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DETERMINATION OF PRESERVATIVES IN FOODSTUFFS:

COLLABORATIVE TRIAL

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The results of a collaborative trial, involving 10 laboratories, to assess a method for the determination of benzoic acid, sorbic acid, and the methyl, ethyl and propyl esters of 4-hydroxybenzoic acid in a range of foodstuffs are reported. In the method tested, foodstuffs are either diluted with methanol or extracted in methanol, followed by centrifugation and filtration, prior to analysis using reversed-phase HPLC with UV detection. For benzoic acid quantitation is carried out at 223 nm and for the other analytes at 258 nm. The method allows for the simultaneous measurement of preservatives E200 to E219, maximum use levels for which are prescribed in Council Directive 95/2 EC.

The trial consisted of two parts. In the first part, participants were asked to carry out a system suitability test using a reference solution consisting of the analytes of interest and possible co-extractants, in order to demonstrate satisfactory chromatographic performance. The second part comprised the trial proper, in which participants analysed 12 test materials of orange squash, cola, beetroot, pie filling and salad cream. The test materials were sent out in the form of blind duplicate and split level samples.

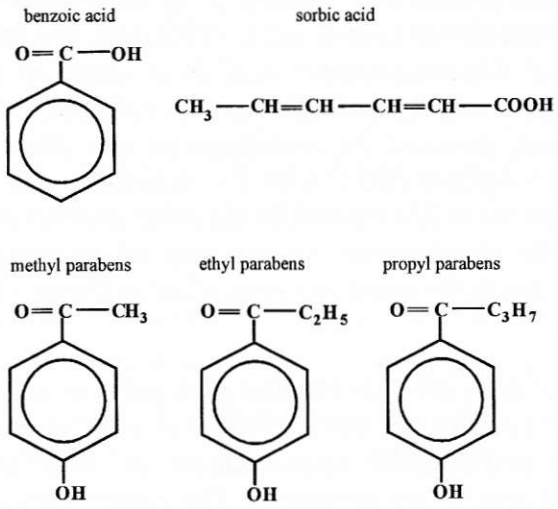
The RSD_r values ranged from 1 to 7 for the samples of orange juice and cola and from 2 to 10 for beetroot, pie filling and salad cream samples. RSD_R values ranged between 4 and 12 for the orange juice and cola samples. For the beetroot, pie filling and salad cream samples RSD_R values ranged between 4 and 16. The Ho_R precision parameter calculated from the trial was satisfactory for the determination of each analyte in the liquid drinks and only marginally outside the theoretically predicted value for 5 determinations involving the more complex matrices.

INTRODUCTION

Benzoic acid, sorbic acid and the methyl-, ethyl- and propyl 4-hydroxybenzoate esters (parabens) are compounds with antimicrobial action, classified as chemical preservatives[1] (Figure 1). Benzoic acid is generally used to inhibit

bacterial development, while sorbic acid is more efficient against yeasts and moulds[2]. The esters of 4-hydroxybenzoic acid are effective up to higher pH than either of the free acids, their antimicrobial activity increasing as the alkyl chain length of the ester group increases[3]. As food additives these compounds, or their salts, have been used widely, either singly or in combination, in processed foods to protect the foodstuffs from deterioration caused by microorganisms[4].

Figure 1. Chemical structures of benzoic acid, sorbic acid and the methyl- ethyl- and propyl- esters of 4-hydroxybenzoic acid.



Many countries have previously set limits for the addition of these compounds to foods, based on considerations of safety-in-use and the concentrations of the compounds needed to achieve antimicrobial effect [2,5-8]. Following the adoption by Member States of EC Directive 95/2 in 1995[9], the use of these preservatives in the United Kingdom is currently controlled by the Miscellaneous Food Additives Regulations 1995[10]. These Regulations specify the categories of food in which the addition of benzoic acid, sorbic acid, the hydroxybenzoate esters and certain salts is permitted, subject to prescribed 'maximum use levels'.

In order to allow the effective enforcement of the prescribed limits, there is a need to have available validated methods of analysis for the determination of these preservatives in foodstuffs. 'Official' methods of analysis have previously been reported for the determination of benzoic acid in either solid or liquid foods based on titration[11], spectrophotometry[12] or thin layer chromatography[13] and for the determination of sorbic acid in dairy products and cheese using spectrophotometry[14] or oxidative determination[15] respectively. An 'official'

method involving the use of gas chromatography for the simultaneous determination of benzoic acid and sorbic acid in foods has also been reported, based on the results of a collaborative study[16,17].

The more recently developed high-performance liquid chromatographic methods for the determination of preservatives in food[1,3,7,18-33] typically have the advantage of reduced sample preparation. The technique permits the simultaneous determination of benzoic acid, sorbic acid and the esters of 4-hydroxybenzoic acid, however some analysts have found it necessary to use an ion-pairing agent[28,33] or gradient conditions[7,19,27] to achieve satisfactory separation, due to the differing polarities of the free acids and the esters.

In the present work, investigations have been carried out to extend the scope of a method, previously validated in the MAFF collaborative trial programme, for the determination of the parabens esters in foodstuffs[34] in order to provide enforcement analysts with a procedure for the simultaneous determination of benzoic and sorbic acids as well as the methyl-, ethyl- and propyl 4-hydroxybenzoate esters in foods. The newly devised procedure, in effect, allows for the determination of the preservatives E200 to E219 on a routine basis (Table 1). This report describes the development studies, carried out at the CSL Food Science Laboratory, Norwich and the formal validation of the candidate method by collaborative trial.

Table 1. The preservatives E200 to E219 listed in Annex III of EC Directive 95/2.

Preservative	E Number
Sorbic acid	E200
Potassium sorbate	E202
Calcium sorbate	E203
Benzoic acid	E210
Sodium benzoate	E211
Potassium benzoate	E212
Calcium benzoate	E213
Ethyl 4-hydroxybenzoate	E214
Sodium ethyl 4-hydroxybenzoate	E215
Propyl 4-hydroxybenzoate	E216
Sodium propyl 4-hydroxybenzoate	E217
Methyl 4-hydroxybenzoate	E218
Sodium methyl 4-hydroxybenzoate	E219

DEVELOPMENT OF THE METHOD OF ANALYSIS UNDER TEST

When developing the candidate method, isocratic liquid chromatographic conditions, as used previously in the work on hydroxybenzoates[34], were initially assessed. This approach highlighted the reported difficulty of achieving separation between benzoic and sorbic acids while at the same time limiting the overall run-time for the more lipophilic parabens compounds[19,28]. By reducing, from 40% to 25%, the proportion of organic modifier in the acetonitrile/citrate buffer (pH 4.8) mobile phase, which was used as in that method in conjunction with a Partisil ODS 2 column, an adequate resolution was at first achieved between benzoic acid and sorbic acid with the compounds of interest all eluting within 45 min. The critical separation between the benzoic and sorbic acids, however, deteriorated with time and although alternative stationary and mobile phases were evaluated no further improvements in isocratic separation could be achieved.

The Kromasil 100-5C18 column which was chosen for further evaluation proved to be robust during the in-house investigations and was well suited to the gradient conditions using acetonitrile/citrate buffer (pH 4.2) which were then devised. The gradient elution reduced the overall run-time for the method and allowed the chromatographic separation to be optimised to take account of reported interferences from synthetic colours, artificial sweeteners[35], caffeine[31], pyrogallol, propyl gallate[27], vanillin and ethyl vanillin[36]. Careful manipulation of the pH of the mobile phase was necessary to achieve satisfactory resolution for benzoic acid/sorbic acid/vanillin and methyl parabens/ethyl vanillin. Other potential interferents, such as colours and the artificial sweeteners saccharin, acesulfame-K and aspartame eluted well before the analytes of interest. The gradient was introduced after the elution of the methyl parabens, terminating prior to the elution of ethyl parabens in order to minimise the effects of the dwell time on the precision of the method.

Details of the instrumental conditions are summarised in Table 2.

Table 2 - Instrumental conditions

Column	Kromasil 100-5C18, 5 μm, 25 cm × 4.6 mm id
Guard column	Kromasil C18, 5 μm, 1 cm × 3.2 mm
Mobile phase A	20% acetonitrile:80% citric acid/sodium citrate buffer (pH 4.2)
Mobile phase B	40% acetonitrile:60% citric acid/sodium citrate buffer (pH 4.2)
Flow rate	1 mL min^{-1}
Gradient system	0 min to 26 min 100% A
	26 min to 31 min go to 100% B
	31 to 45 min 100% B
	45 min to 50 min go to 100 % A
	50 to 55 min 100% A
Temperature	20°C (room temperature)
Injection volume	20 μL
Detection wavelength	223 nm for benzoic acid, 258 nm for sorbic acid and parabens

In view of the potential for interferences in the chromatographic separation, a standard mixture containing the analytes, caffeine, aspartame, vanillin and ethyl vanillin was proposed as a control sample in order to define system suitability. This was subsequently prescribed for use by participants to characterise the chromatographic separation in the collaborative trial. A chromatogram recorded at each of the detection wavelengths, 223 nm and 258 nm, to illustrate the separation achieved using this mixture is shown in Figure 2.

Figure 2.

HPLC separation achieved and recorded in-house for the system suitability test mixture at each of the detection wavelengths using the optimised mobile phase conditions.

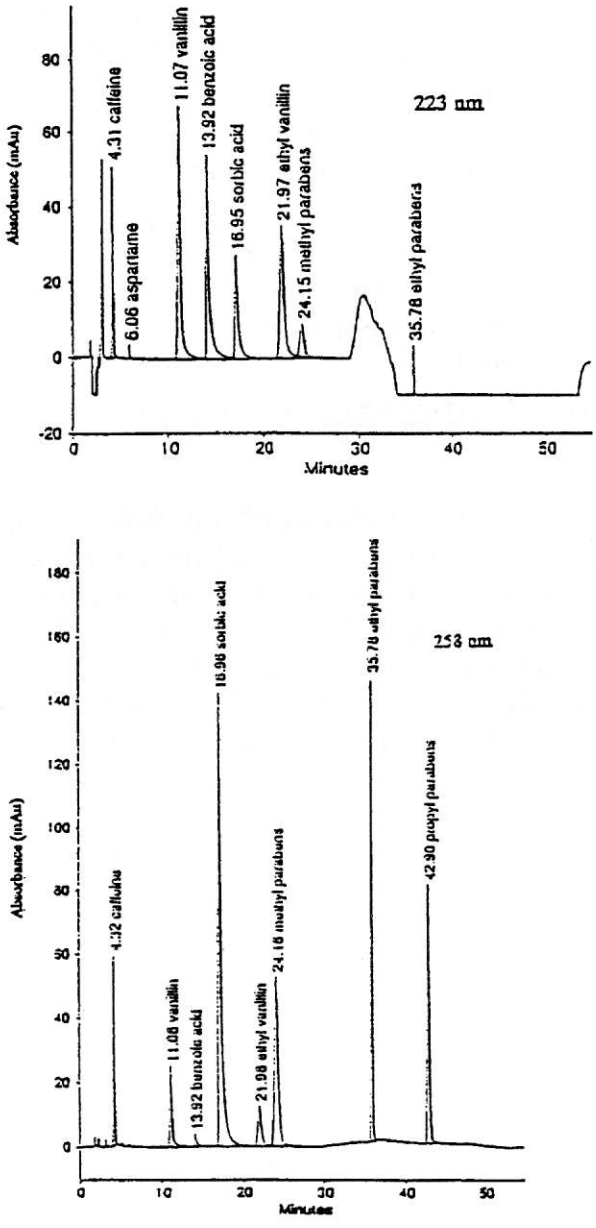
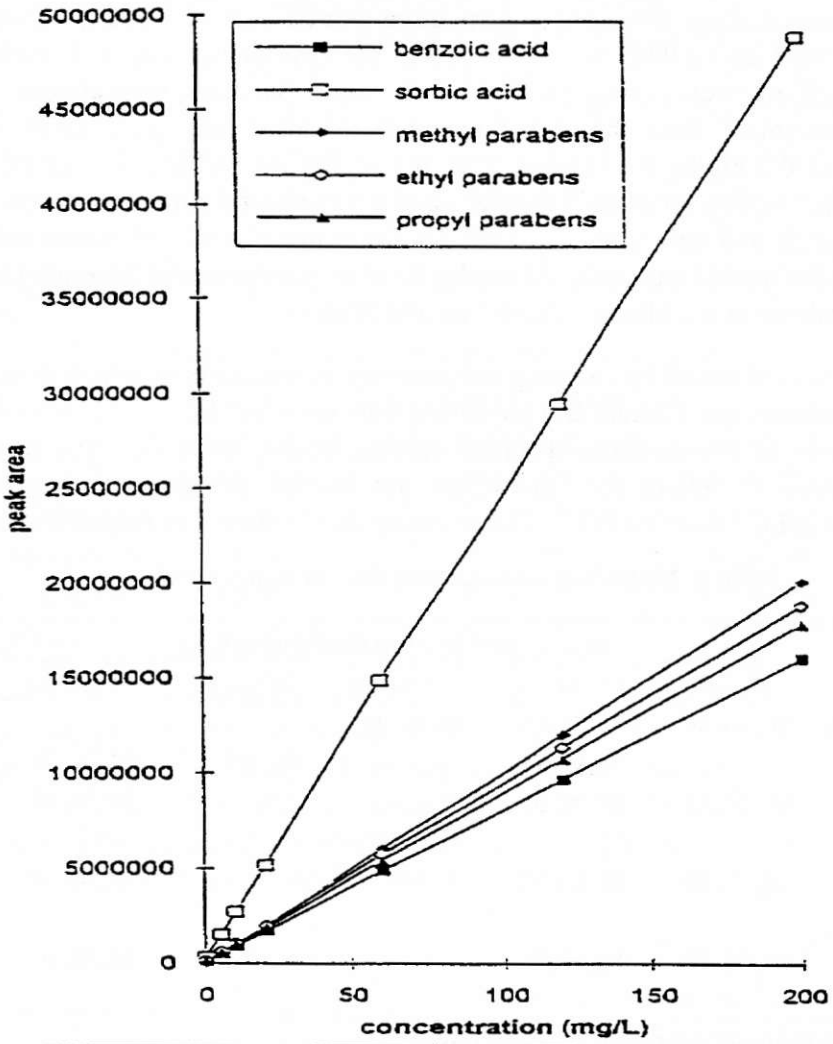


Figure 3. Calibration curves for all analytes from 5 mg/L to 200 mg/L.



Compound	Regession Equation
Benzoic acid	y=79400.643x+120114.086, r=0.9984
Sorbic acid	y=242519.45x+312299.737, r=0.9982
Methyl parabens	y=100027.024x+9105.850, r=0.9999
Ethyl parabens	y=93179.190x+15382.895, r=0.9981
Propyl parabens	y=88554.880x+108977.848, r=0.9985

The concentration versus response relationship for each compound was shown to be linear over the range of standards, from 5 mg/L to 200 mg/L, included in the candidate method (Figure 3). Correlation coefficients of 0.9984, 0.9982, 0.9999, 0.9981 and 0.9985 were obtained in the case of benzoic acid, sorbic acid, methyl parabens, ethyl parabens and propyl parabens respectively. A simple assessment from the signal response to noise ratio gave limits of detection of 0.2 mg/kg for benzoic acid, 0.3 mg/kg for sorbic acid and ethyl parabens, 0.7 mg/kg for methyl parabens and 0.5 mg/kg for propyl parabens in orange squash and cola and of 0.2 mg/kg for benzoic acid and sorbic acid, 0.8 mg/kg for methyl parabens, 0.3 mg/kg for ethyl parabens and 0.5 mg/kg for propyl parabens in pie filling, salad cream and beetroot.

Accuracy was assessed by carrying out recovery experiments in which orange squash, beetroot, salad cream and pie filling were each fortified in turn with the preservatives at one of three specified spiking levels, these different levels being chosen to reflect the "maximum use levels" for these compounds prescribed in EC Directive 95/2[9]. The recovery data is shown in Tables 3 to 6.

Table 3. Method validation recovery data for orange squash

Analyte	Recoveries (%) at specified spiking level				
	125 mg/kg	150 mg/kg	175 mg/kg	250 mg/kg	325 mg/kg
Benzoic acid	86, 86, 84	84, 85, 86	88, 87, 85	-	-
Sorbic acid	-	97, 95, 95	-	96, 100, 97	94, 97, 96
Methyl parabens	92, 93, 92	87, 92, 91	-	-	92, 97, 96
Ethyl parabens	95, 97, 95	91, 96, 95	-	-	93, 99, 98
Propyl parabens	91, 93, 91	86, 92, 91	-	-	88, 92, 91

- recovery experiments not performed at this level

Table 4. Method validation recovery data for beetroot

Analyte	Recoveries (%) at specified spiking level		
	250 mg/kg	1750 mg/kg	2250 mg/kg
Benzoic acid	92, 95, 95	91, 94, 92	91, 92, 93
Sorbic acid	101, 102, 101	97, 98, 100	97, 99, 99
Methyl parabens	105, 106, 105	100, 103, 102	98, 100, 101
Ethyl parabens	101, 99, 98	99, 97, 100	96, 98, 99
Propyl parabens	102, 105, 104	100, 103, 101	99, 101, 101

Table 5. Method validation recovery data for salad cream

Analyte	Recoveries (%) at specified spiking level		
	250 mg/kg	1750 mg/kg	2250 mg/kg
Benzoic acid	104, 100, 102	99, 101, 102	104, 100, 100
Sorbic acid	110, 106, 109	106, 108, 109	110, 107, 106
Methyl parabens	110, 108, 109	107, 110, 110	112, 108, 108
Ethyl parabens	111, 108, 109	105, 107, 108	109, 106, 105
Propyl parabens	111, 106, 107	106, 109, 109	111, 108, 107

Table 6. Method validation recovery data for pie filling

Analyte	Recoveries (%) at specified spiking level		
	250 mg/kg	875 mg/kg	1125 mg/kg
Benzoic acid	84, 81, 87	86, 89, 88	83, 84, 86
Sorbic acid	88, 96, 91	90, 93, 93	86, 87, 89
Methyl parabens	87, 94, 90	88, 92, 91	85, 86, 89
Ethyl parabens	90, 98, 94	91, 94, 94	87, 89, 90
Propyl parabens	85, 92, 89	87, 92, 90	84, 86, 87

Chromatograms obtained for spiked extracts of each food matrix are shown in Figures 4 to 7 respectively. Screening the food matrices to be included in the scope of the method did not identify any matrix interferences of concern, except in the case of salad cream where a small peak was seen preceding, but satisfactorily separated from, benzoic acid.

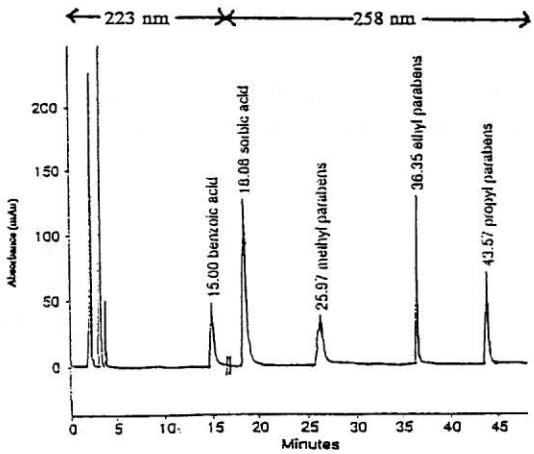


Figure 4. Chromatogram of an orange squash sample spiked with the analytes at 150 mg/kg in each case.

Figure 5. Chromatogram of a beetroot sample spiked with the analytes at 250 mg/kg in each case.

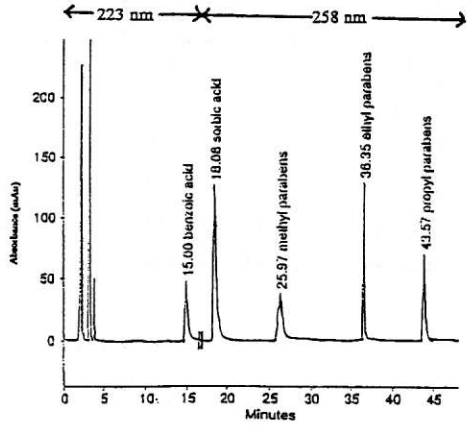


Figure 6. Chromatogram of a salad cream sample spiked with the analytes at 250 mg/kg in each case.

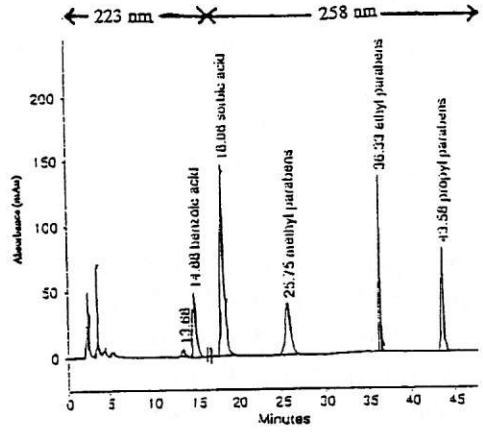
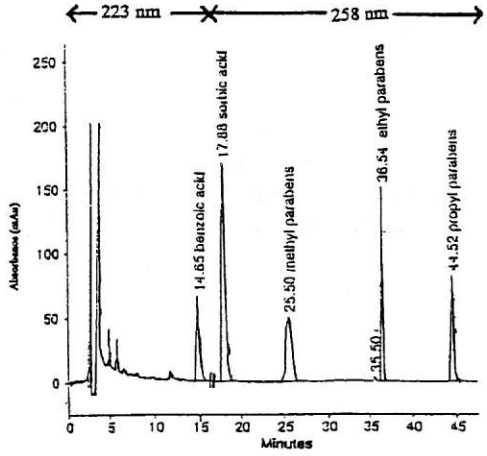


Figure 7. Chromatogram of a pie filling sample spiked with the analytes at 250 mg/kg in each case.



Further evidence of the accuracy and precision of this procedure was provided by the results obtained for the replicate analysis of an external reference material. This material, a cola drink, had previously been analysed as part of the Food Analysis Performance Assessment Scheme (FAPAS®) and had been given assigned values for the content of benzoic acid and sorbic acid respectively. The mean values obtained during the analysis of this material using the candidate method, 603.5 mg/L for benzoic acid and 860.6 mg/L for sorbic acid, corresponded to satisfactory Z-scores of -0.46 and -0.55 respectively (Table 7).

Table 7.
Results obtained from within - batch analysis of the external reference material

Analyte	Assigned value (mg/L)†	Mean (mg/kg)	SD	%CV
Benzoic acid (n=6)	658.80	603.51	29.30	4.86
Sorbic acid (n=6)	922.65	860.58	23.47	2.73

†The Assigned values are expressed in mg/L as originally reported by FAPAS®

In final confirmation of the validity of the proposed procedure, sample test materials were analysed independently by two different techniques, the candidate method and a gradient liquid chromatographic-mass spectrometric procedure. In this latter procedure, separation of the analytes was achieved on the Kromasil 100-5C18 column with 20 mM ammonium acetate (pH 4.2)/acetonitrile mobile phase at a flow rate of 1 mL/min and quantitation of each analyte was based on the deprotonated molecular ion. The agreement between the results obtained using these procedures is shown in Table 8.

Table 8

Comparison of results obtained from the HPLC and HPLC-MS analysis of collaborative trial samples.

Sample	Benzoic acid (mg/kg)		Sorbic acid (mg/kg)		Methyl parabens (mg/kg)		Ethyl parabens (mg/kg)		Propyl parabens (mg/kg)	
	HPLC UV	HPLC MS	HPLC UV	HPLC MS	HPLC UV	HPLC MS	HPLC UV	HPLC MS	HPLC UV	HPLC MS
Orange squash D	460	480	280	310	310	280	210	190	220	200
Beetroot H	1840	1830	380#	410#	<0.8	ND$	1150	1050	650	620
Pie filling A	1540	1560	1050	1020	790	770	380	340	<0.5	ND$
Salad cream L	<0.2	ND$	1580	1500	160	150	480	430	1200	1290

- mean of two determinations.

$ - analyte not detected.

COLLABORATIVE TRIAL ORGANISATION

METHOD OF ANALYSIS TO BE COLLABORATIVELY TESTED

The method tested was that developed at the CSL Food Science Laboratory, Norwich. It comprised dilution of liquid samples with methanol, or, for other foods, extraction with methanol followed by centrifuging and filtering. The concentrations of the analytes in the extracts were measured using gradient reversed-phase liquid chromatography with photometric detection at 223 nm for benzoic acid and 258 nm for the other analytes. Participants were asked to carry out the analysis using a Kromasil 100-5C18 column, but were otherwise allowed a limited degree of flexibility in the choice of instrumental conditions. The method tested is described in Appendix I.

COLLABORATIVE TRIAL ORGANISATION AND SAMPLE PREPARATION

Participants

Ten UK Public Analyst laboratories took part in the study. The method to be used in the trial was sent out to participants for comment, in advance of the trial.

System Suitability Test

Participants were asked to analyse a mixed standard containing 20 mg/L of benzoic acid, sorbic acid, methyl-, ethyl- and propyl 4-hydroxybenzoate, aspartame, caffeine, vanillin and ethyl vanillin using the chromatographic conditions described in the method. Comments were received and minor changes to the written method were made at this stage. The comments received from participants can be found in Appendix II.

Trial Proper

Ten Public Analyst laboratories participated in the trial proper and received twelve test materials, of which 8 were split level samples and 4 were blind duplicates. The orange squash, beetroot, pie filling and salad cream test materials were prepared by adding combinations of the analytes at varying concentrations as shown in Table 9. The cola test materials (Series III, Rounds 10 and 11), which were obtained from FAPAS®, had been used in previous proficiency testing rounds. These samples therefore had assigned values for the analytes present and had been shown to be homogeneous. For each sample type a blank matrix was also supplied to participants to allow them to determine recoveries for all analytes.

Sample Preparation

Table 9.
Test material preparation scheme

Sample	Sample Code	Conc'n of benzoic acid added (mg/kg)	Conc'n of sorbic acid added (mg/kg)	Conc'n of methyl 4-hydroxybenzoic acid added (mg/kg)	Conc'n of ethyl 4-hydroxybenzoic acid added (mg/kg)	Conc'n of propyl 4-hydroxybenzoic acid added (mg/kg)
Orange squash	J	145.2	319.6	349.2	174.8	274.4
(split level)	D	165.2	282.8	314.0	194.8	239.6
Cola*	K	793.6	563.1	none	none	none
(blind duplicate)	W	793.6	563.1	none	none	none
Cola*	G	1383.6	582.2	none	none	none
(blind duplicate)	S	1383.6	582.2	none	none	none
Beetroot	H	2099.2	382.0	none	1200.0	690.0
(split level)	B	1894.0	420.4	none	1068.0	762.4
Pie filling	A	1650.4	1052.4	800.0	353.2	none
(split level)	P	1824.8	1162.4	890.4	312.8	none
Salad cream	C	none	1750.0	226.0	551.2	1352.4
(split level)	L	none	1951.6	198.4	610.4	1489.2

* - concentrations reported in the table are the assigned values for the analytes in the samples obtained from FAPAS®

All of the samples, except the colas which were FAPAS® test materials, were prepared at the Food Science Laboratory, Norwich. Sufficient amounts of each commodity to be analysed were purchased from retail outlets. The sample commodities were homogenised and sub-samples taken for analysis to verify whether they were free of detectable benzoic acid, sorbic acid and the methyl, ethyl and propyl esters of 4-hydroxybenzoic acid. The orange squash sample used was found to contain some benzoic acid, however the content was at a concentration which would not compromise the later recovery determinations performed by participants. Portions of each sample type were fortified with the analytes at the concentrations shown in Table 9 and each fortified material homogenised. Sub-samples for analysis were dispatched to participants after satisfactory homogeneity testing was completed.

The samples were prepared as follows:

Orange Squash samples J & D

250 g of orange squash was placed in a 1 L glass beaker and was stirred for 90 minutes using a magnetic stirrer. 36.3 mg of benzoic acid, 79.9 mg of sorbic acid, 87.3 mg of methyl 4-hydroxybenzoic acid, 43.7 mg of ethyl 4-hydroxybenzoic acid and 68.6 mg of propyl 4-hydroxybenzoic acid for sample J, and 41.3 mg of benzoic acid, 70.7 mg of sorbic acid, 78.5 mg of methyl 4-hydroxybenzoic acid, 48.7 mg of ethyl 4- benzoic acid and 59.9 mg of propyl 4-hydroxybenzoic acid for sample D, were added to the base material which was then stirred for a further 2 hours. 11 g portions were dispensed into a series of 25 mL vials.

Beetroot samples H & B

250 g of pickled beetroot was placed in a 1 L glass beaker and was homogenised for 10 minutes using an Ultra-Turrax blender. 524.8 mg of benzoic acid, 95.5 mg of sorbic acid, 300.0 mg of ethyl 4-hydroxybenzoic acid and 172.5 mg of propyl 4-hydroxybenzoic acid for sample H, and 473.5 mg of benzoic acid, 105.1 mg of sorbic acid, 267.0 mg of ethyl 4-hydroxybenzoic acid and 190.6 mg of propyl 4-hydroxybenzoic acid for sample B, were added to the base material which was then homogenised for a further 30 minutes. 11 g portions were dispensed into a series of 25 mL vials.

Pie filling samples A & P

250 g of cherry pie filling was placed in a 1 L glass beaker and was homogenised for 10 minutes using an Ultra-Turrax blender. 412.6 mg of benzoic acid, 263.1 mg of sorbic acid, 200.0 mg of methyl 4-hydroxybenzoic

acid and 88.3 mg of ethyl 4-hydroxybenzoic acid for sample A, and 456.2 mg of benzoic acid, 290.6 mg of sorbic acid, 222.6 mg of methyl 4-hydroxybenzoic acid and 78.2 mg of ethyl 4-hydroxybenzoic acid for sample P, were added to the base material which was then homogenised for a further 30 minutes. 11 g portions were dispensed into a series of 25 mL vials.

Salad cream samples C & L

250 g of salad cream was placed in a 1 L glass beaker and was homogenised for 10 minutes using an Ultra-Turrax blender. 437.5 mg of sorbic acid, 56.5 mg of methyl 4-hydroxybenzoic acid, 137.8 mg of ethyl 4-hydroxybenzoic acid and 338.1 mg of propyl 4-hydroxybenzoic acid for sample C, and 487.9 mg of sorbic acid, 49.6 mg of methyl 4-hydroxybenzoic acid, 152.6 mg of ethyl 4-hydroxybenzoic acid and 372.3 mg of propyl 4-hydroxybenzoic acid for sample L, were added to the base material which was then homogenised for a further 30 minutes. 11 g portions were dispensed into a series of 25 mL vials.

Cola samples K, W, G &S

The FAPAS® samples, as previously described, were mixed and stirred on a magnetic stirrer for 1 hour. 11 g portions were dispensed into a series of 25 mL vials

Storage of Material

All prepared test materials were stored at +4°C, pending dispatch to participants, for receipt within 48 h.

Verification of Homogeneity

Homogeneity was assessed using procedures described in The International Harmonized Protocol for the Proficiency Testing of (Chemical) Analytical Laboratories[37]. Five randomly selected vials of each test sample were analysed in duplicate using the method to be collaboratively tested.

Results obtained for the verification of homogeneity can be found in Table 10. The samples were found to be homogeneous in the case of each analyte.

Table 10. Homogeneity test results of collaborative trial test materials

Orange squash sample J

Benzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	173	174
2	176	175
3	181	184
4	208	167
5	178	145
Mean	176.1	
F-test	0.696	
F-critical	5.192	

Sorbic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	333	331
2	336	336
3	339	341
4	361	332
5	340	322
Mean	337.1	
F-test	0.749	
F-critical	5.192	

Methyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	362	361
2	363	363
3	366	370
4	287	357
5	364	341
Mean	353.4	
F-test	0.743	
F-critical	5.192	

Ethyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	177	178
2	179	183
3	180	186
4	190	176
5	182	174
Mean	180.5	
F-test	0.500	
F-critical	5.192	

Propyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	268	264
2	259	261
3	275	267
4	304	275
5	267	259
Mean	269.9	
F-test	2.697	
F-critical	5.192	

Orange squash sample D

Benzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	144	156
2	146	152
3	162	166
4	170	145
5	149	171
Mean	156.1	
F-test	0.636	
F-critical	5.192	

Sorbic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	263	268
2	262	266
3	273	275
4	276	261
5	265	278
Mean	268.7	
F-test	0.865	
F-critical	5.192	

Methyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	294	302
2	296	297
3	306	309
4	310	294
5	296	310
Mean	301.4	
F-test	0.791	
F-critical	5.192	

Ethyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	190	189
2	196	194
3	199	201
4	201	191
5	193	201
Mean	195.5	
F-test	1.592	
F-critical	5.192	

Propyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	202	201
2	207	205
3	218	217
4	210	198
5	206	214
Mean	207.8	
F-test	3.940	
F-critical	5.192	

Beetroot sample H

Benzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	1829	1878
2	1919	1896
3	1777	1650
4	1836	1718
5	2077	1754
Mean	1833.4	
F-test	1.103	
F-critical	5.192	

Sorbic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	360	354
2	414	405
3	389	348
4	378	357
5	446	390
Mean	384.1	
F-test	2.822	
F-critical	5.192	

Ethyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	1152	1190
2	1200	1182
3	1130	1157
4	1188	1120
5	1314	1119
Mean	1175.2	
F-test	0.859	
F-critical	5.192	

Propyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	636	666
2	702	667
3	684	639
4	652	604
5	739	649
Mean	663.8	
F-test	0.957	
F-critical	5.192	

Beetroot sample B

Benzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	1611	1813
2	1726	1957
3	1779	1744
4	1631	1761
5	1720	1703
Mean	1744.5	
F-test	0.630	
F-critical	5.192	

Sorbic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	366	408
2	406	458
3	412	405
4	383	402
5	398	396
Mean	403.4	
F-test	1.303	
F-critical	5.192	

Ethyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	997	1108
2	1101	1202
3	1085	1086
4	999	1067
5	1054	1043
Mean	1074.2	
F-test	1.651	
F-critical	5.192	

Propyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	718	771
2	846	764
3	741	819
4	703	735
5	758	724
Mean	757.9	
F-test	2.295	
F-critical	5.192	

Pie filling sample A

Benzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	1569	1612
2	1531	1398
3	1501	1535
4	1636	1618
5	1593	1582
Mean	1557.5	
F-test	4.050	
F-critical	5.192	

Sorbic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	1063	1100
2	1005	934
3	1018	1067
4	1109	1078
5	1079	1068
Mean	1052.1	
F-test	5.089	
F-critical	5.192	

Methyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	787	842
2	754	696
3	758	784
4	828	804
5	808	783
Mean	784.4	
F-test	3.460	
F-critical	5.192	

Ethyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	373	380
2	355	327
3	369	409
4	386	417
5	369	365
Mean	375.0	
F-test	3.184	
F-critical	5.192	

Pie filling sample P

Benzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	2009	1945
2	1977	1892
3	1776	1774
4	1920	1884
5	1840	2064
Mean	1908.1	
F-test	2.004	
F-critical	5.192	

Sorbic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	1303	1261
2	1274	1215
3	1168	1175
4	1254	1224
5	1170	1307
Mean	1235.1	
F-test	1.282	
F-critical	5.192	

Methyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	971	938
2	953	916
3	873	870
4	938	908
5	902	1013
Mean	928.3	
F-test	1.568	
F-critical	5.192	

Ethyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	351	340
2	344	330
3	310	311
4	356	327
5	320	359
Mean	334.8	
F-test	1.907	
F-critical	5.192	

Salad cream sample C

Sorbic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	1458	980
2	1320	1784
3	1415	1272
4	1424	1054
5	2383	1750
Mean	1484.0	
F-test	2.467	
F-critical	5.192	

Methyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	153	99
2	122	174
3	163	141
4	140	126
5	298	220
Mean	163.6	
F-test	4.840	
F-critical	5.192	

Ethyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	504	324
2	396	545
3	509	455
4	462	371
5	681	545
Mean	479.2	
F-test	1.549	
F-critical	5.192	

Propyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	1091	1095
2	804	1317
3	1081	925
4	1041	769
5	1978	1452
Mean	1155.3	
F-test	3.223	
F-critical	5.192	

Salad cream sample L

Sorbic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	1568	1688
2	1630	1651
3	1532	1608
4	1988	1588
5	1450	1584
Mean	1628.7	
F-test	1.047	
F-critical	5.192	

Ethyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	566	673
2	568	540
3	547	463
4	531	442
5	420	445
Mean	519.5	
F-test	3.620	
F-critical	5.192	

Methyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	197	190
2	237	212
3	179	180
4	212	149
5	152	161
Mean	186.9	
F-test	2.613	
F-critical	5.192	

Propyl 4-hydroxybenzoic acid

Vial Number	Portion 1 (mg/kg)	Portion 2 (mg/kg)
1	1076	1079
2	1303	1344
3	1244	1186
4	1507	1113
5	1181	1250
Mean	1228.3	
F-test	1.183	
F-critical	5.192	

Recovery experiments

Participants were asked to determine recoveries for the analytes spiked into each base material supplied. For the main trial, recoveries were determined at two spiking levels as shown in Table 11.

Table 11. Spiking levels for recovery determinations in the trial proper.

Matrix	First Spike Level All analytes	Second Spike Level All analytes
Orange squash	150 mg/kg	325 mg/kg
Cola soft drink	150 mg/kg	325 mg/kg
Beetroot	250 mg/kg	2250 mg/kg
Pie filling	250 mg/kg	2250 mg/kg
Salad cream	250 mg/kg	2250 mg/kg

COLLABORATIVE TRIAL RESULTS

Table 12 gives the retention data reported by participants for the system suitability test mixture. The results obtained by the participants for the main trial are given in Tables 13 to 18.

Table 12. Retention times obtained from participants' standard chromatograms

Laboratory	Column batch number	Analyte retention time (min)				
		Benzoic acid	Sorbic acid	Methyl parabens	Ethyl parabens	Propyl parabens
1	DT0124	13.57	16.84	22.86	33.94	41.33
2	DT0124	14.50	18.06	24.30	35.92	43.05
3	DT0124	14.17	16.85	22.97	37.71	45.03
4	DT0124	16.81	20.74	30.88	41.72	48.61
5	DT0102	14.71	18.34	26.48	37.22	44.83
6	Unknown	18.59	21.87	30.00	37.59	45.99
7	DT0102	13.85	16.41	23.33	35.04	42.59
8	DT0102	15.16	18.44	26.43	37.28	45.05
9	DT0102	14.18	17.45	24.65	36.44	43.96
10	DT0081	14.66	17.86	25.00	37.91	45.47
In-house	DT0102	13.92	16.96	24.16	35.78	42.90

Table 13.. Collaborative trial results for orange squash samples J and D

Benzoic acid (mg/kg)

LABORATORY	(J)	(D)
1	456	466
2	458	456
3(b)	535	462
4	461	472
5	490	513
6(c)	446	453
7	433	457
8	448	476
9	439	459
10	468	491
MEAN	455.4	471.4
n	8	
r	20	
S_r	7.06	
RSD_r	2	
Ho_r	0.4	
R	53	
S_R	18.79	
RSD_R	4	
Ho_R	0.6	

Sorbic acid (mg/kg)

LABORATORY	(J)	(D)
1	324	274
2	294	258
3(b)	250	291
4	319	280
5	344	304
6(c)	321	277
7	302	273
8	322	291
9	304	265
10	322	284
MEAN	316.9	278.4
n	8	
r	13	
S_r	4.48	
RSD_r	4	
Ho_r	0.3	
R	43	
S_R	15.24	
RSD_R	5	
Ho_R	0.8	

Methyl 4-hydroxybenzoic acid (mg/kg)

LABORATORY	(J)	(D)
1	343	300
2[b]	280	284
3	348	310
4	351	312
5	364	330
6[c]	307	0
7	336	300
8[b]	327	317
9	329	294
10	348	317
MEAN	336.2	307.1
n	7	
r	8	
S_r	2.74	
RSD_r	1	
Ho_r	0.2	
R	33	
S_R	11.71	
RSD_R	4	
Ho_R	0.5	

Ethyl 4-hydroxybenzoic acid (mg/kg)

LABORATORY	(J)	(D)
1	162	184
2	146	170
3	169	173
4	163	178
5	166	192
6[c]	167	188
7	157	176
8	149	192
9	140	157
10	167	218
MEAN	158.6	182.8
n	9	
r	28	
S_r	10.13	
RSD_r	6	
Ho_r	1.2	
R	40	
S_R	14.26	
RSD_R	8	
Ho_R	1.1	

Propyl 4-hydroxybenzoic acid (mg/kg)

LABORATORY	(J)	(D)
1	200	186
2	166	158
3	196	179
4	200	179
5	194	160
6(c)	191	166
7	190	167
8	150	173
9	150	126
10	220	189
MEAN	189.6	167.7
n	9	
r	33	
S_r	11.93	
RSD_r	7	
Ho_r	1.4	
R	61	
S_R	21.89	
RSD_R	12	
Ho_R	1.7	

Table 14. Collaborative trial results for cola samples K and W

	Benzoic acid (mg/kg)		Sorbic acid (mg/kg)	
LABORATORY	**(K)**	**(W)**	**(K)**	**(W)**
1	753	757	522	527
2	750	752	520	520
3	694	706	551	561
4	771	766	527	528
5	809	788	566	554
6[c]	739	742	534	528
7[a]	682	742	469	506
8	762	775	542	544
9	739	743	511	513
10[b]	345	390	401	400
MEAN	752.9		534.3	
n	7		7	
r	21		12	
S_r	7.63		4.46	
RSD_r	1		1	
Ho_r	0.3		0.2	
R	86		52	
S_R	30.73		18.75	
RSD_R	4		4	
Ho_R	0.7		0.6	

Table 15. Collaborative trial results for cola samples G and S

Benzoic acid (mg/kg)

LABORATORY	(G)	(S)
1	1268	1267
2[a]	1319	1265
3	1175	1163
4	1265	1278
5	1352	1360
6[c]	1218	1221
7	1078	1087
8	1290	1290
9	1225	1212
10[b]	547	545
MEAN	1234.3	
n	7	
r	19	
S_r	6.71	
RSD_r	1	
Ho_r	0.2	
R	251	
S_R	89.48	
RSD_R	7	
Ho_R	1.3	

Sorbic acid (mg/kg)

LABORATORY	(G)	(S)
1	530	530
2	528	523
3	561	566
4	528	531
5	554	575
6[c]	528	531
7	506	444
8	544	550
9	513	525
10	404	400
MEAN	517.1	
n	9	
r	18	
S_r	6.55	
RSD_r	1	
Ho_r	0.3	
R	157	
S_R	55.94	
RSD_R	11	
Ho_R	1.7	

Table 16. Collaborative trial results for beetroot samples H and B

Benzoic acid (mg/kg)

LABORATORY	(H)	(B)
1	2119	1802
2	1920	1749
3	1924	1813
4	1946	1918
5	2240	1975
6(c)	1823	1715
7	1702	1081
8	1964	1802
9	1938	1687
10	2200	1978
MEAN	1977.6	1752.0
n	9	
r	331	
S_r	118.47	
RSD_r	6	
Ho_r	1.9	
R	631	
S_R	225.25	
RSD_R	12	
Ho_R	2.3	

Sorbic acid (mg/kg)

LABORATORY	(H)	(B)
1	387	394
2	356	381
3	385	444
4	392	412
5	424	442
6(c)	415	435
7(b)	309	228
8	360	413
9	330	355
10	490	516
MEAN	393.2	421.3
n	8	
r	35	
S_r	12.54	
RSD_r	3	
Ho_r	0.7	
R	137	
S_R	49.03	
RSD_R	12	
Ho_R	1.9	

Ethyl 4-hydroxybenzoic acid (mg/kg)

LABORATORY	(H)	(B)
1	1200	1005
2	1004	934
3	1058	1018
4	1053	1067
5	1230	1090
6(c)	1154	1067
7	915	591
8	1122	1040
9	1019	892
10	1253	1137
MEAN	1100.8	984.1
n	9	
r	193	
S_r	69.03	
RSD_r	7	
Ho_r	1.8	
R	393	
S_R	140.42	
RSD_R	14	
Ho_R	2.4	

Propyl 4-hydroxybenzoic acid (mg/kg)

LABORATORY	(H)	(B)
1	671	716
2	589	690
3	661	783
4	558	757
5	718	738
6(c)	643	362
7	489	395
8	672	779
9	569	644
10	687	804
MEAN	625.7	666.8
n	9	
r	162	
S_r	57.86	
RSD_r	9	
Ho_r	2.2	
R	289	
S_R	103.37	
RSD_R	16	
Ho_R	2.6	

Table 17. Collaborative trial results for pie filling samples A and P.

Benzoic acid (mg/kg)

LABORATORY	(A)	(P)
1	1531	1761
2	1542	1764
3	1567	1764
4	1510	1756
5	1697	1916
6[c]	1486	1718
7	1170	1214
8	1512	1665
9	1447	1633
10	1796	1982
MEAN	1565.3	1773.2
n	9	
r	120	
S_r	42.80	
RSD_r	3	
Ho_r	0.8	
R	550	
S_R	196.50	
RSD_R	12	
Ho_R	2.3	

Sorbic acid (mg/kg)

LABORATORY	(A)	(P)
1	990	1168
2	1012	1129
3	985	1138
4	984	1133
5	1094	1234
6[c]	945	1164
7[b]	737	765
8	413	993
9	335	1000
10[b]	516	1265
MEAN	1000.4	1147.3
n	7	
r	70	
S_r	24.91	
RSD_r	2	
Ho_r	0.6	
R	125	
S_R	44.68	
RSD_R	4	
Ho_R	0.7	

Methyl 4-hydroxybenzoic acid (mg/kg)

LABORATORY	(A)	(P)
1	746	850
2	732	810
3	785	808
4	746	854
5	795	928
6(c)	695	688
7	577	597
8	778	834
9	711	818
10	761	873
MEAN	732.6	806.0
n	8	
r	71	
S_r	25.26	
RSD_r	3	
Ho_r	0.8	
R	97	
S_R	34.70	
RSD_R	4	
Ho_R	0.7	

Ethyl 4-hydroxybenzoic acid (mg/kg)

LABORATORY	(A)	(P)
1	317	286
2	306	271
3	352	298
4	317	286
5	354	313
6(c)	342	320
7	245	200
8	340	291
9	287	245
10	354	317
MEAN	321.4	282.7
n	9	
r	16	
S_r	5.59	
RSD_r	2	
Ho_r	0.4	
R	102	
S_R	36.52	
RSD_R	12	
Ho_R	1.8	

Table 18. Collaborative trial results for salad cream samples C and L

Sorbic acid (mg/kg)

LABORATORY	(C)	(L)
1	1534	1948
2	1760	1874
3	1727	1924
4	1527	1881
5	1959	2169
6(c)	1742	1923
7	1491	2209
8	1692	1919
9	1453	1803
10	2157	2365
MEAN	1704.2	2001.5
n	9	
r	356	
S_r	127.18	
RSD_r	7	
Ho_r	2.0	
R	598	
S_R	213.70	
RSD_R	12	
Ho_R	2.2	

Methyl 4-hydroxybenzoic acid (mg/kg)

LABORATORY	(C)	(L)
1	199	199
2	166	169
3	206	163
4	204	197
5	268	214
6(c)	177	156
7	208	194
8	203	136
9	190	208
10	200	196
MEAN	202.1	183.2
n	9	
r	57	
S_r	20.47	
RSD_r	10	
Ho_r	2.2	
R	73	
S_R	26.01	
RSD_R	13	
Ho_R	1.8	

Ethyl 4-hydroxybenzoic acid (mg/kg)

LABORATORY	(J)	(D)
1	518	605
2	496	572
3	551	588
4	505	602
5	582	673
6(c)	534	624
7	496	521
8	570	639
9	484	566
10	608	653
MEAN	534.4	604.3
n	9	
r	51	
S_r	18.24	
RSD_r	3	
Ho_r	0.8	
R	129	
S_R	45.97	
RSD_R	8	
Ho_R	1.3	

Propyl 4-hydroxybenzoic acid (mg/kg)

LABORATORY	(C)	(L)
1	1218	1579
2	1076	1422
3	1163	1426
4	1206	1552
5	1422	1732
6(c)	1135	1562
7	1056	1747
8	1284	1400
9	1153	1478
10	1436	1419
MEAN	1214.9	1531.7
n	9	
r	381	
S_r	136.23	
RSD_r	10	
Ho_r	2.8	
R	381	
S_R	135.11	
RSD_R	10	
Ho_R	1.8	

Statistical analysis of results

The trial results were examined for evidence of individual systematic error ($p<0.01$) using Cochran's and Grubbs' test progressively, by procedures described in the internationally agreed Protocol for the Design, Conduct and Interpretation of Collaborative Studies[38].

Repeatability and Reproducibility

Calculations for repeatability (r) and reproducibility (R) as defined by that protocol[38] were carried out on those results remaining after removal of outliers. These are given in Tables 13 to 18 and summarised in Tables 19 to 23.

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, as predicted by the Horwitz equation, give an indication as to whether the method is sufficiently precise for the level of analyte being measured[39].

The Horwitz predicted value is calculated from the Horwitz equation[39]:

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

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

Horrat Value (Ho)

The Horrat[40] value gives 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 usually 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 given in Tables 13 to 23.

Table 19.

Summary of calculated statistical parameters for benzoic acid in trial proper samples

sample type	Fortification level (mg/kg)	mean (obs)	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
orange squash	456	455.4	8	20	7.06	2	0.4	53	18.79	4	0.6
	476	471.4									
cola soft drink (KW)	794	752.9	7	21	7.63	1	0.3	86	30.73	4	0.7
cola soft drink (GS)	1384	1234.4	7	19	6.71	1	0.2	251	89.48	7	1.3
beetroot	2099	1977.6	9	331	118.47	6	1.9	631	225.25	12	2.3
	1894	1752.0									
pie filling	1650	1565.3	9	120	42.80	3	0.8	550	196.50	12	2.3
	1825	1773.2									

Table 20.

Summary of calculated statistical parameters for sorbic acid in trial proper samples

sample type	Fortification level (mg/kg)	mean (obs)	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
orange squash	320	316.9	8	13	4.48	4	0.3	43	15.24	5	0.8
	283	278.4									
cola soft drink (KW)	563	534.3	7	12	4.46	1	0.2	52	18.75	4	0.6
cola soft drink (GS)	582	517.1	9	18	6.55	1	0.3	157	55.94	11	1.7
beetroot	382	393.2	8	35	12.54	3	0.7	137	49.03	12	1.9
	420	421.3									
pie filling	1052	1000.4	7	70	24.91	2	0.6	125	44.68	4	0.7
	1162	1147.3									
salad cream	1750	1704.2	9	356	127.18	7	2.0	598	213.70	12	2.2
	1952	2011.5									

Table 21.

Summary of calculated statistical parameters for methyl 4-hydroxybenzoic acid in trial proper samples

sample type	Fortification level (mg/kg)	mean (obs)	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
orange	349	336.2	7	8	2.74	1	0.2	33	11.71	4	0.5
squash	314	307.1									
pie filling	800	732.6	8	71	25.26	3	0.8	97	34.70	4	0.7
	890	806.0									
salad cream	226	202.1	9	57	20.47	10	2.2	73	26.01	13	1.8
	198	183.2									

Table 22.

Summary of calculated statistical parameters for ethyl 4-hydroxybenzoic acid in trial proper samples

sample type	Fortification level (mg/kg)	mean (obs)	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
orange squash	175	158.6	9	28	10.13	6	1.2	40	14.26	8	1.1
	195	182.8									
beetroot	1200	1100.8	9	193	69.03	7	1.8	393	140.42	14	2.4
	1068	984.1									
pie filling	353	321.4	9	16	5.59	2	0.4	102	36.52	12	1.8
	313	282.7									
salad cream	551	534.4	9	51	18.24	3	0.8	129	45.97	8	1.3
	610	604.3									

Table 23.

Summary of calculated statistical parameters for propyl 4-hydroxybenzoic acid in trial proper samples

sample type	Fortification level (mg/kg)	mean (obs)	n	r	S_r	RSD_r	Ho_r	R	S_R	RSD_R	Ho_R
orange	274	189.6	9	33	11.93	7	1.4	61	21.89	12	1.7
squash	240	167.7									
beetroot	690	625.7	9	162	57.86	9	2.2	289	103.37	16	2.6
	762	666.8									
salad cream	1352	1214.9	9	381	136.23	10	2.8	381	135.11	10	1.8
	1489	1531.7									

DISCUSSION

In this trial, a system suitability test mixture was used to rigorously define the chromatographic separation which was required for the subsequent analysis of the trial samples. Although it may be possible to gain this separation on an alternative reversed-phase column, participants were asked to use a Kromasil 100-5C18 column, as detailed in the protocol, since this had been shown to be satisfactory during method development. The chromatograms sent in initially by participants for the desired critical separation involving aspartame, caffeine, vanillin, ethyl vanillin and the analytes of interest, in the system suitability test mixture, highlighted various chromatographic problems. Some participants reported a problem with tailing of the peaks, especially benzoic and sorbic acids, whilst others were unable to achieve baseline resolution between all the analytes in the test mixture. In the case of one participant, this lack of suitable separation was as a consequence of not using the HPLC column specified. A minority of participants who did use the Kromasil column unfortunately also experienced difficulties. This problem was investigated and shown to be attributed to a particular batch of column packing. As a result, the participants expressed concern that the method was too reliant on the column, since a batch of nominally the same packing material had given unsatisfactory performance. Whilst such variability can not be ruled out, an indication was given by the column supplier that this particular batch of material had not fully met the suppliers test specifications. The chromatographic problems reported were rectified when participants were supplied with replacement columns or purchased the column specified in the method. All of the participants were then able to meet the system suitability requirements, by using the Kromasil 100-5C18 column, even with minor changes in operating conditions (Table 41). Such a limited degree of flexibility was allowed to participants. From studying the batch numbers of the columns used by participants at this stage it was seen that four different batches of column packing were used and gave comparable results. Participants were then advised to proceed with the analysis of the trial samples proper.

All of the laboratories who participated in this collaborative trial returned results for the analysis of each of the test materials. One participating laboratory (Laboratory No 6) had their results excluded for failing to meet the stipulated deadline for the submission of data. This was due to reported problems of drift in retention time for propyl parabens. Two laboratories (Laboratory Nos. 3 and 7) chose to monitor at one wavelength only (either 230 nm or 235 nm respectively), rather than the two detection wavelengths of 223 nm for benzoic acid and 258 nm in the case of the other analytes, as stated in

the method. These wavelengths, which are close to the respective absorbance maxima for the compounds, were selected during the development of the method in order to achieve maximum sensitivity for the measurements of the preservatives. The results reported by these two laboratories, despite their modifications to the procedure, in general compared well with those reported by the laboratories which employed dual wavelength detection. The loss of sensitivity through use of a single wavelength was not of importance in this case due to the relatively high concentrations of preservatives in the samples.

As evidenced by the Ho_R values of 2 or less, the statistical results reveal the method gave satisfactory precision in each of the blind duplicate samples and in the majority of the split level samples at the concentrations used (456 - 1892 mg/kg for benzoic acid, 283 - 1233 mg/kg for sorbic acid, 314 - 938 mg/kg for methyl parabens, 190 - 1153 mg/kg for ethyl parabens and 218 - 746 mg/kg for propyl parabens) which are representative of the limits set for the analytes in EC Directive 95/2[9]. For the pie filling and salad cream samples analysed, the Ho_R values of 2.3 and 2.2, for benzoic acid and sorbic acid respectively, were marginally above the precision level predicted from the Horwitz equation. Such an increase however, is not unexpected given the more complex nature of these food matrices. In addition, the procedure for salad cream involves an extra defatting step which has the potential for increasing the variability in the analysis. Similarly, the Ho_R values greater than 2 for the determination of benzoic acid, ethyl parabens and propyl parabens in the beetroot samples are indicative of the increased analytical difficulty associated with this matrix. There is no evidence of any interference problems in the chromatography for any of these matrices and recoveries quoted by participants, at analyte levels similar to those used in the test materials, were satisfactory for these compounds. Of these higher Ho_R values in the beetroot matrix, the value of 2.6 for propyl parabens may arise due to poorer peak shape associated with longer elution times for this compound. In the absence of a more precise multi-residue procedure for the simultaneous determination of all these preservatives in such matrices however, the Ho_R values marginally above 2 can be regarded as acceptable. Furthermore, since 'vegetables in vinegar' is not a category of foodstuff for which the addition of hydroxybenzoate esters is permitted, the present procedure could still be used for this matrix to detect the unauthorised addition of these compounds, prior to a more accurate determination.

In the previous MAFF collaborative trial the precision of an isocratic method for the determination of methyl, ethyl and propyl parabens was assessed using orange juice and beetroot samples only[34]. Although the concentrations of analytes added to the samples were different in that investigation and the

present trial, the performance characteristics of the two methods were comparable. Slightly better precision was obtained in the earlier isocratic procedure, for the determination of ethyl and propyl parabens in orange juice and beetroot matrices. This may be accounted for by the fact that although the present method bears similarities to the earlier procedure, in terms of extraction approaches and mobile phase, the incorporation of a gradient elution system to reduce overall run time may have had a slight adverse effect on precision.

Recoveries reported by participants in this trial are given in Tables 24 to 40 and were generally similar to those obtained by the co-ordinating laboratory during the in-house validation work. Poor recoveries (67%) were reported by one laboratory at the low concentration spiking level in beetroot (150 mg/kg) although this does not appear to have affected their results. Participants were instructed not to apply a recovery factor to the results which they reported.

The chromatographic conditions used by participants in the trial proper are given in Table 41. Some participants expressed concern at the overall analysis time of 55 minutes for these five common preservatives in a single run. However using the gradient system developed for this trial greatly shortens the analysis time required for an isocratic separation (>2 hours with excessive peak broadening, especially of the parabens), or as is usual carrying out two separate runs, one for benzoic and sorbic acids and another for the parabens. Moreover, the method which has been developed in this work will in effect allow the simultaneous determination of preservatives E200 to 219, the limits for which are expressed as the free acid in the current regulations[10].

Table 24.

Recovery data for base materials spiked with benzoic acid at 150 and 325 mg/kg for orange squash and cola and 250 and 2250 mg/kg for beetroot, pie filling and salad cream carried out prior to analysing the collaborative trial samples.

Lab	Orange squash (%)		Cola (%)		Beetroot (%)		Pie filling (%)		Salad cream (%)	
	1st level	2nd level	1st level	2nd level	1st level	2nd level	1st level	2nd level	1st level	2nd level
1	102, 104	100, 101	97, 97	98, 98	95, 96	97, 96	98, 98	93, 90	98, 98	100, 99
2	93, 109	108, 79	93, 102	104, 103	86, 83	91, 89	103, 83	102, 106	80, 89	92, 88
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	88 106 98 88	82 105 88	98 90 90							
5	N/R	N/R	100.4, 101.7	100.6, 102.6	105.7, 104.7	94.2, 99.1	111.0, 110.8	99.2, 100.3	102.1, 103.3	97.6, 101.8
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	90.8 - 94.4, 101.6 - 104.8	98.4 - 99.8, 88.4 - 89.0	84.8 - 94.4	98.4 - 102.2	68.4 - 71.6, 80.0 - 82.4	91.8 - 94.1, 85.7 - 87.9	94.4 - 95.6, 96.0 - 97.2	94.8 - 96.7, 93.1 - 95.6	99.2 - 101.6, 94.8 - 98.4	102.8 - 106.8, 93.2 - 99.0
8	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
9	95 - 103	92 - 101	96 - 103	93 - 104	89 - 94	94 - 97	94 - 100	99 - 105	89 - 102	93 - 101
10	99.5, 95.8	98.4, 102.5	81.1, 90.6	92.2, 94.2	92.8, 95.3	96.7, 100.1	95.0, 99.6	102.9, 99.4	93.5, 97.1	101.1, 101.1

Table 25.

Table 25.

Recovery data for base materials spiked with sorbic acid at 150 and 325 mg/kg for orange squash and cola and 250 and 2250 mg/kg for beetroot, pie filling and salad cream carried out prior to analysing the collaborative trial samples.

Lab	Orange squash (%)		Cola (%)		Beetroot (%)		Pie filling (%)		Salad cream (%)	
	1st level	2nd level	1st level	2nd level	1st level	2nd level	1st level	2nd level	1st level	2nd level
1	99, 101	100, 100	99, 99	99, 99	97, 97	96, 96	99, 99	93, 90	98, 98	98, 98
2	83, 87	87, 92	97, 87	98, 101	80, 80	90, 98	81, 87	98, 110	87, 90	93, 90
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	101	86	101	89	95	109	85	99	95	91
5	N/R	N/R	100.4, 101.7	100.6, 102.6	105.7, 104.7	94.2, 99.1	111.0, 110.8	99.2, 100.3	102.1, 103.3	97.6, 101.8
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	90.8 - 94.4, 101.6 - 104.8	98.4 - 99.8, 88.4 - 89.0	84.8 - 94.4	98.4 - 102.2	68.4 - 71.6, 80.0 - 82.4	91.8 - 94.1, 85.7 - 87.9	94.4 - 95.6, 96.0 - 97.2	94.8 - 96.7, 93.1 - 95.6	99.2 - 101.6, 94.8 - 98.4	102.8 - 106.8, 93.2 - 99.0
8	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
9	95 - 103	92 - 101	96 - 103	93 - 104	89 - 94	94 - 97	94 - 100	99 - 105	89 - 102	93 - 101
10	99.5, 95.8	98.4, 102.5	81.1, 90.6	92.2, 94.2	92.8, 95.3	96.7, 100.1	95.0, 99.6	102.9, 99.4	93.5, 97.1	101.1, 101.1

Table 26.

Recovery data for base materials spiked with methyl 4-hydroxybenzoic acid at 150 and 325 mg/kg for orange squash and cola and 250 and 2250 mg/kg for beetroot, pie filling and salad cream carried out prior to analysing the collaborative trial samples..

Lab	Orange squash (%)		Cola (%)		Beetroot (%)		Pie filling (%)		Salad cream (%)	
	1st level	2nd level	1st level	2nd level	1st level	2nd level	1st level	2nd level	1st level	2nd level
1	97, 99	98, 98	98, 98	98, 98	96, 96	97, 96	99, 98	93, 89	97, 98	99, 99
2	82, 92	101, 80	89, 97	101, 92	92, 76	90, 89	101, 81	88, 98	88, 88	84, 83
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	104	98	103	100	95	106	100	99	96	91
5	N/R	N/R	100.4, 101.7	100.6, 102.6	105.7, 104.7	94.2, 99.1	111.0, 110.8	99.2, 100.3	102.1, 103.3	97.6, 101.8
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	90.8 - 94.4, 101.6 - 104.8	98.4 - 99.8, 88.4 - 89.0	84.8 - 94.4	98.4 - 102.2	68.4 - 71.6, 80.0 - 82.4	91.8 - 94.1, 85.7 - 87.9	94.4 - 95.6, 96.0 - 97.2	94.8 - 96.7, 93.1 - 95.6	99.2 - 101.6, 94.8 - 98.4	102.8 - 106.8, 93.2 - 99.0
8	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
9	95 - 103	92 - 101	96 - 103	93 - 104	89 - 94	94 - 97	94 - 100	99 - 105	89 - 102	93 - 101
10	99.5, 95.8	98.4, 102.5	81.1, 90.6	92.2, 94.2	92.8, 95.3	96.7, 100.1	95.0, 99.6	102.9, 99.4	93.5, 97.1	101.1, 101.1

Table 27.

Recovery data for base materials spiked with ethyl 4-hydroxybenzoic acid at 150 and 325 mg/kg for orange squash and cola and 250 and 2250 mg/kg for beetroot, pie filling and salad cream carried out prior to analysing the collaborative trial samples..

Lab	Orange squash (%)		Cola (%)		Beetroot (%)		Pie filling (%)		Salad cream (%)	
	1st level	2nd level	1st level	2nd level	1st level	2nd level	1st level	2nd level	1st level	2nd level
1	99, 101	99, 99	99, 100	100, 101	97, 98	95, 95	100, 100	92, 88	100, 99	99, 99
2	90, 97	94, 92	103, 96	101, 97	91, 90	91, 92	81, 90	93, 103	89, 88	90, 85
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	101	96	100	98	93	106	98	98	93	90
5	N/R	N/R	100.4, 101.7	100.6, 102.6	105.7, 104.7	94.2, 99.1	111.0, 110.8	99.2, 100.3	102.1, 103.3	97.6, 101.8
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	90.8 - 94.4, 101.6 - 104.8	98.4 - 99.8, 88.4 - 89.0	84.8 - 94.4	98.4 - 102.2	68.4 - 71.6, 80.0 - 82.4	91.8 - 94.1, 85.7 - 87.9	94.4 - 95.6, 96.0 - 97.2	94.8 - 96.7, 93.1 - 95.6	99.2 - 101.6, 94.8 - 98.4	102.8 - 106.8, 93.2 - 99.0
8	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
9	95 - 103	92 - 101	96 - 103	93 - 104	89 - 94	94 - 97	94 - 100	99 - 105	89 - 102	93 - 101
10	99.5, 95.8	98.4, 102.5	81.1, 90.6	92.2, 94.2	92.8, 95.3	96.7, 100.1	95.0, 99.6	102.9, 99.4	93.5, 97.1	101.1, 101.1

Table 28.

Recovery data for base materials spiked with propyl 4-hydroxybenzoic acid at 150 and 325 mg/kg for orange squash and cola and 250 and 2250 mg/kg for beetroot, pie filling and salad cream carried out prior to analysing the collaborative trial samples.

Lab	Orange squash (%)		Cola (%)		Beetroot (%)		Pie filling (%)		Salad cream (%)	
	1st level	2nd level	1st level	2nd level	1st level	2nd level	1st level	2nd level	1st level	2nd level
1	97, 99	98, 98	99, 99	99, 99	96, 97	95, 94	99, 99	92, 89	98, 98	99, 99
2	89, 95	94, 90	104, 91	94, 99	86, 87	88, 91	80, 101	94, 103	84, 84	91, 90
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	102	96	101	99	93	107	98	99	94	90
5	N/R	N/R	100.4, 101.7	100.6, 102.6	105.7, 104.7	94.2, 99.1	111.0, 110.8	99.2, 100.3	102.1, 103.3	97.6, 101.8
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	90.8 - 94.4, 101.6 - 104.8	98.4 - 99.8, 88.4 - 89.0	84.8 - 94.4	98.4 - 102.2	68.4 - 71.6, 80.0 - 82.4	91.8 - 94.1, 85.7 - 87.9	94.4 - 95.6, 96.0 - 97.2	94.8 - 96.7, 93.1 - 95.6	99.2 - 101.6, 94.8 - 98.4	102.8 - 106.8, 93.2 - 99.0
8	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
9	95 - 103	92 - 101	96 - 103	93 - 104	89 - 94	94 - 97	94 - 100	99 - 105	89 - 102	93 - 101
10	99.5, 95.8	98.4, 102.5	81.1, 90.6	92.2, 94.2	92.8, 95.3	96.7, 100.1	95.0, 99.6	102.9, 99.4	93.5, 97.1	101.1, 101.1

Table 29.

Trial proper recovery data for orange squash base material spiked at 150 and 325 mg/kg with all analytes and analysed in conjunction with orange squash J.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg
1	102	107	104	107	103	107	106	107	104	106
2	102	84	87	92	100	82	98	93	97	91
3	102	104	85.3	100.6	94.7	93.8	93.3	100.6	N/R	N/R
4	101	98	103	102	103	103	100	100	100	101
5	104.7	107.4	106.7	110.8	103.3	110.8	105.3	112.0	105.3	112.0
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	N/R	104.5	100.9	95.0	98.3	94.4	98.5	93.7	98.9	95.0
8	100.5	104.4	103	111	102	110	105	112	102	111
9	104.7	102.7	101.7	101.5	102.7	103.6	99.0	98.7	96.7	99.3
10	89.0	96.7	90.7	99.7	89.7	99.1	89.4	97.9	96.8	99.7

Table 30.

Trial proper recovery data for orange squash base material spiked at 150 and 325 mg/kg with all analytes and analysed in conjunction with orange squash D.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg
1	102	107	104	107	103	107	106	107	104	106
2	102	84	87	92	100	82	98	93	97	91
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	101	98	103	102	103	103	100	100	100	101
5	104.7	107.4	106.7	110.8	103.3	110.8	105.3	112.0	105.3	112.0
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
8	100.5	104.4	103	111	102	110	105	112	102	111
9	104.7	102.7	101.7	101.5	102.7	103.6	99.0	98.7	96.7	99.3
10	89.0	96.7	90.7	99.7	89.7	99.1	89.4	97.9	96.8	99.7

Table 31

Trial proper recovery data for cola base material spiked at 150 and 325 mg/kg with all analytes and analysed in conjunction with cola K.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg
1	105	106	104	106	103	106	104	106	104	106
2	102	103	85	101	91	90	97	97	97	94
3	100	100.9	98	102.5	88	93.8	96.6	99.0	90	95
4	99	99	104	103	105	104	103	102	104	103
5	118.7	116.8	120.0	117.5	118.7	116.0	120.7	117.8	123.3	119.4
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	94.0	90.8	94.9	91.9	95.5	92.6	94.4	91.3	97.1	92.8
8	106	109	109	107	108	105	111	108	106	106
9	99.4	102.5	97.0	100.8	98.4	101.8	94.7	98.2	95.0	99.1
10	65.8	77.7	79.3	86.8	91.7	96.4	93.4	95.4	91.7	94.4

Table 32.

Trial proper recovery data for cola base material spiked at 150 and 325 mg/kg with all analytes and analysed in conjunction with cola W.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg
1	105	106	104	106	103	106	104	106	104	106
2	102	103	85	101	91	90	97	97	97	94
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	99	99	104	103	105	104	103	102	104	103
5	112.0	114.2	116.7	117.2	114.7	116.0	116.7	116.9	116.0	117.2
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
8	106	109	109	107	108	105	111	108	106	106
9	99.4	102.5	97.0	100.8	98.4	101.8	94.7	98.2	95.0	99.1
10	65.8	77.7	79.3	86.8	91.7	96.4	93.4	95.4	91.7	94.4

Table 33.

Trial proper recovery data for cola base material spiked at 150 and 325 mg/kg with all analytes and analysed in conjunction with cola G.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg
1	105	106	104	106	103	106	104	106	104	106
2	102	103	85	101	91	90	97	97	97	94
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	99	99	104	103	105	104	103	102	104	103
5	118.7	116.8	120.0	117.5	118.7	116.0	120.7	117.8	123.3	119.4
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
8	106	109	109	107	108	105	111	108	106	106
9	99.4	102.5	97.0	100.8	98.4	101.8	94.7	98.2	95.0	99.1
10	65.8	77.7	79.3	86.8	91.7	96.4	93.4	95.4	91.7	94.4

Table 34.

Trial proper recovery data for cola base material spiked at 150 and 325 mg/kg with all analytes and analysed in conjunction with cola S.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg	150 mg/kg	325 mg/kg
1	105	106	104	106	103	106	104	106	104	106
2	102	103	85	101	91	90	97	97	97	94
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	99	99	104	103	105	104	103	102	104	103
5	112.0	114.2	116.7	117.2	114.7	116.0	116.7	116.9	116.0	117.2
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
8	106	109	109	107	108	105	111	108	106	106
9	99.4	102.5	97.0	100.8	98.4	101.8	94.7	98.2	95.0	99.1
10	65.8	77.7	79.3	86.8	91.7	96.4	93.4	95.4	91.7	94.4

Table 35.

Trial proper recovery data for beetroot base material spiked at 250 and 2250 mg/kg with all analytes and analysed in conjunction with beetroot H.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg
1	106	97	107	97	106	96	108	97	107	96
2	91	89	80	98	90	89	90	92	87	91
3	107.6	100.9	109	101	98	106	102	94	94	94
4	101	98	98	98	100	99	97	97	98	98
5	114.0	108.6	115.2	107.2	114.0	108.7	115.6	108.4	116.4	110.5
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	67.2	71.5	67.3	84.6	67.2	85.5	67.4	85.8	68.1	85.7
8	108	101	108	102	105	104	109	101	107	103
9	94.8	96.5	93.6	96.0	94.8	96.5	90.0	95.6	90.8	95.2
10	94.2	96.9	94.4	105.6	94.2	94.9	93.7	97.1	91.5	93.7

Table 36.

Trial proper recovery data for beetroot base material spiked at 250 and 2250 mg/kg with all analytes and analysed in conjunction with beetroot B.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg
1	106	97	107	97	106	96	108	97	107	96
2	91	89	80	98	90	89	90	92	87	91
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	101	98	98	98	100	99	97	97	98	98
5	114.0	108.6	115.2	107.2	114.0	108.7	115.6	108.4	116.4	110.5
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
8	108	101	108	102	105	104	109	101	107	103
9	94.8	96.5	93.6	96.0	94.8	96.5	90.0	95.6	90.8	95.2
10	94.2	96.9	94.4	105.6	94.2	94.9	93.7	97.1	91.5	93.7

Table 37.

Trial proper recovery data for pie filling base material spiked at 250 and 2250 mg/kg with all analytes and analysed in conjunction with pie filling A.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg
1	109	100	110	100	108	99	110	100	109	99
2	103	106	87	110	81	98	92	103	88	103
3	111.6	98.4	112	98	106.4	97.6	104	93.3	104.8	95.4
4	144	101	139	101	137	102	139	100	139	101
5	114.8	111.6	114.8	106.9	114.0	110.4	115.6	109.8	115.2	111.8
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	77.0	66.3	75.5	78.9	76.1	80.3	75.7	79.5	81.6	79.6
8	96	100	104	99	99	100	104	99	101	100
9	101.8	103.9	100.4	104.9	101.8	106.0	97.2	104.9	98.6	103.6
10	96.1	104.8	93.2	113.1	90.8	99.5	92.1	102.5	90.0	98.5

Table 38.

Trial proper recovery data for pie filling base material spiked at 250 and 2250 mg/kg with all analytes and analysed in conjunction with pie filling P.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg
1	109	100	110	100	108	99	110	100	109	99
2	103	106	87	110	81	98	92	103	88	103
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	144	101	139	101	137	102	139	100	139	101
5	114.8	111.6	114.8	106.9	114.0	110.4	115.6	109.8	115.2	111.8
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
8	96	100	104	99	99	100	104	99	101	100
9	101.8	103.9	100.4	104.9	101.8	106.0	97.2	104.9	98.6	103.6
10	96.1	104.8	93.2	113.1	90.8	99.5	92.1	102.5	90.0	98.5

Table 39.

Trial proper recovery data for salad cream base material spiked at 250 and 2250 mg/kg with all analytes and analysed in conjunction with salad cream C.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg
1	109	98	108	97	108	97	110	98	108	97
2	89	88	90	90	88	83	88	85	90	84
3	94.4	103	98.4	100	97.6	102.3	100.0	94.2	94.8	95.7
4	101	102	98	102	99	103	97	101	97	102
5	107.6	102.6	119.6	108.4	114.4	109.2	116.8	108.6	117.6	110.7
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	81.1	68.9	84.6	88.3	85.4	88.1	83.5	86.9	81.1	85.7
8	106	104	116	104	113	106	116	104	113	105
9	94.8	99.4	95.1	100.3	94.5	100.6	90.5	99.2	87.9	97.6
10	90.7	103.0	90.4	113.5	87.7	100.5	88.9	104.1	88.5	99.5

Table 40.

Trial proper recovery data for salad cream base material spiked at 250 and 2250 mg/kg with all analytes and analysed in conjunction with salad cream L.

Lab	Benzoic acid (%)		Sorbic acid (%)		Methyl parabens (%)		Ethyl parabens (%)		Propyl parabens (%)	
	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg	250 mg/kg	2250 mg/kg
1	109	98	108	97	108	97	110	98	108	97
2	89	88	90	90	88	83	88	85	90	84
3	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
4	101	102	98	102	99	103	97	101	97	102
5	107.6	102.6	119.6	108.4	114.4	109.2	116.8	108.6	117.6	110.7
6	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
7	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
8	106	104	116	104	113	106	116	104	113	105
9	94.8	99.4	95.1	100.3	94.5	100.6	90.5	99.2	87.9	97.6
10	90.7	103.0	90.4	113.5	87.7	100.5	88.9	104.1	88.5	99.5

KEY TO TABLES 13 TO 40.

N/R Participant did not report any data.

(a) An outlying result as determined by Cochran's Test at $P<0.01$ level, not used in calculation of statistical parameters.

(b) An outlying result as determined by Grubb's Test

(c) Result excluded from the statistical analysis due to non compliance.

obs. The observed mean, the mean obtained from the collaborative trial data.

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 $r = 0.66_R$.

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.

Table 41.

Chromatography conditions used by participants for analysis of the trial samples.

Lab	Column and dimensions	Flow rate (mL/min)	Changes made to gradient system
1	Kromasil 100 5 C18, 250 × 4.6 mm (Hichrom)	1.0	0-24 min 100% A, 24-29 min→100%B, 29-45 min 100% B, 45-50 min →100% A, 50-55 min 100%A
2	Kromasil 100 5 C18, 250 × 4.6 mm (Hichrom)	1.0	None
3	Kromasil 100 5 C18, 250 × 4.6 mm (Hichrom)	1.0	None
4	Kromasil 100 5 C18, 250 × 4.6 mm (Hichrom)	1.0	0-31 min 100% A, 31-36 min→100%B, 36-50 min 100% B, 50-55 min →100% A
5	Kromasil 100 5 C18, 250 × 4.6 mm (Hichrom)	1.0	None
6	Kromasil 100 5 C18, 250 × 4.6 mm (Phenomenex)	1.0	None
7	Kromasil 100 5 C18, 250 × 4.6 mm (Hichrom)	1.1	23 min 100% B, 28 min 100% B, 44 min 100% B
8	Kromasil 100 5 C18, 250 × 4.6 mm (Hichrom)	1.0	None
9	Kromasil 100 5 C18, 250 × 4.6 mm (Hichrom)	1.0	None
10	Kromasil 100 5 C18, 250 × 4.6 mm (Hichrom)	1.0	None

CONCLUSION

A robust method for the determination of benzoic, sorbic and the methyl, ethyl and propyl esters of 4-hydroxybenzoic acid has been validated by a collaborative trial conducted to internationally agreed procedures.

The method is recommended for publication as a MAFF Validated Method.

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APPENDIX I:

THE DETERMINATION OF BENZOIC ACID, SORBIC ACID AND HYDROXYBENZOATES IN FOOD BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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 describes the determination of benzoic acid, sorbic acid and the methyl, ethyl and propyl esters of 4-hydroxybenzoic acid in foods, whose content of each analyte is in the range 50 - 2000 mg/kg.

2. DEFINITION

Benzoic acid, sorbic acid and the methyl, ethyl and propyl esters of 4-hydroxybenzoic acid are determined as individual entities. Each analyte content is the concentration as determined under the specified conditions.

3. PRINCIPLE

Liquid foods not containing insoluble matter are diluted with methanol. Other foods are extracted by shaking with methanol, centrifuging and filtering. The concentrations of the analytes in the clear extracts are measured using reversed-phase liquid chromatography with UV detection.

4. REAGENTS

4.1 All reagents should be of laboratory or analytical reagent grade unless otherwise stated.

4.2 Water should be de-ionised, distilled or of similar quality, filtered through a 0.45 μm filter before use.

4.3 Methanol, HPLC grade, filtered through a 0.45 μm filter before use.

4.4 Acetonitrile, HPLC grade, filtered through a 0.45 μm filter before use.

4.5 Mobile Phase.

4.5.1 0.1 M tri-sodium citrate solution. Dissolve analytical grade tri-sodium citrate (29.41 g) in water (1 L). Filter through a 0.45 μm filter.

4.5.2 0.1 M citric acid solution. Dissolve analytical grade citric acid (21.01 g) in water (1 L). Filter through a 0.45 µm filter.

4.5.3 Mobile phase A

Place 40 mL of 0.1M tri-sodium citrate solution (**4.5.1**), 60 mL of 0.1 M citric acid solution (**4.5.2**) in a measuring cylinder and make up to 800 mL with water, mix thoroughly. Add 200 mL of acetonitrile (**4.4**) and mix thoroughly.

De-gas the mobile phase before use by passing a slow stream of helium gas through it. Alternatively de-gassing may be carried out by sonication in an ultrasonic bath.

4.5.4 Mobile phase B

Place 40 mL of 0.1M tri-sodium citrate solution (**4.5.1**), 60 mL of 0.1 M citric acid solution (4.5.2) in a measuring cylinder and make up to 600 mL with water, mix thoroughly. Add 400 mL of acetonitrile (**4.4**) and mix thoroughly.

De-gas the mobile phase before use by passing a slow stream of helium gas through it. Alternatively de-gassing may be carried out by sonication in an ultrasonic bath.

4.6 Benzoic acid, (minimum 99% purity).

4.7 Sorbic acid, (minimum 99% purity).

4.8 Methyl 4-hydroxybenzoate, (minimum 99% purity).

4.9 Ethyl 4-hydroxybenzoate, (minimum 99% purity).

4.10 Propyl 4-hydroxybenzoate, (minimum 99% purity).

4.11 Standard Solutions

4.11.1 Prepare a separate standard solution of each of the five analytes by weighing accurately 0.500 g of each compound (**4.6, 4.7, 4.8, 4.9, 4.10**) into a separate beaker. Dissolve each compound in methanol (**4.3**) and quantitatively transfer the solutions to 100 mL volumetric flasks using further portions (10 mL each) of methanol (**4.3**) to rinse the beakers. Adjust the volume of the solution in each flask to the calibration mark by the addition of methanol (**4.3**) and mix. Each solution contains a concentration of 5000 mg/L.

4.11.2 Prepare a combined standard solution containing all five analytes by pipetting 20.0 mL of each analyte solution (**4.11.1**) into a 200 mL volumetric flask, adding mobile phase A (**4.5.3**) up to the calibration mark, and mixing. This solution contains a concentration of 500 mg/L of each of the five analytes.

4.11.3 Using a burette or pipettes measure 1.0, 2.0, 5.0, 15.0, 25.0 and 40.0 mL of the combined standard solution (**4.11.2**) into separate 100 mL volumetric flasks, adding mobile phase A (**4.5.3**) up to the calibration

marks and mixing. These solutions respectively contain 5, 10, 25, 75, 125 and 200 mg/L of each analyte.

NOTE: Standard solutions prepared at 4.11.3 are stable for up to 5 days when stored at 4 °C

5. APPARATUS

5.1 Normal laboratory glassware and apparatus.

5.2 Analytical balance accurate to 0.005 g.

5.3 HPLC system with UV detector, recording integrator or recorder.

5.4

The following conditions have been shown to be satisfactory.

Guard Column	*Kromasil C18, 5 µm, 10 × 3.2 mm with cartridge holder*	
Column	*Kromasil 100-5C18, 250 × 4.6 mm*	
Detector	*UV detector*	
Wavelength	*223 nm for benzoic acid and 258 nm for sorbic acid, methyl 4-, ethyl 4- and propyl 4-hydroxybenzoate*	
Mobile Phase	*80% Citric Acid/Sodium Citrate Buffer 20% Acetonitrile (A)*	
	60% Citric Acid/Sodium Citrate Buffer 40% Acetonitrile (B)	
Gradient system	*0 min - 26 min*	*100% A*
	26 min - 31 min	*go to 100% B*
	31 min - 45 min	*100% B*
	45 min - 50 min	*go to 100% A*
	50 min - 55 min	*100% A*
Flow Rate	*1.0 mL/min.*	
Injection Volume	*20 µL*	
Column Temperature	*Ambient*	

Under these conditions the analytes elute in the order

1. benzoic acid
2. sorbic acid
3. methyl 4-hydroxybenzoate
4. ethyl 4-hydroxybenzoate
5. propyl 4-hydroxybenzoate

The approximate retention times are 13.9, 17.0, 24.2, 35.8 and 42.9 min respectively.

5.4 Centrifuge with appropriate centrifuge tubes (approximately 50 mL capacity) with screw caps or other suitable closures.

5.5 0.45 µm filtration apparatus.

5.6 Whatman Filter Paper No.1 or equivalent.

5.7 Vortex mixer.

6. PROCEDURE

6.1 Preparation of Calibration Graphs

Inject 20 µL of each of the standard solutions (**4.11.3**). Plot the peak area obtained for each analyte in each standard solution on the vertical

axis versus the corresponding analyte concentration in mg/L, along the horizontal axis to give the five calibration graphs.

6.2 Sample Preparation

Homogenise the sample. The portion of prepared sample not immediately required for analysis should be placed in an air-tight container and stored in such a way that deterioration and change in composition is prevented.

6.3 Liquid Samples Not Containing Insoluble Matter.

6.3.1 Weigh, to the nearest 0.001 g, about 10 g of prepared sample (**6.2**) and dilute with methanol (**4.3**) to 100 mL in a volumetric flask and mix. Pass this solution through a 0.45 µm filter (**5.5**) to eliminate any particulate matter.

6.3.2 Confirm that the HPLC system is operating correctly by injecting the combined 20 mg/L standard solution (**4.11.3**), then inject 20 µL of the sample filtrate (**6.3.1**) on to the HPLC column (**5.3**). After the analyte peak or peaks have been eluted and a steady base-line is re-attained repeat the injection. Inject 20 µL of a combined standard (**4.11.3**) solution after every 4th injection. If the amount of analyte(s) in the extract is high an aliquot of the extract should be diluted with mobile phase A (**4.5.3**) such that the concentration in the diluted extract is within the range used in the calibration graphs and an appropriate dilution factor used in the calculation.

6.4 Other Samples

6.4.1 Weigh, to the nearest 0.001 g, about 10 g of prepared sample (**6.2**) into a centrifuge tube (**5.4**).

6.4.2 Add methanol (**4.3**) (20 mL) and close the tube. Vortex mix (**5.7**) the sample and methanol (4.3) to ensure a uniform suspension and then extract the sample by shaking vigorously for 2 minutes.

6.4.3 Centrifuge (5.4) at a relative centrifugal force (RCF) of approximately 2630 for 5 minutes and decant off the methanol layer into a 100 ml volumetric flask.

NOTE: Since the centrifuge is to be used with methanolic extracts it should be emphasised that tubes with screw caps or other suitable closures are required.

6.4.4 Repeat steps **6.4.2** and **6.4.3** twice with further portions of methanol (**4.3**) (20 mL each). It is particularly important to vortex mix during re-extraction as the solid matter can be difficult to disperse. Care is also needed in decanting the methanol (**4.3**) layer from a sample containing a high oil content to ensure that none of the oil layer is decanted with the methanol (**4.3**).

6.4.5 Combine the extracts in the 100 ml volumetric flask and make up to the calibration mark by the addition of methanol (**4.3**). Shake to obtain a homogeneous solution.

NOTE: For high fat percentage foodstuffs it is advisable to include a freezing out stage for the combined extracts at the end of the extraction procedure (after 6.4.4). This can be performed by placing the sample in dry ice for approximately 20 minutes until the fat has solidified, decanting the methanolic solution and then proceeding by making to volume with methanol, as in 6.4.5.

6.4.6 Filter the solution through a filter paper (**5.6**), rejecting the first few mL and collect about 15 mL. Filter this through a 0.45 μm filter (**5.5**).

6.4.7 Carry out the chromatographic analysis on the filtered extract as in **6.3.2**.

6.4.8 A reagent blank should be determined with each batch of samples. If the blank is 2 mg/kg or more the determination should be repeated using fresh reagents, otherwise ignore it.

6.5 Recovery Check

This should be carried out on at least one in every ten samples to be analysed. Using a standard solution (**4.11**) of the five analytes add an appropriate volume (dependant on sample type) to a further portion of a prepared sample to be analysed, homogenise and apply the method procedure commencing at **6.2**.

7. CALCULATION

7.1 Determine the mean value of the two peak areas for each analyte obtained from the two injections made for each sample extract. Using this mean value obtain from the calibration graph (6.1) the concentration of each of the analytes in the extract and hence calculate the concentration of each analyte in the sample from the formula given in **7.2**.

7.2 The concentration of each analyte in the sample is given by:

$$\text{Analyte (mg/kg)} \quad \frac{C \times 100}{M} \times f$$

where C = concentration of analyte in extract, mg/L

M = mass of sample, g

f = dilution factor for extract (if applied at 6.3.2)

8. EXPRESSION OF RESULTS

Report the results as mg/kg.

APPENDIX II
COMMENTS FROM PARTICIPANTS

GENERAL COMMENTS RECEIVED ON METHOD FOR TRIAL 103

LABORATORY 5

50 milligram is a very small weight of standard to weigh out. Would it not be preferable to take a larger quantity and include a further dilution step at section 5.11.27?

I am concerned (from a health and safety point of view) at the amount of methanol used to prepare the standard solutions. In particular is it necessary to prepare the combined standard at 5.11.2 in methanol? A consequence of this is that the working standards at 5.11.3 contain widely varying ratios of methanol to mobile phase.

Similarly is it essential to use methanol for all the sample extraction steps? Could mobile phase not be used instead?

Since the centrifuge is to be used with methanol extracts it should be specified as being explosion-proof (i.e. the vital need to use screw cap tubes or other suitable closures should be made clearer).

LABORATORY 7

I am surprised that the calibration standards are made up with a significant volume of mobile phase when no attempt is made to matrix match the sample extracts. Surely band broadening will be evident in the samples but not in the standards.

I am never happy with a gradient system running from 100% A to 100% B because of the demands placed upon the pumps (we operate high pressure mixing) to perform consistently at the extremes of the gradient profile.

COMMENTS RECEIVED FROM PARTICIPANTS ON TRIAL 103

LABORATORY 1

Standard solutions were injected every fifth injection.

When centrifuging pie fillings (6.4.4) the 'plug' could not be broken up by centrifuging alone. The plug was broken up with a glass rod prior to vortex mixing.

Benzoic acid was quantified at 224 nm, other analytes were quantified at 258 nm.

LABORATORY 3

The base material labelled orange and cola contained benzoic acid at 834 mg/L and 158 mg/l respectively these values were allowed for when calculating recoveries.

LABORATORY 4

The chromatographic method is considered too long to allow the use of this as a routine method.

It was necessary to discard the first 10 mL of filtrate to be used for the chromatography to ensure recovery of benzoic and sorbic acids.

The pie filling formed a solid mass at the bottom of the centrifuge tube which could not be broken up by vortexing and shaking after the first extraction cycle.

Some peak shifting was observed.

The column performance (in theoretical plates) fell during the course of the trial.

As shown by the pre-trial the column is inconsistent from batch to batch.

LABORATORY 5

Run times are long making the method unsuitable for routine use.

Method is not robust with regard to the HPLC column. It seems that only the column specified is satisfactory and even this is batch specific.

Problems were experienced during the analysis of orange samples used in the "pre-trial". Unidentified peaks were found to interfere with the benzoic acid, and virtually co-elute with the sorbic acid. An example of a sample used was Safeway High Juice Orange Squash.

Working standards should more closely match the samples by being prepared in methanol.

Pipetting small volumes (<1 mL) of a methanolic spiking solution is not satisfactory.

LABORATORY 6

Propyl parabens seriously affected by drift of retention time. Insufficient time to remedy the problem. It is possible some peaks were lost completely because of this, and some results came from the subsequent chromatogram.

LABORATORY 7

The problem with the original column was only one of our problems. A lab which varies by as much as 10-15°C over 24 hours does not make for consistent chromatography and circuit breakers which trip 3 hours into a 36 hour run do not help.

LABORATORY 8

We obtained the specified column from Hichrom as recommended. However, the test chromatogram did not give the required separation/peak shape.

The method appears to be so highly dependant on the column that even another batch of nominally the same packing material gives completely different (and unsatisfactory) performance. In my opinion such a criticality is unacceptable, whether the method is intended for routine or regulatory purposes.

55 minutes is an extremely long run time. Even with automated equipment only 24 injections can be made in a day, which translates to substantially less than this once standards and QA samples have been included in the batch.

Without automated equipment an 8-hour day allows just 5 sample injections even if only a single standard is used and QA limited to one duplicate and one spike.

Given the fact that a gradient is already in use, and that there is a long delay before elution of the hydroxybenzoates, I would recommend a change in gradient during the run to bring the latter off faster and complete the run in less time.

We encountered problems in dispensing the small volumes of strong spiking solution, primarily due to the use of methanol as solvent. In my opinion it would be better to use a less volatile solvent - perhaps aqueous methanol, or even fully aqueous solutions of the sodium salts.