twice - once to quantify oranges and yellows, second time to quantify oranges and reds because a programmable wavelength detector was used - not diode array or equivalent.
3. Experienced poor resolution between analytes and variable retention times.

Lab No 4

Pre-trial:

1. Both mobile phases were filtered through 0.45 µm filters prior to use.
2. Mobile phase A was changed to 50 (acetonitrile/methanol) : 50 CPC pH 7.5 in order to reduce the possibility of dissolved gases releasing into the mobile phase, especially when mixing takes place. No helium sparge was available at that time.
3. A separating funnel was used to carry out the extractions instead of centrifuging the sample.
4. Chromatography problems were encountered when a aliquot of butanol was added to an equal volume of mobile phase: split peaks were produced for each colour component. The problem was overcome by making a further dilution 1 : 1 with the mobile phase (i.e. butanol content 25% by volume).

5. The HPLC column specified was found to be unsatisfactory with respect to the very high back pressure experienced at a flow rate of 1 mL/min, which caused problems with leaks with the autosampler, and also poor selectivity which resulted in poor resolution between the selected food colours.

   The chromatography was improved by the use of 100 mm × 4.6 mm ODS 5 μm column, which resulted in increased resolution between the respective colours and an affordable back pressure at 0.65 mL/min.

6. The samples were passed through a 0.45 μm filter prior to the HPLC analysis.

Main Trial:

1. Mobile phase A was changed to 50 (acetonitrile/methanol) : 50 CPC pH 7.5, due to the absence of a helium sparge.
2. Chromatography problems were encountered when a aliquot of butanol extract was added to an equal volume of mobile phase and then chromatographed, the problem was resolved by adding three volumes of mobile phase to one volume of butanol extract.
3. A Spherisorb C_8 5 μm 250 × 4.6 mm column was found to be unsatisfactory with respect to high back pressure and poor resolution. A 100 × 4.6 mm ODS2 5 μm column was found to be suitable with respect to resolution and affordable back pressure.
4. When a Nalgene (191 - 2045) 0.45 μm filter was used, due to the aggressive nature of the solvent, a yellow colouration was extracted which has a similar retention time to Sunset Yellow.

Lab No 5

Pre-trial:

1. Monitored at 450 and 520 nm instead of 430, 480, 520 nm.
2. Initially had a problem with split peaks for Sunset Yellow and Red 2 G, modified the mobile phase gradient slightly to rectify this.
3. The first dilution of Red 2 G in butan-1-ol did not give a completely clear solution but this cleared on further dilution.

Lab No 7

Main Trial:

1. The paragraph detailing preparation of standards is ambiguous.

2. Extra extractions with butan-1-ol were carried out on the flour based products as some colour remained after the four extractions suggested.

3. It was found that dilution of the butan-1-ol extract resulted in precipitation of the sample requiring further filtration.

Lab No 8

Main Trial:

1. Encountered problems with the chromatography of Erythrosine- sometimes no peak and at other times a very large peak, also variable retention times.

Lab No 9

Pre-trial:

1. The composition of the mobile phase differed in that the gradient used was 70% to 90% B instead of 60% to 80% B.
2. Calibration graphs of the peak area for the standards did not always pass through the origin and a different result is obtained depending on whether the calculation is based on the standard of concentration above or below the sample concentration.
3. An emulsion was formed during the extraction when using water saturated butan-1-ol, this was overcome by using butan-1-ol without pretreatment.

Main Trial:

Samples J and X (skimmed milk) formed emulsions after the initial extraction, the emulsion was washed with 2 mL aliquots of solvent and recentrifuged until the final volume was reached

Lab No 10

Pre-trial:

Experienced split peaks for standards.

APPENDIX IV
TEST MATERIAL HOMOGENEITY RESULTS

a: Pre-Trial Samples

PT i	1	2	3	4	5	6	Mean	Std. Devn.	C.V %
Sunset Yellow	27.3	27.7	2.46	26.7	-	-	26.6	1.4	5.2
Red 2 G	27.5	25.1	23.1	24.4	-	-	25.0	1.9	7.4
Tartrazine	27.5	26.9	24.2	25.8	-	-	26.1	1.5	5.6
Amaranth	29.1	25.9	22.9	24.7	-	-	25.1	2.6	10.2
Carmoisine	29.3	27.6	21.7	24.5	-	-	25.8	3.4	13.1

PT ii	1	2	3	4	5	6	Mean	Std. Devn.	C.V %
Sunset Yellow	37.8	40.0	37.0	42.1	41.2	39.1	39.5	2.0	5.0
Red 2 G	36.3	37.6	34.3	38.6	38.6	35.8	36.9	1.7	4.6
Tartrazine	37.5	38.8	35.6	41.6	41.6	38.0	38.9	2.4	6.1
Amaranth	38.2	38.8	35.4	40.6	40.6	36.9	38.4	2.1	5.4
Carmoisine	37.2	40.0	36.5	40.9	40.9	36.6	38.7	2.1	5.5

b. Trial Samples Proper

	Tartrazine mg/kg	Carmoisine mg/kg	Erythrosine mg/kg
Cake"A"	81.6	60.1	75.5
Cake"A"	81.2	61.9	75.9
Cake"A"	80.6	62.2	83.9
Cake"A"	80.8	56.4	77.6
Cake"A"	78.8	57.5	74.5
Cake"A"	87.8	59.6	81.8
Mean	*81.8*	*59.6*	*78.2*
Standard deviation	*3.1*	*2.3*	*3.8*
CV %	*3.8*	*3.9*	*4.2*

	Tartrazine mg/kg	Carmoisine mg/kg	Erythrosine mg/kg
Cake"D"	98.4	80.4	104.8
Cake"D"	100.6	85.3	104.3
Cake"D"	101.9	84.7	102.0
Cake"D"	104.3	80.0	104.7
Cake"D"	109.5	81.6	112.4
Cake"D"	106.7	87.8	107.7
Mean	*103.6*	*83.3*	*106.0*
Standard deviation	*4.1*	*3.1*	*3.6*
CV %	*4.0*	*3.7*	*3.4*

	Tartrazine mg/kg	Carmoisine mg/kg	Erythrosine mg/kg
Skimmed Milk "J"	94.7	76.5	99.5
Skimmed Milk "J"	93.5	81.9	99.6
Skimmed Milk "J"	91.5	74.1	100.7
Skimmed Milk "J"	92.0	85.4	101.3
Skimmed Milk "J"	93.3	80.2	104.0
Skimmed Milk "J"	NR	NR	NR
Mean	*93.00*	*79.6*	*101.0*
Standard deviation	*1.3*	*4.5*	*1.8*
CV %	*1.4*	*5.6*	*1.8*

	Tartrazine mg/kg	Amaranth mg/kg	Carmoisine mg/kg
Skimmed Milk "X"	79.2	67.9	78.7
Skimmed Milk "X"	77.1	62.9	84.2
Skimmed Milk "X"	78.0	67.9	80.6
Skimmed Milk "X"	79.3	68.8	81.4
Skimmed Milk "X"	79.5	69.6	82.8
Skimmed Milk "X"	80.6	69.6	81.4
Mean	*79.0*	*67.8*	*81.5*
Standard deviation	*1.2*	*2.5*	*1.9*
CV %	*1.5*	*3.7*	*2.3*

	Red 2 G mg/kg	**Tartrazine mg/kg**	**Amaranth mg/kg**
Lemonade "Q"	48.7	86.9	51.5
Lemonade "Q"	47.7	86.7	50.7
Lemonade "Q"	48.4	87.4	51.3
Lemonade "Q"	46.7	85.0	49.8
Lemonade "Q"	47.2	85.2	50.5
Lemonade "Q"	47.3	87.9	51.1
Mean	*47.7*	*86.5*	*50.8*
Standard deviation	*0.8*	*1.2*	*0.6*
CV %	*1.7*	*1.4*	*1.2*

	Red 2 G mg/kg	**Tartrazine mg/kg**	**Amaranth mg/kg**
Lemonade "R"	57.5	62.8	59.4
Lemonade "R"	56.8	61.6	60.2
Lemonade "R"	55.5	60.3	58.6
Lemonade "R"	54.9	60.7	58.5
Lemonade "R"	55.5	60.2	58.7
Lemonade "R"	55.7	61.8	59.0
Mean	*56.0*	*61.2*	*59.1*
Standard deviation	*1.0*	*1*	*0.6*
CV %	*1.8*	*1.6*	*1.0*

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