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Analytical methods for food additives

Roger Wood, Lucy Foster, Andrew Damant and Pauline Key

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Contents

Introduction xi

1 E110: Sunset yellow 1
1.1 Introduction 1
1.2 Methods of analysis 1
1.3 Recommendations 2
1.4 References 2
1.5 Appendix: method procedure summaries 4
Table 1.1 Summary of methods for sunset yellow in foods 6
Table 1.2 Summary of statistical parameters for sunset yellow in foods 10
Table 1.3 Performance characteristics for sunset yellow in lemonade (pre-trial samples) 14
Table 1.4 Performance characteristics for sunset yellow in bitter samples 14

2 E122: Azorubine (carmoisine) 15
2.1 Introduction 15
2.2 Methods of analysis 15
2.3 Recommendations 16
2.4 References 16
2.5 Appendix: method procedure summaries 17
Table 2.1 Summary of methods for azorubine in foods 19
Table 2.2 Summary of statistical parameters for azorubine in foods 21
Table 2.3 Performance characteristics for azorubine in collaborative trial samples 23
Table 2.4 Performance characteristics for azorubine in bitter samples 23

3 E141: Copper complexes of chlorophylls and chlorophyllins 24
3.1 Introduction 24
3.2 Methods of analysis 24
3.3 Recommendations 25
3.4 References 25
Table 3.1 Summary of methods for Cu complexes of chlorophylls and chlorophyllins in foods 26
Table 3.2 Summary of statistical parameters for Cu complexes of chlorophylls and chlorophyllins in foods 26

4 E150c: Caramel class III 27
4.1 Introduction 27
4.2 Methods of analysis 27
4.3 Recommendations 27
4.4 References 28
Table 4.1 Summary of methods for caramel (class III) 29

5 E160b: Annatto extracts 30
5.1 Introduction 30
5.2 Methods of analysis 30
5.3 Recommendations 31
5.4 References 31
Table 5.1 Summary of methods for annatto extracts in foods . . 32
Table 5.2 Summary of statistical parameters for annatto extracts in foods 34

6 E200–3: Sorbic acid and its salts 35
6.1 Introduction 35
6.2 Methods of analysis 35
6.3 Recommendations 36
6.4 References 36
6.5 Appendix: method procedure summaries 37
Table 6.1 Summary of methods for sorbic acid in foods 42
Table 6.2 Summary of statistical parameters for sorbic acid in foods 48
Table 6.3 Performance characteristics for sorbic acid in almond paste, fish homogenate and apple juice 52
Table 6.4 Performance characteristics for sorbic acid in orange squash, cola drinks, beetroot, pie filling and salad cream 53

7 E210–13: Benzoic acid 54
7.1 Introduction 54
7.2 Methods of analysis 54

7.3 Recommendations 55
7.4 References 55
7.5 Appendix: method procedure summaries 57
Table 7.1 Summary of methods for benzoic acid in foods 62
Table 7.2 Summary of statistical parameters for benzoic acid in foods 67
Table 7.3 Performance characteristics for benzoic acid in almond paste, fish homogenate and apple juice 70
Table 7.4 Performance characteristics for benzoic acid in orange juice 71
Table 7.5 Performance characteristics for benzoic acid in orange squash, cola drinks, beetroot and pie filling 72

8 E220–8: Sulphites 73
8.1 Introduction 73
8.2 Methods of analysis 73
8.3 Recommendations 75
8.4 References 76
8.5 Appendix: method procedure summaries 77
Table 8.1 Summary of methods for sulphites in foods 87
Table 8.2 Summary of statistical parameters for sulphites in foods 90
Table 8.3 Performance characteristics for sulphites in hominy, fruit juice and seafood 92
Table 8.4 Performance characteristics for sulphites in wine, dried apples, lemon juice, potato flakes, sultanas and beer 92
Table 8.5 Performance characteristics for total sulphite in shrimp, orange juice, dried apricots, dehydrated potato flakes and peas . . 94
Table 8.6 Performance characteristics for total sulphite in starch, lemon juice, wine cooler, dehydrated seafood and instant mashed potatoes 95
Table 8.7 Performance characteristics for total sulphite in shrimp, potatoes, pineapple and wine 96
Table 8.8 Performance characteristics for free sulphite in wine . 97

9 E249–50: Nitrites 98
9.1 Introduction 98
9.2 Methods of analysis 98
9.3 Recommendations 100
9.4 References 100
9.5 Appendix 1: method procedure summaries (meat – DD ENV 12014) 101
9.6 Appendix 2: method procedure summaries (milk and milk products – BS EN ISO 14673) 106

Table 9.1 Summary of methods for nitrites in foods 118
Table 9.2 Summary of statistical parameters for nitrites in foods 122
Table 9.3 Performance characteristics for nitrite in meat products 126
Table 9.4 Performance characteristics for nitrite in foods 127

10 E297: Fumaric acid and its salts 128
10.1 Introduction 128
10.2 Methods of analysis 128
10.3 Recommendations 129
10.4 References 129
10.5 Appendix: method procedure summaries 131
Table 10.1 Summary of methods for fumaric acid in foods . . . 132
Table 10.2 Summary of statistical parameters for fumaric acid in foods 138
Table 10.3 Performance characteristics for fumaric acid in
Table 10.4 Performance characteristics for fumaric acid in lager beers 141

11 E310–12: Gallates 142
11.1 Introduction 142
11.2 Methods of analysis 142
11.3 Recommendations 142
11.4 References 143
11.5 Appendix: method procedure summaries 144
Table 11.1 Summary of methods for gallates in foods 146
Table 11.2 Summary of statistical parameters for gallates in foods 150
Table 11.3 Performance characteristics for gallates in oils, lard and butter oil 152

12 E320: BHA 153
12.1 Introduction 153
12.2 Methods of analysis 153
12.3 Recommendations 154
12.4 References 154
12.5 Appendix: method procedure summaries 155
Table 12.1 Summary of methods for BHA in foods 157
Table 12.2 Summary of statistical parameters for BHA in foods 162
Table 12.3 Performance characteristics for BHA in oils, lard and butter oil 165

13 E334–7, E354: L-tartaric acid and its salts 166
13.1 Introduction 166
13.2 Methods of analysis 166
13.3 Recommendations 167
13.4 References 167
13.5 Appendix: method procedure summaries 167
Table 13.1 Summary of methods for L-tartaric acid in foods . . 169
Table 13.2 Summary of statistical parameters for L-tartaric acid in foods 172
Table 13.3 Performance characteristics for L-tartaric acid in grape juices 173

14 E355–7, E359: Adipic acid and its salts 174
14.1 Introduction 174
14.2 Methods of analysis 174
14.3 Recommendations 175
14.4 References 175
14.5 Appendix 1: method procedure summaries (analysis of orange drinks) 176
14.6 Appendix 2: method procedure summaries: analysis of starch 177
Table 14.1 Summary of methods for adipic acid in foods 179
Table 14.2 Summary of statistical parameters for adipic acid in foods 181
Table 14.3 Performance characteristics for adipic acid in orange drink samples 182
Table 14.4 Performance characteristics for adipic acid in acetylated adipyl cross-linked starches 182

15 E405, E477: Propylene glycol (propan-1,2-diol) 183
15.1 Introduction 183
15.2 Methods of analysis 183
15.3 Recommendations 184
15.4 References 184
Table 15.1 Summary of methods for propylene glycol in foods 185
Table 15.2 Summary of statistical parameters for propylene glycol in foods 186

16 E416: Karaya gum 187
16.1 Introduction 187
16.2 Methods of analysis 187
16.3 Recommendations 188
16.4 References 188
Table 16.1 Summary of methods for karaya gum 189

17 E432–6: Polysorbates 190
17.1 Introduction 190
17.2 Methods of analysis 190
17.3 Recommendations 191
17.4 References 191
Table 17.1 Summary of methods for polysorbates in foods . . . 192
Table 17.2 Summary of statistical parameters for polysorbates in foods 194

18 E442: Ammonium phosphatides 196
18.1 Introduction 196
18.2 Methods of analysis 197
18.3 Recommendations 197
18.4 References 197
Table 18.1 Summary of methods for phosphorus in foods 198
Table 18.2 Summary of statistical parameters for phosphorus in foods 199
Table 18.3 Performance characteristics for total phosphorus in collaborative trial samples 200

19 E444: Sucrose acetate isobutyrate 201
19.1 Introduction 201
19.2 Methods of analysis 201
19.3 Recommendations 201
19.4 References 202
19.5 Appendix: method procedure summary 202
Table 19.1 Summary of methods for sucrose acetate isobutyrate in foods 204
Table 19.2 Summary of statistical parameters for sucrose acetate isobutyrate in foods 204

20 E472e: Mono/diacetyl tartaric acid esters of mono/diglycerides of fatty acids 205
20.1 Introduction 205
20.2 Methods of analysis 205
20.3 Recommendations 206
20.4 References 206
Table 20.1 Summary of methods for mono/diacetyl tartaric acid esters of mono/diglycerides of fatty acids in foods 207
Table 20.2 Summary of statistical parameters for mono/diacetyl tartaric acid esters of mono/diglycerides of fatty acids in foods 208

21 E476: Polyglycerol esters of polycondensed fatty acids of castor oil 209
21.1 Introduction 209
21.2 Methods of analysis 209
21.3 Recommendations 209
21.4 References 210
Table 21.1 Summary of methods for polyglycerol polyricinoleate in foods 211

22 E481–2: Stearoyl lactylates 212
22.1 Introduction 212
22.2 Methods of analysis 212
22.3 Recommendations 213
22.4 References 213
Table 22.1 Summary of methods for stearoyl lactylates in foods 214
Table 22.2 Summary of statistical parameters for stearoyl lactylates in foods 214

23 E483: Stearyl tartrate 215
23.1 Introduction 215
23.2 Methods of analysis 215
23.3 Recommendations 215

24 E491–2, E493–4, E495: Sorbitan esters 216
24.1 Introduction 216
24.2 Methods of analysis 216
24.3 Recommendations 216
24.4 References 217
Table 24.1 Summary of methods for sorbitan esters in foods . 218
Table 24.2 Summary of statistical parameters for sorbitan esters in foods 219

25 E520–3, E541, E554–9, E573: Aluminium 220
25.1 Introduction 220
25.2 Methods of analysis 220
25.3 Recommendations 221
25.4 References 221
Table 25.1 Summary of methods for aluminium in foods 223
Table 25.2 Summary of statistical parameters for aluminium in foods 226
Table 25.3 Performance characteristics for aluminium in milk powder 228
Table 25.4 Summary of key steps of procedures used in IUPAC sample survey 229

26 E954: Saccharin 230
26.1 Introduction 230
26.2 Methods of analysis 230
26.3 Recommendations 231
26.4 References 232
26.5 Appendix: method procedure summaries 233
Table 26.1 Summary of methods for saccharin in foods 241
Table 26.2 Summary of statistical parameters for saccharin in foods 246
Table 26.3 Performance characteristics for saccharin in sweetener tablets 248
Table 26.4 Performance characteristics for saccharin in liquid sweetener 249
Table 26.5 Performance characteristics for sodium saccharin in marzipan, yogurt, orange juice, cream, cola and jam 250
Table 26.6 Performance characteristics for sodium saccharin in juice, soft drink and sweets 251
Table 26.7 Performance characteristics for sodium saccharin in juice, soft drink and dessert 252

Index 253

Introduction

Additives are added to food to perform different technological functions, for example, to increase shelf life (preservatives), or to protect against rancidity (antioxidants). The use of additives in food is controlled by separate legislation relating to, for example, colours in food, sweeteners, miscellaneous additives (other than colours and sweeteners) and flavourings. Most areas of food additives legislation (with the exception of additives in flavourings, additives in other additives (i.e. other than carriers/solvents) and controls on enzymes/processing aids) have been fully harmonised throughout the European Union for a number of years. The initial groundwork for this was laid down by the Food Additives Framework Directive (89/107/EEC). Indeed, UK legislation covering the main groups of food additives is based on European Community Directives, which were agreed during 1994 and 1995. Under these legislative requirements (including amendments), most additives are permitted only in certain specified foods, at specified maximum levels (although some are generally permitted at levels of '*quantum satis*'). However, only additives that have been approved for safety by the European Commission's Scientific Committee on Food are included in the legislation and are identifiable by their designated E number in the relevant Directives.

Food additive-based research and surveillance carried out by organisations such as The Food Standards Agency aims to support consumer protection by providing the best possible scientific evidence to ensure that the use of food additives does not prejudice food safety. Much of the Agency's work has concentrated on developing and validating appropriate methodology to measure levels of additives in food. This work has ranged from feasibility studies to acquire a better understanding of factors affecting additive intakes to the development of appropriate test protocols. Development of food surveillance methodology is also integral to improving understanding of additive exposure through collation of

information on additive levels and usage. This information is needed to monitor additive levels in foods, changes in dietary behaviour and patterns of additive use, and to fulfil European Community legislation requirements for Member States to monitor food intakes. A preliminary European Commission monitoring exercise carried out in the European Union has identified several additives or additive groups that require further review by Member States.*

To ensure consumer safety, existing intake estimations and safety monitoring of additives need refining, and information is required to compare actual levels of additive use and consumption with safety guidelines (acceptable daily intakes) set by the EU Scientific Committee on Food. To obtain this information, robust quantitative methods of analysis are required to measure levels of additives in a broad range of food matrices, as several additives or groups of additives with similar functions may coexist within a single food matrix. A variety of published analytical methods are available in the literature, particularly for artificial food colours, preservatives and sweeteners. However, the availability of reliable methodology for some of the more analytically complex additives, such as emulsifiers, natural colours and polysaccharide gums is limited by the inherent compositional complexity of these substances and the variability of food matrices in which they occur.

To meet this problem, a review of published analytical methods has been compiled which seeks to identify those additives for which methods are incomplete, i.e. protocols which only cover a limited range of permitted foods, or are missing. For this exercise, selection of additives for review was based on additive use in foods (at permitted levels and *quantum satis*), availability of dietary intake information and analyte complexity (chemical form). Additives selected were those where more information is required in terms of additive level and usage to refine intake estimates. However, information is generally lacking for these additives because robust methods are not available for analysis due to the complexity of the additive/matrix. Therefore the law cannot be enforced.

The additives listed below have been identified as requiring more information in terms of their level and usage. The E number and name are given below:

E110	Sunset yellow
E122	Azorubine
E141	Copper complexes of chlorophylls and chlorophyllins
E150c	Caramel class III
E160b	Annatto extracts
E200–3	Sorbic acid and its salts
E210–13	Benzoic acid
E220–8	Sulphites
E249–50	Nitrites
E297	Fumaric acid and its salts

*Council of the European Union, Report from the Commission on dietary food additive intake in the European Union, document DENLEG 47, 2001.

E310–12	Gallates
E320	BHA
E334–7, E354	L-tartaric acid and its salts
E355–7, E359	Adipic acid and its salts
E405, E477	Propylene glycol
E416	Karaya gum
E432–6	Polysorbates
E442	Ammonium phosphatides
E444	Sucrose acetate isobutyrate
E472e	Mono/diacetyl tartaric acid ester of mono/diglycerides of fatty acids
E476	Polyglycerol esters of polycondensed fatty acids of castor oil
E481–2	Stearoyl lactylates (including calcium and sodium stearoyl lactylate)
E483	Stearyl tartrate
E491–2, E493–4 and E495	Sorbitan esters
E520–3, E541, E554–9 and E573	Aluminium
E954	Saccharin

This review considers the published methodology available for the extraction and analysis of a specific additive or group of additives. The present status of the methodology is also assessed for each additive and information on the most widely used available methods for the determination of the additive in specified foods is detailed, including the performance characteristics where these are available. Some recommendations for future research to improve method availability are also given. For each of the additives an introduction, a summary of the available methods of analysis, any recommendations and appropriate references are given. There are also tables which summarise the available methods, the available statistical performance parameters for the methods and results of any collaborative trials that may have been carried out on the method. Provision of this information should help analysts estimate the concentration of any of the additives of interest in foods. Where 'gaps' in methodology have been identified, then these are mentioned in the recommendations and may lead to research being carried out to develop appropriate methods for these additives. It is becoming increasingly common for method criteria to be incorporated in legislation rather than particular methods of analysis being prescribed. This means that methods of analysis used for control purposes, or for due diligence purposes, should meet certain specified minimum analysis requirements. It will then become increasingly helpful to food analysts for information in this format to be made readily available.

It should be noted that the contents of the book reflect the authors' views and not those of the Food Standards Agency.

1

E110: Sunset yellow

1.1 Introduction

The major food groups contributing to dietary intake of sunset yellow are confectionery, emulsified sauces, soft drinks and chocolate products; the maximum permitted level of 500 mg/kg is allowed in sauces, seasonings, pickles, relishes, chutney, piccalilli; decorations and coatings; salmon substitutes; surimi. The acceptable daily intake (ADI) for sunset yellow is 2.5 mg/kg body weight.

1.2 Methods of analysis

The general scheme for identifying coal-tar dyes present in foods normally involves:[1]

1 Preliminary treatment of the food.
2 Extraction and purification of the dye from the prepared solution or extract of the food.
3 Separation of mixed colours if more than one is present.
4 Identification of the separated dyes.

There are numerous methods published for the determination of sunset yellow in foodstuffs. The majority of these methods are for the determination of various water-soluble dyes, including sunset yellow, in foodstuffs. The early workers on the development of methods for food colours used paper chromatography and TLC but over the last 20 years HPLC,[2–8] spectrophotometric,[9–15,22] voltammetric[20,21] and more recently capillary zone electrophoresis[16–19] methods have been developed and a summary of these is given in Table 1.1, together with the matrices to which the methods apply. If statistical parameters for these methods are available they are

summarised in Table 1.2. The majority of published methods are for the determination of sunset yellow in liquid matrices i.e. drinks, therefore further development of extraction procedures is necessary to adapt methods for other food matrices i.e. chocolate products.

A suitable method for the analysis of sunset yellow in soft drinks was collaboratively trialled.[2] The method consisted of a quantitative extraction, as ion pairs with cetylpyridinium chloride, from aqueous solutions into *n*-butanol. The sunset yellow was analysed using reversed phase, ion pair gradient elution HPLC with diode array detection. A summary of the procedure for this method is given in the Appendix and the performance characteristics are given in Table 1.3.

A reverse phase HPLC method for the analysis of six dyes including sunset yellow was applied to a number of food samples (three beverages, gelatin dessert and a strawberry flavoured syrup) and found to be suitable.[3] Separation was performed on a Nova-Pak C18 column using methanol–NaH_2PO_4/Na_2HPO_4, pH 7, buffer solution (0.1 M) as mobile phase with an elution gradient system and UV–vis detection at 520 nm. Under optimum conditions (details given in the Appendix) dyes were eluted in 4 min. A summary of the procedure for this method is given in the Appendix and a summary of the statistical parameters in Table 1.4. This method has also been used to compare the results for the simultaneous determination of dyes in foodstuffs when new methods have been developed i.e. by capillary zone electrophoresis.[16]

1.3 Recommendations

For sunset yellow analytical methods using extraction followed by spectoroscopy[1] are in place for a full range of beverages, sauces, starchy and fatty foods. There are no recent publications for sunset yellow in chocolate products, therefore this is an area that requires method development.

1.4 References

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1.5 Appendix: method procedure summaries

Analysis of soft drinks[2]

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.

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. Add 5 mL 0.1 mol/L cetylpyridinium chloride in water, mix and add 10 mL of water-saturated *n*-butanol. Shake vigorously for 10 min on mechanical shaker. Centrifuge at 1000 g for 5 min and transfer upper organic layer to a 25 mL volumetric flask using a Pasteur pipette. Repeat the procedure with three 5 mL portions of water-saturated *n*-butanol.

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

Quantitative determination: HPLC
Load 20 µL of sample extract onto column and use gradient (linear) elution to achieve optimum separation.

Column	Spherisorb C8, 250 × 4.6 mm, 5 µm
Guard column	packed with 40 µm reverse phase material (e.g. Perisorb RP8 30–40 µm
Mobile phase	60 % Solution B and 40 % Solution A linear gradient to 80 % Solution B and 20 % Solution A after 20 min
Flow rate	1.5 mL/min
Detector	430 nm
Solution A	Phosphate buffer 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 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 to avoid frothing.
Solution B	Cetylpyridinium chloride solution is diluted 50 mL to 1 L with a 1 L + 1 L dilution of acetonitrile and methanol.

Analysis of beverages[3]

Sample preparation
The samples were prepared as follows:

1 Quantitative determination by direct preparation using calibration graphs: 5 mL of the sample was transferred to a 25 mL flask and diluted with deionised water to the mark.
2 Quantitative determination by standard addition: to 5 mL of the beverage sample were added different amounts (2, 4, 6, 8 mg/L) of the dye to determine and proceed as before.

Analysis of beverages
The samples were filtered through a Millipore filter before being injected into the chromatographic system and all the experiments were carried out in duplicate.

HPLC conditions

Column	Nova-Pak C18	
Mobile phase	Eluent A	Methanol
	Eluent B	NaH_2PO_4/Na_2HPO_4 buffer solution 0.1 M pH=7
Gradient profile	t_0(initial)	20 % eluent A, 80 % eluent B
	t_1(2 min)	100 % eluent A
	t_2(4 min)	100 % eluent A
	t_0(5 min)	20 % eluent A, 80 % eluent B
Flow rate	2 mL/min	
Injection volume	20 µL	
Detection	520 nm	

Table 1.1 Summary of methods for sunset yellow in foods

(a)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
IP-RP-HPLC	Lemonade	Ion pairs with cetylpyridinium chloride from aqueous solutions into *n*-butanol	Spherisorb C8	Gradient elution (1.5 mL/min) with phosphate buffer containing cetylpyridinium chloride, acetonitrile and methanol	Diode-array at 430 nm	2
RP-HPLC	Bitter	Diluted with water and filtered	Nova-Pak C18	Gradient elution (2 mL/min) using methanol and 0.1 M sodium phosphate buffer at pH 7	520 nm	3
Ion-pair reversed-phase HPLC	Fruit juice soy sauce	Neutralised with aq 50 % NH_3 and centrifuged	Zorbax ODS	Gradient elution (1 mL/min) MeOH–CH_3CN–0.02 M-triammonium citrate (10:1:39), to methanol (1:1)	254 nm	4
HPLC	Beverages and foods		Altex Ultra-sphere TM ODS	Gradient elution with 0.2 N ammonium acetate and 18 to 100 % methanol		5
HPLC	Beverages	Neutralised with aq NH_3 and filtered	μBondapak C18	Gradient elution (2 mL/min) with 20 mM ammonium acetate aq and methanol	230 nm	6
High-performance ion chromatography	Drinks and instant powder drinks	Diluted with water and filtered	Dionex Ion Pac AS11	Gradient elution (1.5 mL/min) with HCl:water:acetonitrile, 50 μL injection	480 nm	7

(b)

Method	Matrix	Sample preparation	Extraction	Detection	Reference
Ion-pair HPLC	Beverages, gelatine, syrups	Diluted with water and filtered	Nova-Pak C18 column with gradient elution (1.5 mL/min) with methanol-phosphate buffer of pH 7 containing 5 mM tetra-butylammonium bromide	520 nm	8
Spectro-photometry visible	Commercially available dyes	Diluted with water and ultrasonicated	Computer program that determines concentration of mixtures of 4 compounds by comparing their spectra with standard spectra	MULTv3.0 Quimio program	9
Solid-phase spectrophotometry	Soft drinks, fruit liqueurs and ice-cream	Filtered food samples were diluted to 100 mL with the addition of 5 mL 1 M acetate buffer at pH 5 and 10 mL ethanol	The mixture was agitated with 50 mg Sephadex DEAE A-25gel. The solid phase was extracted and packed into 1 mm cells for spectrophotometric determination	487 nm	10
Spectro-photometric	Soft drinks	Ion-pair formation with octadecyl-trimethylammonium bromide at pH 5.6	Extraction of the ion-pair into *n*-butanol	485 nm	11
Solid-phase spectrophotometry	Soft drinks, sweets and fruit jellies	Samples dissolved in water and filtered	The colourants were fixed in Sephadex DEAE A-25gel at pH 2.0 and packed into 1 mm cells for spectrophotometric determination	Between 400 and 800 nm. Partial least squares (PLS) multivariate calibration used	12

Table 1.1 cont'd

(c)

Method	Matrix	Sample preparation	Method conditions	Detection	Reference
First-derivative spectrophotometry	Confectionery products	Samples diluted 5–20 g in 100 mL water		350–700 nm. First derivative spectrum was obtained	13
Simultaneous spectrophotometry	Candy and carbonated drinks	Food samples were diluted to 25 mL with the addition of 5 mL acetate buffer at pH 4.5 and water	The colourants were isolated from the food matrices by SPE using polyamide sorbent packed into 1 mm cells for spectrophotometric determination	300–700 nm in 5 nm intervals. First and, second derivatives were analysed by (PLS) multivariate calibration	14
Derivative spectrophotometric ratio spectrum-zero crossing	Commercial products	This method is applied to samples containing 3 dyes to determine each dye under optimum conditions	No separation step is required. Method was used to determine synthetic mixtures of these dyes in different ratios from 1:1:1 to 1:5:5 or even higher		15
Capillary zone electrophoresis (CZE)	Non-alcoholic beverages and fruit-flavoured syrups	Samples used as is or diluted with water	A background solution consisting of 15 mM borate buffer at pH 10.5, hydrodynamic injection and a 20 kV separation voltage	216 nm	16
CZE	Beverages – strawberry and orange drinks	Sample, either concentrated or directly after filtration was applied for determination by CZE	Uncoated quartz column operated at separation voltage 28 kV with 10 mM $KH_2PO_4/Na_2B_4O_7$/3 % ethanol at pH 11 as background electrolyte	254 nm	17

High-performance capillary electrophoretic (HPCE)	Ice-cream bars and fruit soda drinks	Direct injection of liquids	pH 9.5 borax–NaOH buffer containing 5 mM β-cyclodextrin	Diode-array	18
Micellar electrokinetic capillary chromatography (MECC)	Cordials and confectionery	5 g sample was extracted with 25 mL water–methanol (4:1). 1 mL 0.05 M tetrabutylammonium phosphate was added and extracted by adsorption onto C18 Sep-Pak cartridge and elution with methanol	Fused-silica capillary column operated at 30 kV with a buffer of 0.05 M sodium deoxycholate in 5 mM NaH_2PO_4/5 mM sodium borate at pH 8.6/acetonitrile (17:3)	214 nm	19
Ratio derivative voltammetry	Soft drinks	Samples were dissolved in water, warmed to dissolve completely and filtered	Measurements were carried out directly using an HMDE (hanging mercury dropping electrode)		20
Square wave adsorptive voltammetry	Refreshing drinks	Samples were diluted with water	Measurements were made directly. Sunset yellow in 0.5 M NH_4Cl/NH_3 buffer solution gave an adsorptive stripping voltammetric peak at the hanging mercury drop electrode at: –0.60V using an accumulation potential of –0.40V		21
Integrated solid phase spectrophotometric-FIA	Drinks	Samples (3 mL) into a 10 mL volumetric flask made up to volume with carrier solution and analysed by the flow procedure at 4 mL/min	When the flow cell contains C18 silica the sunset yellow is transported across the filled cell measuring the absorbance increase at 487 nm		22

Table 1.2 Summary of statistical parameters for sunset yellow in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
IP-RP-HPLC	Lemonade	Full collaborative trial	see Table 1.3	2
RP-HPLC	Bitter	Performance of method established with standards (n=9) and validated with real samples	Linear range of calibration 2–10 mg/L Determination limit 4 ng Recoveries 88.1–106.0 % CV 3.5 % Bitter sample (n=9) see Table 1.4	3
SP spectro-photometry	Soft drinks, sweets, fruit jellies	Performance of method established and applied to 7 real samples (n=5)	Linear range 50–650 ng/mL SD 5.5267 RSD 6.07 % square of correlation coefficient 0.9977 RSD 1.8–7.6 % for commercial samples Orange drinks: 3.68 mg/L RSD 3.5 % (n=5) Pineapple jelly: 3.68 mg/L RSD 3.5 % (n=5) Orange drink: 3.20 mg/L RSD 4.7 % (n=5) Honey sweet: 0.19 mg/L RSD 1.8 % (n=5) Colourant: 845.0 mg/L RSD 2.5 % (n=5) Fruit jelly: 0.66 mg/L RSD 7.6 % (n=5) Melon drink: 23.60 mg/L RSD 6.3 % (n=5)	12
IP-HPLC	Commercial products	Performance of method established with standards (n=9) and validated with commercial food products	Calibration graph linear from 2–10 mg/L SD 0.071 mg/L RSD 4.22 % Detection limit 1.4 ng Recovery 99.1 % (n=5) Real samples: Bitter: 16.7±0.3 mg/L Grenadine: 29.5±0.3 mg/L Gelatine: 160.0±0.4 mg/kg	8
HPIC	Drinks	Performance of method established and validated with 3 real samples	Linear range 2.0–40 µg/mL Recoveries of spiked samples 94.7–109 % (n=4) RSD 2.01 % at 20 µg/mL (n=7) Detection limit 2.0 µg/mL Real samples: 42.5±1.0 µg/mL, 67.0±1.4 µg/mL, 176.0±4.0 µg/mL (n=4).	7

Square wave adsorptive voltammetry	Refreshing drinks	Performance of method established and applied to 3 real samples (n=5)	Calibration graph linear in the range 5–90 µg/L RSD = 2.2 % for a solution of 30 µg/L (n=10) in the same day. The determination limit was 5 µg/L Tof (lemon) 192±4 µg/L (n=5) Gatorade (lemon) 5790±116 µg/L (n=5) Refreshing drink (orange) 2142±42 µg/L (n=5)	21
CZE cf HPLC[3]	Non-alcoholic beverages and flavoured syrups	Performance of method established and applied to real samples	Calibration graph linear up to 4–200 mg/L Detection limit 0.38 mg/L Recoveries were 92.3–111.3 % for 4–60 mg/L dyes from synthetic mixtures Real samples: Ice lolly: 11.0±0.2 mg/L by CZE (n=3) 10.7±0.2 mg/L by HPLC (n=3)	16
Spectro-photometric	Soft drinks	Performance of method established and applied to real samples	Linear range 0–60 µg/mL Recovery 99 % (n=6) RSD 1.9 % for 8 µg/mL (n=10) Sparkling orange drink: 9.32 µg/mL (n=3) {9.6} RSD 0.1 % Results agree with manufacturers' values {}	11
Integrated solid phase spectrophoto-metric-FIA	Drinks	Performance of method established and applied to a real sample	Concentration range 0.5–20 mg/L Detection limit 0.2 mg/L RSD = 1.6 % Mango liqueur 39.44±1.334 µg/L (n=3) Results for sample compare with HPLC data for this sample	22
Derivative spectrophoto-metric ratio spectrum-zero crossing	Commercial products	Performance of method established and applied to real samples	Calibration graph linear up to 40 mg/L SD 0.8 % at 8 mg/L Recovery 94–105 % Results for samples compare with HPLC data for these samples	15
SP spectro-photometry	Soft drinks, liqueurs, ice-cream	Performance of method established and applied to real samples	Linear range 15–500 ng/mL Detection limit 3.5 ng/mL RSD 2.8 % for 150 ng/mL	10

Table 1.2 cont'd

Method	Matrix	Extent of validation	Statistical parameters				Reference
Spectro-photometric	Commercial dyes	Performance of method established on standards	Recovery 93.81–106.1 % SD 4.03 mg/L RSD 4.0 % for 100 mg/L				9
CZE	Beverages	Performance of method established and applied to a soft drink sample	Calibration graph linear Recoveries 95–103 % RSD 2.2–5.8 %				17
First derivative spectro-photometry	Confectionery products	Method applied to 2 real samples (n=5)	Recovery 92.1–107.9 % Real samples:	Sugar candy: Jelly:	122.0±1.8 µg/g 3.2±0.2 µg/g	(n=5) (n=5)	13
Ratio derivative voltammetry	Soft drinks	Method applied to 3 commercial products (n=3)	Calibration graph linear (r = 0.9997) Recoveries 88–110 % Orange juice Fruit juice Merida orangeade	32.4 µg/mL 8.9 µg/mL 49.9 µg/mL	SD = 0.8 SD = 0.5 SD = 1.5	(n=3) (n=3) (n=3)	20
Simultaneous spectro-photometry	Candy and carbonated drinks	Method applied to 2 real samples (n=3)	Real samples: Results agree with manufacturers' values {}	Soft drink: Candy:	13.71 mg/L (n=3) {14.00} 8.45 mg/kg (n=3) {8.51}		14

HPCE	Ice-cream bars and soda drinks	Method applied to a real sample (n=3)	Calibration graph linear Commercial soda drink:	RSD of migration time 0.49 % 9.34 µg/mL		RSD 3.81 %	(n=7) (n=3)	18
MECC	Cordials and confectionery	Method applied to commercial products	Calibration graph linear up to 100 µg/mL Reporting limit 5 mg/kg Results for samples compare with HPLC data for these samples			RSD 1.9–4.3 %		19
IP-RP-HPLC	Fruit juice, soy sauce	Method applied to soy sauce	Recoveries 91–113 %	CV 0.4–3.7 %				4
HPLC	Beverages and foods	Method applied to commercial products	Recoveries 96.7–101 %					5
HPLC	Beverages	Method applied to beverages	Recoveries 92–108 %	CV 0.4–4.0 %				6

Table 1.3 Performance characteristics for sunset yellow in lemonade (pre-trial samples)[2]

Sample	Lemonade
Analyte	Sunset yellow
No. of laboratories	10
Units	mg/kg
Mean value	23.9 34.4
S_r	1.67
RSD_r	5.7 %
r	4.69
S_R	3.57
RSD_R	12.2 %
R	10.0
Ho_R	1.3

Key

Mean	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).
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 the reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

Table 1.4 Performance characteristics for sunset yellow in bitter samples[3]

Sample	Bitter kalty	
Analyte	Sunset yellow	
Quantification method	Direct measurement	Standard addition
Number of determinations	2	2
Units	mg/L	mg/L
Mean value	7.8±0.2	7.3±0.3
	Statistical parameters for assay	
Number of determinations	9	
Calculated by	Peak height	Peak area
Units	mg/L	
SD	0.056	0.046
RSD	±3.72	±2.85
Detection limit	25.3	4.0

2

E122: Azorubine (carmoisine)

2.1 Introduction

The major food groups contributing to dietary intake of azorubine are chocolate products, confectionery, emulsified sauces and soft drinks with the maximum permitted level of 500 mg/kg being allowed in the same matrices as for sunset yellow i.e. sauces, seasonings, pickles, relishes, chutney and piccalilli; decorations and coatings; salmon substitutes; surimi. The ADI for azorubine is 4 mg/kg body weight/day.

2.2 Methods of analysis

Azorubine is also a coal-tar dye and the general scheme for identifying these dyes present in foods is the same as for sunset yellow.[1]

There are many methods published for the determination of azorubine in foodstuffs. The majority of these are for the determination of various water-soluble dyes, including azorubine, in foodstuffs and some of these methods are the same as for sunset yellow. The early workers on the development of methods for food colours used paper chromatography and TLC but over the last 20 years HPLC,[2–4,6,7] spectrophotometric[8–11] and more recently capillary zone electrophoresis[5] methods have been developed and a summary of these is given in Table 2.1, together with the matrices to which they apply. If statistical parameters for these methods were available these have been summarised in Table 2.2. The majority of published methods are for the determination of azorubine in liquid matrices i.e. drinks, therefore further development of extraction procedures would be necessary to adapt methods for other food matrices i.e. chocolate products.

A suitable method for the analysis of azorubine in soft drinks and flour-based products was collaboratively trialled.[2] The method consisted of a quantitative extraction, as ion pairs with cetylpyridinium chloride, from aqueous solutions into

n-butanol. The azorubine was analysed using reversed phase, ion pair gradient elution HPLC with diode array detection. A summary of the procedure for this method is given in the Appendix for this chapter and the performance characteristics are given in Table 2.3. The method was also used for skimmed milk using the sample preparation and extraction procedure as for soft drinks. If the extraction procedure had been followed for flour-based products the performance characteristics would probably have been improved.

A reverse phase HPLC method for the analysis of six dyes including azorubine (carmoisine) was applied to a number of food samples (three beverages, gelatin dessert and a strawberry-flavoured syrup and found to be suitable.[3] Separation was performed on a Nova-Pak C18 column using methanol–NaH_2PO_4/Na_2HPO_4, pH 7, buffer solution (0.1 M) as mobile phase with an elution gradient system and UV–vis detection at 520 nm. Under optimum conditions (details given in the Appendix) dyes were eluted in 4 min. The procedure for this method is given in the Appendix with a summary of the statistical parameters being given in Table 2.4. This method has also been used to compare the results for the simultaneous determination of dyes in foodstuffs when new methods have been developed i.e. by capillary zone electrophoresis.[5]

2.3 Recommendations

For azorubine, analytical methods using extraction followed by spectoroscopy[1] are in place for a full range of beverages, sauces and starchy and fatty foods. There are no recent publications for azorubine in chocolate products, therefore this is an area that requires method development.

2.4 References

1 *Pearson's Composition and Analysis of Foods*, 9 ed. Kirk R and Sawyer R, Longman Scientific, Harlow (1989).
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2.5 Appendix: method procedure summaries

For the analysis of soft drinks the method is the same as for sunset yellow but sample preparation and extraction are modified for flour-based products.

Analysis of flour-based products[2]

Sample preparation
Accurately weigh 5 g of sample into a 50 mL beaker. De-fat the sample by stirring and decanting with 3 × 50 mL portions of petroleum spirit 40–60 at a temperature no greater than 40 ºC. Discard petroleum spirit and air-dry the sample at ambient temperature under a fume hood with occasional stirring.

Extraction
Transfer the air-dried de-fatted sample to centrifuge tube. Add 10 mL 0.05 mol/L phosphate buffer pH 7.0. Add 100 mg α-amylase and incubate at 40 ºC for 2 h in a shaking water bath or by regular manual shaking. Add 5 mL 0.1 mol/L cetylpyridinium chloride in water, mix and add 10 mL of water-saturated *n*-butanol. Shake vigorously for 10 min on mechanical shaker. Centrifuge at 1000 g for 10 min. If a gel forms in the upper organic layer, add 2 mL water-saturated *n*-butanol and gently stir into the upper layer, with a glass rod, until emulsion breaks. Transfer upper organic layer to a 25 mL volumetric flask using a Pasteur pipette. Repeat the extraction procedure with three further 5 mL portions of water-saturated *n*-butanol. Make the combined *n*-butanol extracts up to 25 mL with water-saturated *n*-butanol. Accurately dilute an aliquot of the filtrate with an equal volume of mobile phase (1L + 1L dilution of mobile phase A and solution B). Mix and filter a portion through a filter.

Quantitative determination: HPLC
Load 20 μL of sample extract onto column and use gradient (linear) elution to achieve optimum separation. The same HPLC conditions were used as for sunset yellow in soft drinks but the detector was set at 520 nm for azorubine.

Analysis of beverages[3]
The same sample preparation, analysis and HPLC conditions as used for sunset yellow (Chapter 1, Appendix) were used to determine azorubine.

Table 2.1 Summary of methods for azorubine in foods

(a)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
IP-RP-HPLC	Lemonade, cake crumb, skimmed milk	Ion pairs with cetylpyridinium chloride from aqueous solutions into *n*-butanol	Spherisorb C8	Gradient elution (1.5 mL/min) with phosphate buffer containing cetylpyridinium chloride, acetonitrile and methanol	Diode-array at 520 nm	2
RP-HPLC	Bitters	Diluted with water and filtered	Nova-Pak C18	Gradient elution (2 mL/min) using methanol and 0.1 M sodium phosphate buffer at pH 7	520 nm	3
HPLC	Beverages, gelatine, syrups	Diluted with water and filtered	Nova-Pak C18	Gradient elution (1.5 mL/min) with methanol–phosphate buffer at pH 7 (1:4) containing 5 mM tetrabutyl ammonium bromide	520 nm	4
HPLC	Yogurt	Shaken with 5 % NH_3. Acetone added and shaken. Centrifuged supernatant concentrated to remove acetone. Adjust to pH 4. Shake with polyamide. Centrifuge. The polyamide washed 3× with water and then shaken with MeOH–aqNH_3 (19:1)	MicroPak MCH-10	Gradient elution using TBA in methanol diluted with methanol–phosphate buffer at pH 7±0.05	254 nm	6
HPLC	Confect-ionery	Sweets stirred in methanol. Methanol extract diluted (1:10) in water and filtered 0.45 µm before injection	Spherisorb ODS-2 with LiChrospher RP-18 guard column	Water–acetonitrile (7:3) containing 5 mM octylamine/ orthophosphoric acid at pH 6.4 (1 mL/min)	520 nm	7

Table 2.1 cont'd

(b)

Method	Matrix	Sample preparation	Method conditions	Detection	Reference
Capillary zone electrophoresis (CZE)	Non-alcoholic beverages and fruit flavoured syrups	Samples used as is or diluted with water	A background solution consisting of 15 mM borate buffer at pH 10.5, hydrodynamic injection and a 20 kV separation voltage	216 nm	5
Spectro-photometric	Beverages, gelatine, syrups	Samples diluted in 5 mL acetate buffer and diluted to 25 mL with water	Analysed by spectrophotometry using a Beckman DU-70 instrument	427 nm	8
Solid-phase spectro-photometry	Colourings caramel, confectionery	Sample solution mixed with 1 M HCl, ethanol sufficient for a 10 % conc., water and Sephadex DEAE A-25 gel	The mixture was shaken for 15 min then the gel beads were filtered off, packed into a 1 mm cell and absorbance measured	Absorbance measured at 525 nm and 800 nm	9
Rapid clean-up method for spectro-photometric and TLC methods	Various foods	Liquid samples as is. Solid samples dissolved in water and filtered through sintered glass filter	Colour separated on reverse phase C18 Sep-Pak cartridge and eluted with aqueous isopropanol solutions	TLC or spectrophotometric	10
Spectro-photometric	Soft drinks	Ion-pair formation with octadecyl-trimethylammonium bromide at pH 5.6	Extraction of the ion-pair into *n*-butanol	550 nm	11

Table 2.2 Summary of statistical parameters for azorubine in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
Rapid clean-up method for spectro-photometric and TLC methods	Various foods	AOAC Official Method 988.13	Ref. *JAOAC* (1988), **71**, 458.	10
IP-RP-HPLC	Lemonade, cake crumb, skimmed milk	Full collaborative trial	see Table 2.3	2
RP-HPLC	Bitter	Performance of method established with standards (n=9) and validated with real samples	Linear range of calibration 2–10 mg/L, Recoveries 93.6–106.3 % CV 4.7 % Bitter sample (n=9) see Table 2.4	3
IP HPLC	Commercial products	Performance of method established with standards (n=9) and validated with commercial food products	Calibration graph linear from 2–10 mg/L SD 0.039 mg/L RSD 2.32 % Detection limit 7.6 ng Recovery 99.54 % (n=5) Real samples: Bitter: 34.3±0.1 mg/L Syrup: 146.2±0.3 mg/kg	4

Table 2.2 cont'd

Method	Matrix	Extent of validation	Statistical parameters	Reference
CZE cf HPLC[3]	Non-alcoholic beverages and flavoured syrups	Performance of method established and applied to real samples	Calibration graph linear up to 4–200 mg/L Detection limit 0.60 mg/L Recoveries were 92.3–111.3 % for 4–60 mg/L dyes from synthetic mixtures Real samples: Bitter: 37.5±0.2 mg/L (CZE), 35.0±0.2 mg/L (HPLC) (n=3) Strawberry syrup: 141.9±0.4 mg/kg (CZE), 137.9±0.3 mg/kg (HPLC) (n=3)	5
Spectro-photometric	Soft drinks	Performance of method established and applied to real samples	Linear range 0–40 µg/mL Recovery 98 % (n=6) RSD 1.1 % for 8 µg/mL (n=10) Strawberry flavoured drink: 3.90 µg/mL (n=3) {4} RSD 0.1 % Results agree with manufacturers' values {}	11
SP spectro-photometry	Colourings, caramel, confectionery	Performance of method established and applied to 4 real samples (n=3)	Concentration range 12–650 µg/L Detection limit 3.38 µg/L RSD 1.3 % for samples containing 250 µg/L Caramel: 107.99±0.3 mg/L	9
Spectro-photometric	Beverages, gelatine, syrups	Performance of method established and applied to real samples	Calibration graph linear up to 32 mg/L Replicate samples 8 mg/L (n=9) RSD 3.44 % Detection limit 0.72 mg/L Recovery 95.3 % (n=10)	8
HPLC	Confectionery	Method applied to confectionery	Detection limit <12 µg/L	7
HPLC	Yogurt	Method specific for yogurt	Recovery 98 %	6

Table 2.3 Performance characteristics for azorubine in collaborative trial samples[2]

Sample	Lemonade	Cake crumb	Skimmed milk
Analyte	Azorubine	Azorubine	Azorubine
No. of laboratories	10	9	9
Units	mg/kg	mg/kg	mg/kg
Mean value	24.5 35.1	51.5 72.8	84.4 81.1
S_r	1.64	3.68	14.81
RSD_r	5.5 %	5.92 %	17.89 %
r	4.59	10.31	41.46
S_R	2.05	7.69	20.32
RSD_R	6.87 %	12.37 %	24.56 %
R	5.73	21.53	56.91
Ho_R	10.72	1.44	2.98

Key

Mean	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).
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 the reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

Table 2.4 Performance characteristics for azorubine in bitter samples[3]

Sample	Bitter kas		Bitter kalty	
Analyte	Azorubine		Azorubine	
Quantification method	Direct measurement	Standard addition	Direct measurement	Standard addition
Number of determinations	2	2	2	2
Units	mg/L		mg/L	
Mean value	33.3±0.1	32.8±0.2	18.5±0.1	17.5±0.3
	Statistical parameters for assay			
Number of determinations	9			
Calculated by	Peak height		Peak area	
Units	mg/L			
SD	0.041		0.040	
RSD	±2.40		±2.44	
Detection limit	4.1		1.9	

3

E141: Copper complexes of chlorophylls and chlorophyllins

3.1 Introduction

The major food groups contributing to dietary intake of copper complexes of chlorophylls and chlorophyllins are sugar confectionery, desserts, sauces and condiments, cheese and soups and soft drinks. The ADI for copper complexes of chlorophylls and chlorophyllins is 15 mg/kg body weight/day.

Sodium copper chlorophyllin (Cu-Chl-Na) is not a single substance but a mixture mainly consisting of copper chlorin e_6 and copper chlorin e_4. Copper chlorin e_6 is less stable and in some cases disappears as a result of pH and heat treatment during the manufacturing process of foods, whereas copper chlorin e_4 is relatively stable under these conditions and can be used as an indicator substance for the analysis of Cu-Chl-Na.[1]

3.2 Methods of analysis

The only references that could be found for copper complexes of chlorophylls and chlorophyllins were in Japanese[1,2] and both are HPLC methods. A summary of them is given in Table 3.1, together with the matrices for which the method is applicable. Statistical parameters for these methods, if available, are summarised in Table 3.2.

3.3 Recommendations

There are no recent methods published for copper complexes of chlorophylls and chlorophyllins in foods; therefore these need to be developed and validated by collaborative trial.

3.4 References

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Table 3.1 Summary of methods for Cu complexes of chlorophylls and chlorophyllins in foods

Method	Matrix	Sample preparation/extraction	Method conditions	Detection	Reference
HPLC	Boiled bracken, agar-agar, chewing gum	Sample homogenised after pH adjustment to 3–4 with 0.1 M HCl and extracted with ethyl ether, concentrated to dryness. Residue dissolved in MeOH	Inertsil ODS-2 column with MeOH–H_2O (97:3) mobile phase containing 1 % acetic acid	Photodiode array at 405 nm	1
HPLC	Chewing gum, candies, processed seaweeds, processed edible wild plants, chocolate	Sample was suspended in citrate buffer (pH 2.6), homogenised after adding ethyl acetate–acetone (5:1). Extracted with 1 % aq ammonia solution. Ethanol added to aqueous layer	Chemcosorb 5-ODS-UH column with MeOH–H_2O–acetic acid (100:2:0.5) mobile phase	Photodiode array at 625 nm	2

Table 3.2 Summary of statistical parameters for Cu complexes of chlorophylls and chlorophyllins in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
HPLC	Chewing gum, candies, processed seaweeds, processed edible wild plants, chocolate	Requires further validation	Determination limit 5 ng/g Recoveries in spiked food samples 90.7–102.5 % Sodium copper chlorophyllin detected at levels of 4.3–85.3 ng/g in 2 types of chewing gum and 2 types of candy produced in the UK	2

4

E150c: Caramel class III

4.1 Introduction

The major food groups containing caramel (Class III) are sauces and condiments, soft and carbonated drinks, pies and pastries, desserts, soup and cakes. The ADI for ammonia caramel is 200 mg/kg body weight/day. There are four classes of caramel colours used as food additives and they are defined by the reactant added to the carbohydrate during production. The reactant used in the production of Class III caramels is ammonia and so the product is sometimes called ammonia caramel.[1]

4.2 Methods of analysis

No references could be found for the analysis of caramel colour (Class III) in foods. The only reference that could be found was for the analysis of caramel colour (Class III) in general. This was an ion-pair HPLC and capillary electrophoresis method, developed to distinguish Class III caramels from Classes I and IV.[1] A summary of this method is given in Table 4.1.

4.3 Recommendations

This method produced a fingerprint peak that was present in only Class III samples and the observation of this fingerprint peak in foods could be used to indicate the presence of Class III caramel and permit a semi-quantitative estimation of the level of caramel in the foods. Therefore this method[1] needs to be further developed and applied to foods.

4.4 References

1 'Analysis for caramel colour (Class III)', Coffey J S, Castle L. *Food Chemistry* (1994) **51**, 413–416.

Table 4.1 Summary of methods for caramel (class III)

Method	Matrix	Sample preparation/extraction	Method conditions	Detection	Reference
IP-HPLC followed by CE	Caramels	Sample dissolved in distilled water used as is for HPLC method. For CE filtered through 2 µm syringe filter before analysis.	HPLC: ODS-2 column with gradient of 5 mM pentane-sulphonic acid in MeOH–H_2O (5:95) [A] and MeOH [B] mobile phases at 1 mL/min, 20 µL injection Capillary electrophoresis: Open bore capillary column. 30 mM phosphate buffer (pH 1.9) at 20 kV and 35 ºC. Injections in hydrokinetic mode, loading 1s.	Photodiode array at 275 nm	1

5

E160b: Annatto extracts

5.1 Introduction

The major food groups contributing to dietary intake of annatto extracts are such items as various cheeses, and snacks. The maximum permitted level of 50 mg/kg is allowed in Red Leicester cheese, 10–25 mg/kg in snacks and 10 mg/kg in liqueurs. The acceptable daily intake (ADI) for annatto extracts (as bixin) is 0.065 mg/kg body weight.

5.2 Methods of analysis

Annatto is a natural food colour and can be identified by characteristic colour reactions. In 'flavoured' milk it can be detected by pouring a few millilitres of milk into a flat dish, adding sodium bicarbonate solution and then inserting a strip of filter paper. After a few hours the paper is stained brown in the presence of annatto and turns pink on the addition of a drop of stannous chloride solution. In butter, annatto can be detected by the following method: divide an ethereal solution of isolated butterfat into two tubes. To one tube (A) is added 1–2 mL hydrochloric acid (1+1) and to (B) 1–2 mL 10 % sodium hydroxide solution. If annatto or other vegetable colour is present there is no colour in A, but a yellow colour appear in B.[1]

There are several methods published for the determination of annatto in foodstuffs. The traditional methods developed for annatto depend on its characteristic colour reactions.[1,2] More recently HPLC,[2–7] TLC[8,9] and photoacoustic spectrometry (PAS)[10] methods have been developed. A summary of these methods is given in Table 5.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 5.2.

5.3 Recommendations

Colorimetric methods and various HPLC methods have been developed for specific foods but these methods require validation and further development to adapt them for use with all relevant foodstuffs where annatto is permitted.

5.4 References

1 *Pearson's Composition and Analysis of Foods*, 9 ed. Kirk R and Sawyer R, Longman Scientific, Harlow (1989).

2 'AOAC Official Method 925.13. Coloring matter in macaroni products', *AOAC Official Method of Analysis* (2000) 32.5.15 p 55.

3 'Determination of annatto in high-fat dairy products, margarine and hard candy by solvent extraction followed by high-performance liquid chromatography', Lancaster F E, Lawrence J F. *Food Additives and Contaminants* (1995) **12**(1), 9–19.

4 'Analysis of annatto (*Bixa orellana*) food coloring formulations. 1. Determination of coloring components and colored degradation products by high-performance liquid chromatography with photodiode array detection', Scotter M J, Wilson L A, Appleton G P, Castle L. *Journal of Agricultural and Food Chemistry*. (1998) **46**(3), 1031–1038.

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6 'Identification of natural dyes added to food products', Tricard C, Cazabeil J M, Medina B. *Sciences Des Aliments* (1998) **18**(1), 25–40. [French]

7 'Supercritical fluid carbon dioxide extraction of annatto seeds and quantification of trans-bixin by high pressure liquid chromatography', Anderson S G, Nair M G, Chandra A, Morrison E. *Phytochemical Analysis* (1997) **8**(5), 247–249.

8 'Analysis of turmeric oleoresin, gardenia yellow and annatto extract in foods using reversed-phase thin layer chromatography/scanning densitometry', Ozeki L, Ueno E, Ito Y, Hayashi T, Itakura Y, Yamada S, Matsumoto H, Ito T, Maruyama T, Tsuruta M, Miyazawa T. *Journal of the Food Hygienic Society of Japan* (2000) **41**(6), 347–352. [Japanese]

9 Validation of Enforcement Methods Service (VEMS) Method 0240: *TLC method for colours, annatto and curcumin in foods, general.*

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Table 5.1 Summary of methods for annatto extracts in foods
(a)

Method	Matrix	Principle of method	Reference
Colour reaction	Macaroni products	80 % alcohol added to ground sample to extract colour, left overnight to precipitate proteins, filtered, evaporated, 25 % NaCl solution and slight excess of NH_4OH was added to filtrate. Transferred to separating funnel and extracted with petroleum ether. Combined petroleum ether extracts were washed with NH_4OH and acidified with CH_3COOH. In presence of $SnCl_2$ annatto produced a purple stain	2
Spectroscopic	Commercial annatto formulations	Oil-soluble annatto as bixin: 0.1 g to 200 mL 10 % acetic acid in chloroform. Diluted 1 in 10 with 3 % acetic acid in chloroform. Absorbance read at 505 and 474 nm. Water-soluble annatto as norbixin: 0.1 g to 200 mL 5 % acetic acid in chloroform. Diluted 1 in 10 with chloroform. Absorbance read at 503 and 473 nm	4
RP TLC/scanning densitometry	Foods	1 Clean-up with C18 cartridge. 2 Separation by reverse-phase C18-TLC using acetonitrile–THF–0.1 mol/L oxalic acid (7:8:7) as solvent system. 3 Measurement of visible absorption spectra using scanning densitometry	8
Photoacoustic spectrometry (PAS)	Commercial seasoning products	PAS was employed to determine the content of annatto via the intensity of an absorption peak compared with the absorption standard samples with a known content of annatto. Owing to strong absorption and saturation of the signal of the pigment in UV and vis regions, a peak of weak absorption in near-IR region was used, guaranteeing a linear relationship between peak intensity and annatto content for the usually applied low to medium levels of dye contents in commercial products	10

(b)

Method	Matrix	Sample preparation	Method conditions	Detection	Reference
HPLC	Cheese, butter, margarine and hard candy	20 g crushed candy dissolved in 50 mL water. Annatto extracted into 0.5 % acetic acid in chloroform. 20 g sample taken through extraction procedure Fig. 2.[3]	Supelco LC-18 column, mobile phase MeOH–2 % acetic acid (9:1)	500 nm	3
HPLC	Commercial annatto formulations	Solvent extraction of annatto depends on formulation of annatto. Final extraction into methanol and filtered through a 0.2 µm membrane filter prior to analysis	Hichrom RPB column, mobile phase: 65 % A (acetonitrile) and 35 % B (0.4 % aq acetic acid)	435 nm with 40 nm bandwidth	4
HPLC	Foods	None specified	Supelco LC-18 column, mobile phase MeOH and 6 % aq acetic acid	493 nm	5
HPLC	Cheese	10 g cheese extracted with water THF(1:1), centrifuged. Aqueous phase contained norbixin and organic phase contained bixin. Aqueous phase filtered through 0.45 µm membrane	ODS column, mobile phase: A (phosphate buffer) B (acetonitrile), gradient. Flow rate 1 mL/min	450 nm	6
HPLC	Annatto seeds	Bixin was extracted using supercritical carbon dioxide containing acetonitrile (0.05 % trifluoroacetic acid) as modifier at 60.62 MPa and 40 ºC. Sample extracts filtered through a 0.22 µm filter prior to injection	Capcell-Pak C18 column, mobile phase: acetonitrile–0.01 % trifluoroacetic acid aq. (90:10), isocratic. Flow rate 1 mL/min	460 nm	7

Table 5.2 Summary of statistical parameters for annatto extracts in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
HPLC	Cheese, butter, margarine and hard candy	Performance of method established and recovery determined. Method applied to commercial samples	Recovery: norbixin from spiked cheese samples av. 92.6 % (1–110 µg/g) bixin from spiked butter samples av. 93.2 % (0.1–445 µg/g) norbixin from hard candies av 88 %. Commercial cheese samples contained 1.1–68.8 µg/g total norbixin and 2 samples contained 5.1–5.6 µg/g total bixin. 0.2 µg/g total bixin and 0.91 µg/g total norbixin were found in one commercial butter sample	3
HPLC	Foods	Performance of method not stated. A simple, reliable method that was applied to food products such as fruit beverages, yogurt and candies	Detection limit 100 ng/g for annatto	5
RP TLC/ scanning densitometry	Foods	Performance of method not stated. Applied to commercial foods	89 commercial foods analysed and their chromatographic behaviour and spectra were observed. The separation and the spectra obtained were not affected by coexisting substances in foods. The spots always gave the same RF values and spectra as the standards with good reproducibility	8

6

E200–3: Sorbic acid and its salts

6.1 Introduction

Sorbic acid is used as a preservative in a wide variety of foods. Sorbic acid retards the growth of yeast and moulds and is usually added to foods as a salt. The major food groups contributing to dietary intake of sorbic acid constitute a wide variety permitted at the following levels: various foods 200–2000 mg/kg (liquid egg 5000 mg/kg, cooked seafood 6000 mg/kg) and soft drinks, wine etc. 200–300 mg/kg (Sacramental grape juice 2000 mg/kg, liquid tea concentrates 600 mg/kg). The acceptable daily intake (ADI) for sorbic acid is 25 mg/kg body weight.

6.2 Methods of analysis

There are numerous methods published for the determination of sorbic acid in foodstuffs. The majority of these methods are separation methods. Methods that have been developed for sorbic acid in foodstuffs include gas chromatography (GC),[1–7] high pressure liquid chromatography (HPLC),[8–14] spectrophotometric,[15–21] high performance thin layer chromatography (HPTLC)[22] and micellar electrokinetic chromatography (MECC).[23] A summary of these methods is given in Table 6.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 6.2. Three of these methods[1,15,16] are AOAC Official Methods of Analysis and one[1] has been collaboratively tested.

The NMKL-AOAC method[1] was collaboratively tested on apple juice, almond paste and fish homogenate [at 0.5–2 g/kg levels], representing carbohydrate-rich, pasty, rich in fat and carbohydrates, and protein-rich foods. In this method sorbic acid is isolated from food by extraction with ether and successive partitioning into

aqueous NaOH and CH_2Cl_2. Acids are converted to trimethylsilyl (TMS) esters and determined by GC. Phenylacetic acid is used as internal standard for benzoic acid. A summary of the procedure for this method is given in the Appendix and the performance characteristics are given in Table 6.3.

A suitable HPLC method for sorbic acid in foodstuffs was collaboratively tested on orange squash, cola drinks, beetroot, pie filling and salad cream and is applicable to the determination of 50–2000 mg/kg sorbic acid in foodstuffs.[11] In this method liquid foods not containing insoluble matter are diluted with methanol. Other foods are extracted by shaking with methanol, centrifuging and filtering. The concentration of sorbic acid in the clear extract is measured using reverse-phase liquid chromatography with UV detection. A summary of the procedure for this method is given in the Appendix and a summary of the statistical parameters in Table 6.4.

6.3 Recommendations

There are many methods available for the analysis of sorbic acid in foods and the decision as to which one should be used depends on the matrix to be analysed. The majority of methods are for liquids such as beverages, sauces and yogurt; further method development may be required to adapt these methods to be applicable for all matrices.

6.4 References

1 'AOAC Official Method 983.16. Benzoic acid and sorbic acid in food, gas-chromatographic method. NMLK–AOAC Method', *AOAC Official Method of Analysis* (2000) 47.3.05 p 9.
2 'Simultaneous determination of sorbic acid, benzoic acid and parabens in foods: a new gas chromatography–mass spectrometry technique adopted in a survey on Italian foods and beverages', De Luca C, Passi S, Quattrucci E. *Food Additives and Contaminants* (1995), **12**(1), 1–7.
3 'Simple and rapid method for the determination of sorbic acid and benzoic acid in foods', Choong Y-M, Ku K-L, Wang M-L, Lee M-H. *J Chinese Agricultural Chemical Society* (1995) **33**(2) 247–261. [Chinese]
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phase high-performance liquid-chromatography', Ivanovic D, Medenica M, Nivaudguernet E, Guernet M. *Chromatographia* (1995) **40**(11–12), 652–656.
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10 'Analysis of additives in fruit juice using HPLC', Kantasubrata J, Imamkhasani S. *ASEAN Food Journal* (1991) **6**(4), 155–158.
11 'Determination of preservatives in foodstuffs: collaborative trial', Willetts P, Anderson S, Brereton P, Wood R. *J. Assoc. Publ. Analysts*. (1996) **32**, 109–175.
12 'Determination of benzoic and sorbic acids in labaneh by high-performance liquid chromatography', Mihyar G F, Yousif A K, Yamani M I. *Journal of Food Composition and Analysis* (1999) **12**, 53–61.
13 'Rapid high-performance liquid chromatographic method of analysis of sodium benzoate and potassium sorbate in foods', Pylypiw H M, Grether M T. *Journal of Chromatography A* (2000) **883**(1–2), 299–304.
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15 'AOAC Official Method 971.15. Sorbic acid in cheese, oxidation method', *AOAC Official Method of Analysis* (2000) 47.3.36 p 24.
16 'AOAC Official Method 974.10. Sorbic acid in dairy products, spectrophotometric method', *AOAC Official Method of Analysis* (2000) 47.3.37 p 25.
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6.5 Appendix: method procedure summaries

Gas chromatographic method – NMKL–AOAC method[1]

Preparation of test sample

Homogenise test sample in mechanical mixer. If consistency of laboratory sample makes mixing difficult, use any technique to ensure that the material will be homogeneous.

Extraction

(a) *General method* – Accurately weigh 5.0 g homogenised test portion into 30 mL centrifuge tube with Teflon-lined screw cap. Add 3.00 mL internal standard solution, 1.5 mL H_2SO_4 (1 + 5), 5 g sand, and 15 mL ether. Screw cap on tightly to avoid leakage. Mechanically shake 5 min and centrifuge 10 min at 1500 g. Transfer ether layer with disposable pipette to 250 mL separator. Repeat extraction twice with 15 mL ether each time.

Extract combined ether phases twice with 15 mL 0.5 M NaOH and 10 mL saturated NaC1 solution each time. Collect aqueous layers in 250 mL separator, add 2 drops of methyl orange, and acidify to pH 1 with HC1 (1 + 1). Extract with CH_2C1_2, using successive portions of 75, 50, and 50 mL. If emulsion forms, add 10 mL saturated NaC1 solution. Drain CH_2C1_2 extracts through filter containing 15 g anhydrous Na_2SO_4 into 250 mL round-bottom flask. Evaporate CH_2C1_2 solution in rotary evaporator at 40 ºC just to dryness.

(b) *Cheese and food products with paste-like consistency* – Accurately weigh 5.0 g homogenised test portion into 200 mL centrifuge flask. Add 15 mL H_2O and stir with glass rod until test portion is suspended into aqueous phase. Add 3.00 mL internal standard solution, 1.5 mL H_2SO_4 (1 + 5), and 25 mL ether. Stopper flask carefully and check for leakage. Mechanically shake 5 min and centrifuge 10 min at 2000 g. Transfer ether layer with disposable pipette to 250 mL separator. Repeat extraction twice with 25 mL ether each time. Continue as in (a), beginning 'Extract combined ether phases. . .'

Derivatisation and gas chromatography

Add 10.0 mL $CHC1_3$ to residue in 250 mL round-bottom flask. Stopper and shake manually 2 min. Transfer 1.00 mL $CHC1_3$ solution to 8 mL test tube with Teflon-lined screw cap and add 0.20 mL silylating agent. Cap and let stand 15 min in oven or H_2O bath at 60 ºC. Inject duplicate 1 µL portions of residue solution into gas chromatograph. Start temperature program when solvent peak emerges. Measure peak heights and calculate peak height ratios of sorbic acid/caproic acid. Use average of duplicate ratios. Peak height ratios for duplicate injections should differ ≤5 %.

Preparation of standard curves

Transfer 1.00 mL standard solutions to five 8 mL test tubes with Teflon-lined screw caps. Add 0.20 mL silylating agent to each tube, cap, and let stand 15 min in oven or H_2O bath at 60 ºC. Inject duplicate 1 µL portions of standard solutions into gas chromatograph. Use same conditions as for test portion solution. Measure peak heights and calculate peak height ratios of sorbic acid/caproic acid. Peak height ratios for duplicate injections should differ ≤5 %. Plot weight ratios (x) vs. average peak height ratios (y) for each preservative. Calculate slope and intercept of standard curve by method of least squares.

Calculation

$$\text{Preservative, mg/kg} = \frac{y - a}{b} \times \frac{W'}{W} \times 1000 \qquad [6.1]$$

where
b = slope of standard curve
a = intercept
y = average peak height ratio of preservative/internal standard
W = weight of test portion in g
W′ = weight of internal standard in mg.

HPLC method for sorbic acid applicable for foodstuffs containing sorbic acid in the range 50–2000 mg/kg[11]

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	0–26 min	100 % A
	26–31 min	go to 100 % B
	31–45 min	100 % B
	45–50 min	go to 100 % A
	50–55 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.

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

Preparation of calibration graphs

Inject 20 µL of each of the standard solutions. 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.

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 are prevented.

Liquid samples not containing insoluble matter
Weigh, to the nearest 0.001 g, about 10 g of prepared sample and dilute with methanol to 100 mL in a volumetric flask and mix. Pass this solution through a 0.45 μm filter to eliminate any particulate matter.

Confirm that the HPLC system is operating correctly by injecting the combined 20 mg/L standard solution, then inject 20 μL of the sample filtrate onto the HPLC column. 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 solution after every fourth injection. If the amount of analyte(s) in the extract is high an aliquot of the extract should be diluted with mobile phase A 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.

Other samples
Weigh, to the nearest 0.001 g, about 10 g of prepared sample into a centrifuge tube. Add methanol (20 mL) and close the tube. Vortex mix the sample and methanol to ensure a uniform suspension and then extract the sample by shaking vigorously for 2 min. Centrifuge at a relative centrifugal force (RCF) of approximately 2630 for 5 min 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.)

Repeat steps twice with further portions of methanol (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 layer from a sample containing a high oil content to ensure that none of the oil layer is decanted with the methanol. Combine the extracts in the 100 mL volumetric flask and make up to the calibration mark by the addition of methanol. 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. This can be performed by placing the sample in dry ice for approximately 20 min until the fat has solidified, decanting the methanolic solution and then proceeding by making to volume with methanol.)

Filter the solution through a filter paper, rejecting the first few mL and collect about 15 mL. Filter this through a 0.45 μm filter. Carry out the chromatographic analysis on the filtered extract. 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.

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

Calculation
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 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 below.

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

$$\text{Analyte (mg/kg)} = \frac{C \times 100}{M} \times f \qquad [6.2]$$

where
C = concentration of analyte in extract, mg/L
M = mass of sample, g
f = distribution factor for extract

Expression of results
Report the results as mg/kg.

Table 6.1 Summary of methods for sorbic acid in foods

(a)

Method	Matrix	Sample preparation/extraction	Column	Conditions	Detection	Reference
GC	Foods	Extracted with ether and into aq NaOH and CH_2Cl_2. Converted to TMS esters	1.8 mm × 2 mm (i.d.) coiled glass with 3 % OV-1 on 100–20 mesh Varaport 30	Oven temp: 80–210 ºC, 8 ºC/min; injection port 200 ºC, N_2 carrier 20 mL/min	FID at 280 ºC	1
GC–MS (SIM)	Foods	Homogenised with water at pH 1. Extracted into ether, evaporated, added acetonitrile. For GC evaporated acetonitrile and formed TMS esters	Ultra 1 (cross-linked methyl-silicone gum phase, 25 m × 0.2 mm × 0.33 µm)	Injection 1 µL, temp 250 ºC; splitless flow (helium) 11 psi. Oven temp programmed 90–270 ºC	MS selected ion monitoring (SIM) mode. Electron multiplier voltage 2200–2400 emV	2
GC	Foods	Solvent extraction with heptanoic acid as an internal standard	DB-Wax (30 m × 0.53 mm, 1 µm)	Splitless GC, direct injection 0.5 µL. Oven temperature programmed 140–220 ºC	FID	3
GC-MS (SIM)	Foods	Solid phase extraction (SPE) with a polymer-based cartridge and pH adjustment of sample (pH = 3.5) in pre-treatment	HP-INNOWax (30 m × 0.25 mm i.d. 0.25 µm)	Splitless GC. temp 220 ºC; splitless flow (helium) 11 psi. Oven temperature programmed 100–240 ºC	MS selected ion monitoring (SIM) mode. m/z 97	4

GC	Fatty foods	Samples manually extracted with a mixture of solvents then subjected to continuous SPE system	Fused-silica capillary column HP-5 (30 m × 0.32 mm, 1 µm)	Oven temp:125–315 ºC, 10 ºC/min; injection port 250 ºC, N_2 carrier 1 mL/min	FID at 310 ºC, ionisation energy 70 eV MS from 50–500 m/z (105 m/z)	5
GC	Foods	Solid samples require pre-treatment: liquid–liquid extraction, evaporation of extract and residue dissolved in 0.1 M HNO_3. Samples inserted to SPE (XAD-2 column) flow system at pH 1. Elution with 150 µL ethyl acetate	Two columns (15m × 0.53 mm i.d.) (i) 5 % diphenyl-95 % dimethylsiloxane, 3 µm (HP-5) (ii) 50 % diphenyl-50 % dimethylsiloxane, 1 µm (HP-50)	2 µL aliquots of eluate are manually injected. Oven temperature programmed 70–160 ºC, injection port 250 ºC, nitrogen carrier at 14.7 mL/min	FID at 250 ºC	6
GC	Vinegar, pickle condiment liquid, soy sauce, fish sauce	Sample (1 mL) transferred to 7 mL vial. 0.5 mL 0.2 % (1,4-dihydroxybenzene (IS) dissolved in 20 % MeOH) was added. Mixture acidified with 5 % HCl and vortexed	CP-SIL 8CB (30 m × 0.53 mm, 1.5 µm)	0.1 µL direct injection. Oven temperature programmed 100–300 ºC, injection port 290 ºC, helium carrier at 4 mL/min	FID at 290 ºC	7

Table 6.1 cont'd
(b)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
RP-HPLC	Pharmaceutical formulations	Sample filtered through 0.45 µm filter	LiChrosorb RP 18 250 mm × 4.6 mm, 7 µm	MeOH–H_2O + 1 % acetic acid, flow rate 1 mL/min, injection 10 µL	UV at 254 nm	8
HPLC	Beverages and jams	Beverages diluted 10 fold. Jams (5 g) diluted with water (65 mL), sonicate make up to 100 mL. Filter and inject 20 µL	C18 Spherisorb ODS-1 (250 mm × 4.6 mm, 5 µm)	8 % MeOH in phosphate buffer at pH 6.7	UV at 227 nm	9
HPLC	Fruit juices	Sample filtered through 0.45 µm filter	µ-Bondapak CN	2 % acetic acid–MeOH (95:5), flow rate 1.5 mL/min at room temperature	UV at 240 and 254 nm	10
HPLC	Foods	Extracted by shaking with methanol, centrifuging and filtering	Kromasil 100-5C18	Citric acid–sodium citrate buffer:acetonitrile, programmed	UV at 258 nm	11
HPLC	Labaneh (concentrated set yogurt)	Proteins were precipitated, methanol added and filtered	ODS C-18 (150 mm × 4.6 mm, 5 µm)	Phosphate–methanol (90:10), flow rate 1.2 mL/min at room	UV at 227 nm	12

				temperature		
HPLC	Foods	Liquid samples dilute 10 fold in acetonitrile/ammonium acetate buffer solution. Solid samples blended with same buffer solution 1:5 followed by dilution as for liquid samples	Supelcosil LC-18 (250 mm × 4.6 mm, 5 µm)	90 % ammonium acetate buffer with 10 % acetonitrile	UV at 255 nm	13
HPLC	Yogurt, non-alcoholic beverages and fruit juices	Yogurt samples treated with potassium ferricyanide (III) and zinc sulphate. Non-alcoholic beverages and fruit juices diluted and filtered	Divinyl benzene-styrene copolymer (DVB-H)	0.01 NH_2SO_4–CH_3CN (75:25)	UV at 258 nm	14

Table 6.1 cont'd

(c)

Method	Matrix	Sample preparation	Method conditions	Reference
Oxidation	Cheese	Steam distil sample with 1 M H_2SO_4 and $MgSO_4$. Collect distillate in volumetric flask	Make to volume. Pipette aliquot into test tube, add 1.0 mL 0.15 M H_2SO_4 + 1.0 mL $K_2Cr_2O_7$ and heat in boiling water bath for 5 min. Cool. Add thiobarbituric acid soln. Replace on boiling water bath for 10 min. Cool. Determine *A* at 532 nm against blank	15
Spectro-photometric	Dairy products	Blend with HPO_3 soln for 1 min. Filter through Whatman No. 3 paper	Transfer 10 mL filtrate to separator containing 100 mL mixed ethers and shake for 1 min. Discard aqueous layer and dry ether extract with 5 g Na_2SO_4. Determine *A* at 250 nm against reference soln	16
Colorimetric	Raw beef	Ground beef mixed thoroughly and homogenised with water and pH adjusted to 5.0 with phosphoric acid solution	Modification of AOAC oxidation method.[15] Extraction by steam distillation was improved through dispersion of the meat matrix with sand in a ratio of meat to sand 1:3, followed by oxidation and reaction with thiobarbituric acid to form a red pigment, with absorption measured at 532 nm	17
Spectro-photometric flow-injection	Wines	1.0 mL wine diluted to 25 mL with water and injected into FI system	A simple rapid and accurate method based on oxidation of sorbic acid with $K_2Cr_2O_7/H_2SO_4$ at 100 ºC, followed by reaction of resulting malonaldehyde with thiobarbituric acid, at 100 ºC to give a red product, the absorbance measured at 532 nm	18

4 spectro-photometric and a GC	Prunes	Mixed thoroughly	Two of the spectrophotometric methods based on measurements in visible region; one utilised a 2-step extraction and the other used a simple water extraction. Two methods employ measurement in UV region at 235 nm; one includes distillation step and the other includes a 2-step extraction. Fifth method was a GC–MS method	19
Enzymatic	Foods	Samples treated with Carrez 1 and 2 if necessary. Blended with water, sonicated and filtered	Sorbic acid converted to sorbyl coenzyme A with acyl CoA synthetase in the presence of coenzyme A and adenosine-5′-triphosphate. Pyrophosphate is hydrolysed with inorganic pyrophosphate to give inorganic phosphate. Sorbyl CoA is determined spectrophotometrically at 300 nm	20
Diffusivity	Cheeses	Potassium sorbate concentration in cheese was determined by AOAC method[16]	To determine diffusivity the concentration of potassium sorbate in sliced cheese was measured by penetration time and distance from surface	21
HPTLC	Beverages	No extraction or clean-up required	Aliquots of samples and standards are chromatographed on preadsorbent silica gel of C18 bonded silica gel plates containing fluorescent indicator and the zones, which quench fluorescence, are compared by scanning densitometry	22
Micellar electrokinetic chromatography (MECC)	Cola beverages and jams	Butyl paraben was used as an internal marker	Additives were separated using a 20 mM berate buffer with 35 mM sodium cholate, 15 mM sodium dodecyl sulphate and 10 % methanol added at pH 9.3	23

Table 6.2 Summary of statistical parameters for sorbic acid in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
GC	Foods	Full collaborative trial	see Table 6.3	1
HPLC	Foods	Full collaborative trial	see Table 6.4	11
GC–MS (SIM)	Foods	Precision of method established and applied to real samples (n=249)	Detection limit 100–200 pg. Mean recovery 97.2 % for cheese spiked at levels from 50–500 mg/kg (n=3) for each level. Method applied to 249 samples of foods and beverages on sale in markets in Rome	2
HPLC	Foods	Precision of method established and applied to real samples (n=65)	Linear range 2.5–100 mg/L. Detection limit 10 mg/L in a juice matrix. Samples spiked at 0.10 and 0.05 % gave recoveries of 82–96 %	13
GC	Foods	Precision of method established and applied to real samples (n=37)	Detection limit lower than 0.5 ppm Recoveries: Spiked vinegar at 200 µL 97.7 % CV 4.9 % (n=3) Spiked soy sauce at 200 µL 99.4 % CV 3.8 % (n=3) Method applied to 37 liquid food samples	7
GC	Foods	Precision of method established and applied to real samples (n=36)	Detection limit 2 ppm Recovery studies performed on various foods spiked with sorbic acid at levels of 200–1000 µg Recoveries 94.3–102.9 %, CV <6.8 %	3

HPLC	Labaneh (concentrated set yogurt)	Precision of method established and applied to real samples (n=25)	Linear range 32–300 mg/L Recoveries added at 28.0 and 56.0 mg/100 g to labaneh, averaged 101.1 and 97.4 % with CV of 0.5 % and 0.8 %, respectively	12
Enzymatic	Foods	Precision of method established and applied to real samples (n=6)	Repeatability carried out on apricot preserve Mean value 467±20 mg/kg (n=5) r_{lab} 35 mg/kg Recovery of spiked samples 95.5–100.6 % (n=9) Method compared well with HPLC method	20
			Sample — *Enzymatic method* — *HPLC* Apricot preserve — 467 mg/kg — 488 mg/kg Alcoholic beverage — 190 mg/L — 198 mg/L Alcoholic beverage — 174 mg/L — 180 mg/L Raspberry syrup — 314 mg/kg — 319 mg/kg Tomato ketchup — 324 mg/kg — 337 mg/kg Chilli spice — 657 mg/kg — 678 mg/kg	
Spectro-photometric flow-injection	Wines	Precision of method established and applied to real samples (n=5)	Calibration graph linear 0–15 µg/mL, detection limit was 0.14 µg/mL. RSD 1.58 % (n=10). Applied to both red and white wine: White wine 1 — 85.7±0.6 µg/mL White wine 2 — 8.5±0.5 µg/mL White wine 3 — 7.3±1.0 µg/mL Red wine 1 — 58.8±1.3 µg/mL Red wine 2 — 9.3±0.3 µg/mL	18

Table 6.2 cont'd

Method	Matrix	Extent of validation	Statistical parameters	Reference
GC	Fatty foods	Precision of method established and applied to real samples	Linear range (μg/mL) 0.5–100 (FID), 2–500 (MS) (n=3) LOD (μg/mL) 0.2 (FID), 1.0 (MS) RSD (%) 3.7 (FID), 4.0 (MS) Checked with real samples for 5 samples of each analysed in triplicate by SPE–GC–FID: light mayonnaise 400 mg/kg (RSD 3.8 % n=15), pâté 225 mg/kg (RSD 5.3 % n=15), cheese 745 mg/kg (RSD 4.0 % n=15), corn margarine 390 mg/kg (RSD 5.1 % n=15)	5
HPLC	Beverages and jams	Precision of method established and applied to real samples	Recovery data for 4 spiked beverage samples ranged from 98.4 to 104.8 %. Linear range 0 to 100 mg/L. Results of jam samples using this method compared favourably with NKLM method	9
GC	Foods	Precision of method established and applied to real samples	Linear range 0.3–25 mg/L. Detection limit 0.10 mg/L RSD 3.8 % The method was applied to non-fatty foods i.e. soft drinks, jams, and sauces	6
HPTLC	Beverages	Precision of method established and applied to commercial samples	Recoveries of sorbic acid from wine and juices spiked at 50–300 ppm averaged 98 %, CV ranged from 2–5 %	22
GC–MS (SIM)	Foods	Precision of method established and applied to real samples	Recovery of spiked fruit vinegar (n=6) 86 % (RSD=1.6 %) Detection limit 0.1 ppb	4

HPLC	Yogurt, non-alcoholic beverages and fruit juices	Precision of method established	The method showed good precision and accuracy without interferences with other components of the samples	14
Colorimetric	Raw beef	Precision of method established	Spiked samples: 99± 2.1 ppm (n=10) 2.1 %CV Recovery 99 %, 2651±181.9 ppm (n=15) 6.9 %CV Recovery 102 %	17
HPLC	Fruit juices	Method applied to commercial samples in Indonesia (n=16)	Absorbance ratio (254/240 nm) for sorbic acid = 1.951±0.061	10
Micellar electrokinetic chromatography (MECC)	Cola beverages and jams	Method applied to jam samples (n=1)	Low-joule jam: sorbic acid 1.38 mg/g RSD 1.4 % (n=3), recovery 98.9 % RSD 0.5 % (n=3)	23
4 spectro-photometric and a GC	Prunes	Methods applied to prune sample (n=1)	1 spectral (visible), chloroform extraction 300 ppm (n=2) 2 spectral (visible), filtered aqueous extract 350 ppm (n=2) 3 spectral (UV), chloroform extraction 330 ppm (n=2) 4 spectral (UV), distillation 360 ppm (n=2) 5 gas chromatographic, DCM extraction 370 ppm (n=2)	19

Table 6.3 Performance characteristics for sorbic acid in almond paste, fish homogenate and apple juice[1]

Samples	Almond paste		Fish homogenate		Apple juice	
No. of laboratories	8	8	8	8	6	8
Units	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Mean value	983	2029	484	1008	45	530
S_r	41	84	34	27	2.3	18
RSD_r	4.1 %	4.1 %	7.0 %	2.6 %	5.1 %	3.4 %
S_R	74	65	27	60	10.2	29
RSD_R	8.5 %	5.2 %	9.0 %	6.5 %	23.3 %	6.4 %
Av recovery	99.1 %	103.4 %	96.4 %	98.4 %	109.1 %	105.8 %

Key

Mean 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).

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).

Table 6.4 Performance characteristics for sorbic acid in orange squash, cola drinks, beetroot, pie filling and salad cream[11]

Sample	Orange squash		Cola drink	
No. of laboratories	8		7	9
Units	mg/kg		mg/kg	mg/kg
Mean	316.9	278.4	534.3	517.1
Mean recovery %	99	98.4	94.9	88.8
S_r	4.48		4.46	6.55
RSD_r	4 %		1 %	1 %
r	13		12	18
S_R	15.24		18.75	55.94
RSD_R	5 %		4 %	11 %
R	43		52	157
Ho_R	0.8		0.6	1.7

Sample	Beetroot		Pie filling		Salad cream	
No. of laboratories	8		7		9	
Units	mg/kg		mg/kg		mg/kg	
Mean	393.2	421.3	1000.4	1147.3	1704.2	2011.5
Mean recovery %	102.9	100.3	95.1	98.7	97.4	103
S_r	12.54		24.91		127.18	
RSD_r	3 %		2 %		7 %	
r	35		70		356	
S_R	49.03		44.68		213.70	
RSD_R	12 %		4 %		12 %	
R	137		125		598	
Ho_R	1.9		0.7		2.2	

Key

Mean 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).

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).

7

E210–13: Benzoic acid

7.1 Introduction

Benzoic acid is used as a preservative in a wide variety of foods. Benzoic acid retards the growth of yeast and moulds, the effective agent being the undissociated acid. The major food groups contributing to dietary intake of benzoic acid are a wide variety of foods permitted at the following levels; various foods 200–1000 mg/kg (prepared salads, confectionery, etc. 1500 mg/kg; food supplements, preserved vegetables 2000 mg/kg; liquid egg 5000 mg/kg; cooked seafood 2000–6000 mg/kg) and soft drinks 150 mg/kg, alcohol-free beer 200 mg/kg (Sacramental grape juice 2000 mg/kg, liquid tea concentrates 600 mg/kg). The acceptable daily intake (ADI) for benzoic acid is 5 mg/kg body weight.

7.2 Methods of analysis

There are numerous methods published for the determination of benzoic acid in foodstuffs. The majority of these methods are separation methods. Methods that have been developed for benzoic acid in foodstuffs include gas chromatography (GC),[1–7] high pressure liquid chromatography (HPLC),[8–19] micellar electrokinetic chromatography(MECC),[20] the use of lanthanide-sensitised luminescence,[21] spectrophotometric,[22] high performance thin layer chromatography (HPTLC),[23] and potentiometric.[24] A summary of these methods is given in Table 7.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 7.2. Two of these methods[1,8] are AOAC Official Methods of Analysis and both have been collaboratively tested.

The NMKL–AOAC method[1] was collaboratively tested on apple juice, almond paste and fish homogenate [at 0.5–2 g/kg levels], representing carbohydrate-rich, pasty, fat-rich, carbohydrate-rich and protein-rich foods. In this method benzoic acid is isolated from food by extraction with ether and successive partitioning into aqueous NaOH and CH_2Cl_2. Acids are converted to trimethylsilyl (TMS) esters and determined by GC. Phenylacetic acid is used as internal standard for benzoic acid. A summary of the procedure for this method is given in the Appendix and the performance characteristics are given in Table 7.3.

The AOAC liquid chromatographic method[8] was collaboratively tested on orange juice and is applicable to the determination of 0.5–10 ppm benzoic acid in orange juice. In this method benzoic acid in solid-phase extracted orange juice is separated by liquid chromatography on C18 column, detected by ultraviolet absorbance at 230 nm, and quantitated by external standard. A summary of the procedure for this method is given in the Appendix and a summary of the statistical parameters in Table 7.4.

A suitable HPLC method for benzoic acids in foodstuffs was collaboratively tested on orange squash, cola drinks, beetroot and pie filling and is applicable to the determination of 50–2000 mg/kg benzoic acid in foodstuffs.[11] In this method liquid foods not containing insoluble matter are diluted with methanol. Other foods are extracted by shaking with methanol, centrifuging and filtering. The concentration of benzoic acid in the clear extract is measured using reverse-phase liquid chromatography with UV detection. A summary of the procedure for this method is given in the Appendix and a summary of the statistical parameters in Table 7.5.

7.3 Recommendations

There are many methods available for the analysis of benzoic acids in foods and the decision as to which should be used depends on the matrix to be analysed. The majority of methods are for liquids i.e. beverages, sauces, yogurt etc. and further method development may be required to adapt them to be applicable for all matrices.

7.4 References

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7.5 Appendix: method procedure summaries

Gas chromatographic method – NMKL–AOAC method[1]

Preparation of test sample
Homogenise test sample in mechanical mixer. If consistency of laboratory sample makes mixing difficult, use any technique to ensure that the material will be homogeneous.

Extraction

(a) *General method* – Accurately weigh 5.0 g homogenised test portion into 30 mL centrifuge tube with Teflon-lined screw cap. Add 3.00 mL internal standard solution, 1.5 mL H_2SO_4 (1 + 5), 5 g sand, and 15 mL ether. Screw cap on tightly to avoid leakage. Mechanically shake 5 min and centrifuge 10 min at 1500 g. Transfer ether layer with disposable pipette to 250 mL separator. Repeat extraction twice with 15 mL ether each time.

Extract combined ether phases twice with 15 mL 0.5M NaOH and 10 mL saturated NaC1 solution each time. Collect aqueous layers in 250 mL separator, add 2 drops of methyl orange, and acidify to pH 1 with HC1 (1 + 1). Extract with CH_2C1_2, using successive portions of 75, 50, and 50 mL. If emulsion forms, add 10 mL saturated NaC1 solution. Drain CH_2C1_2 extracts through filter containing 15 g anhydrous Na_2SO_4 into 250 mL round-bottom flask. Evaporate CH_2C1_2 solution in rotary evaporator at 40 ºC just to dryness.

(b) *Cheese and food products with paste-like consistency* – Accurately weigh 5.0 g homogenised test portion into 200 mL centrifuge flask. Add 15 mL H_2O and stir with glass rod until test portion is suspended into aqueous phase. Add 3.00 mL internal standard solution, 1.5 mL H_2SO_4 (1 + 5), and 25 mL ether. Stopper flask carefully and check for leakage. Mechanically shake 5 min and centrifuge 10 min at 2000 g. Transfer ether layer with disposable pipette to 250 mL separator. Repeat extraction twice with 25 mL ether each time. Continue as in (a), beginning 'Extract combined ether phases. . .'

Derivatisation and gas chromatography
Add 10.0 mL $CHC1_3$ to residue in 250 mL round-bottom flask. Stopper and shake manually 2 min. Transfer 1.00 mL $CHC1_3$ solution to 8 mL test tube with Teflon-lined screw cap and add 0.20 mL silylating agent. Cap and let stand 15 min in oven or H_2O bath at 60 ºC. Inject duplicate 1 µl portions of residue solution into gas

chromatograph. Start temperature program when solvent peak emerges. Measure peak heights and calculate peak height ratios of benzoic acid/phenylacetic acid. Use average of duplicate ratios. Peak height ratios for duplicate injections should differ ≤5 %.

Preparation of standard curves
Transfer 1.00 mL standard solutions to five 8 mL test tubes with Teflon-lined screw caps. Add 0.20 mL silylating agent to each tube, cap, and let stand 15 min in oven or H_2O bath at 60 ºC. Inject duplicate 1 µL portions of standard solutions into gas chromatograph. Use same conditions as for test portion solution. Measure peak heights and calculate peak height ratios of benzoic acid/phenylacetic acid. Peak height ratios for duplicate injections should differ ≤5 %. Plot weight ratios (x) versus average peak height ratios (y) for each preservative. Calculate slope and intercept of standard curve by method of least squares.

Calculation

$$\text{Preservative, mg/kg } \frac{y - a}{b} \times \frac{W'}{W} \times 1000 \qquad [7.1]$$

where:
b = slope of standard curve
a = intercept
y = average peak height ratio of preservative/internal standard
W = weight of test portion in g
W′ = weight of internal standard in mg.

AOAC liquid chromatographic method for benzoic acid in orange juice[8]

Preparation of test samples for HPLC
Place 10.0 mL orange juice sample into 50 mL centrifuge tube and centrifuge 5 min at 1500 g. Using 10 mL syringe, precondition C18 cartridge by passing 2 mL methanol through cartridge, followed by 5 mL H_2O. Pipette 1.0 mL portion of test sample supernate into syringe and through conditioned cartridge. Slowly wash cartridge (let eluate drip by slowly pushing plunger) with 3.0 mL 2 % acetonitrile in hexane and discard eluate. Push syringe plunger 3x, blowing air through cartridge to eliminate excess hexane in cartridge. Add 3.0 mL methanol to syringe. Slowly elute cartridge with 3 mL methanol and collect eluate in 5 mL graduated centrifuge tube. Adjust eluate final volume to 3.0 mL with methanol. Pass eluate through 0.45 µm filter into vial.

Recovery test
Determine recovery of benzoic acid in juices by dividing spiked juice sample into 2 equal portions. Filter 1 portion through 0.45 µm filter, as control sample, while passing other portion through C18 cartridge and 0.45 µm filter. Calculate recoveries

based on difference between amount determined in control and amount obtained after C18 cartridge clean-up.

LC determination of benzoic acid
Make duplicate 10 µL injections of eluted test sample to LC, after injecting standard. Calculate concentration of benzoic acid in sample as follows:

$$\text{Benzoic acid, µg/mL} = (A/A') \times C \times 3 \times (1/R) \quad [7.2]$$

where
A, A′ = peak area of test sample and standard, respectively
C = concentration of standard, µg/mL
3 = dilution factor
R = recovery rate.

HPLC method for benzoic acid applicable for foodstuffs containing benzoic acid in the range 50–2000 mg/kg[11]
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	0–26 min	100 % A
	26–31 min	go to 100 % B
	31–45 min	100 % B
	45–50 min	go to 100 % A
	50–55 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. Centrifuge with appropriate centrifuge tubes (approximately 50 mL capacity) with screw caps or other suitable closures.

Preparation of calibration graphs
Inject 20 μL of each of the standard solutions. 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.

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 are prevented.

Liquid samples not containing insoluble matter
Weigh, to the nearest 0.001 g, about 10 g of prepared sample and dilute with methanol to 100 mL in a volumetric flask and mix. Pass this solution through a 0.45 μm filter to eliminate any particulate matter.

Confirm that the HPLC system is operating correctly by injecting the combined 20 mg/L standard solution, then inject 20 μL of the sample filtrate onto the HPLC column. 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 solution after every fourth injection. If the amount of analyte(s) in the extract is high an aliquot of the extract should be diluted with mobile phase A 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.

Other samples
Weigh, to the nearest 0.001 g, about 10 g of prepared sample into a centrifuge tube. Add methanol (20 mL) and close the tube. Vortex mix the sample and methanol to ensure a uniform suspension and then extract the sample by shaking vigorously for 2 min. Centrifuge at a relative centrifugal force (RCF) of approximately 2630 for 5 min 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.)

Repeat steps twice with further portions of methanol (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 layer from a sample containing a high oil content to ensure that none of the oil layer is decanted with the methanol. Combine the extracts in the 100 ml volumetric flask and make up to the calibration mark by the addition of methanol. 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. This can be performed by placing the sample in dry ice for approximately 20 min until the fat has solidified, decanting the methanolic solution and then proceeding by making to volume with methanol.)

Filter the solution through a filter paper, rejecting the first few mL and collect about 15 mL. Filter this through a 0.45 μm filter. Carry out the chromatographic

analysis on the filtered extract. 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.

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

Calculation
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 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 below.

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

$$\text{Analyte (mg/kg)} = \frac{C \times 100}{M} \times f \qquad [7.3]$$

where
C = concentration of analyte in extract, mg/L
M = mass of sample, g
f = distribution factor for extract

Expression of results
Report the results as mg/kg.

Table 7.1 Summary of methods for benzoic acid in foods

(a)

Method	Matrix	Sample preparation/extraction	Column	Conditions	Detection	Reference
GC	Foods	Extracted with ether and into aq NaOH and CH_2Cl_2. Converted to TMS esters	1.8 mm × 2 mm (i.d.) coiled glass with 3 % OV-1 on 100–200 mesh Varaport 30.	Oven temp: 80–210 ºC, 8 ºC/min; injection port 200 ºC, N_2 carrier 20 mL/min	FID at 280 ºC	1
GC–MS (SIM)	Foods	Homogenised with water at pH 1. Extracted into ether, evaporated, added acetonitrile. For GC evaporated acetonitrile and formed TMS esters	Ultra 1 (cross-linked methyl-silicone gum phase, 25 m × 0.2 mm × 0.33 µm)	Injection 1 µL, temp 250 ºC; splitless flow (helium) 11 psi. Oven temp programmed 90–270 ºC.	MS selected ion monitoring (SIM) mode. electron multiplier voltage 2200–2400 emV	2
GC	Foods	Solvent extraction with heptanoic acid as an internal standard	DB-Wax (30 m × 0.53 mm, 1 µm)	Splitless GC, direct injection 0.5 µL. Oven temperature programmed 140–220 ºC	FID	3
GC–MS (SIM)	Foods	Solid phase extraction (SPE) with a polymer-based cartridge and pH adjustment of sample (pH = 3.5) in pre-treatment	HP-INNOWax (30 m × 0.25 mm i.d. 0.25 µm)	Splitless GC, temp 220 ºC; splitless flow (helium) 11 psi. Oven temperature programmed from 100–240 ºC	MS selected ion monitoring (SIM) mode. m/z 105	4

GC	Fatty foods	Samples manually extracted with a mixture of solvents then subjected to continuous SPE system	Fused-silica capillary column HP-5 (30 m × 0.32 mm, 1 µm)	Oven temp:125–315 ºC, 10 ºC/min; injection port 250 ºC, N_2 carrier 1 mL/min	FID at 310 ºC, ionisation energy 70 eV MS 50–500 m/z (105 m/z)	5
GC	Foods	Solid samples require pretreatment: liquid–liquid extraction, evaporation of extract and residue dissolved in 0.1 M HNO_3. Samples inserted to SPE (XAD-2 column) flow system at pH 1. Elution with 150 µL ethyl acetate	Two columns (15 m × 0.53 mm i.d.) (i) 5 % diphenyl-95 % dimethylsiloxane, 3 µm (HP-5) (ii) 50 % diphenyl-50 % dimethylsiloxane, 1 µm (HP-50)	2 µL aliquots of eluate manually injected. Oven temperature programmed 70–160 ºC, injection port 250 ºC, nitrogen carrier at 14.7 mL/min	FID at 250 ºC	6
GC	Vinegar, pickle condiment liquid, soy sauce, fish sauce	Sample (1 mL) transferred to 7 mL vial. 0.5 mL 0.2 % (1,4-dihydro-xybenzene (IS) dissolved in 20 % MeOH) was added. Mixture acidified with 5 % HCl and vortexed	CP-SIL 8CB (30 m × 0.53 mm, 1.5 µm)	0.1 µL direct injection. Oven temperature programmed 100–300 ºC, injection port 290 ºC, helium carrier at 4 mL/min	FID at 290 ºC	7

Table 7.1 cont'd

(b)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
HPLC	Orange juice	SPE extraction using C18 cartridge (SEP-PAK)	PRP-1 (250 mm × 4.1 mm, 10 µm)	Acetonitrile–phosphate buffer (40+60)	UV at 230 nm	8
HPLC	Beverages and jams	Beverages diluted 10 fold. Jams (5 g) diluted with water (65 mL), sonicate made up to 100 mL. Filter and inject 20 µL	C18 Spherisorb ODS-1 (250 mm × 4.6 mm, 5 µm)	8 % MeOH in phosphate buffer at pH 6.7	UV at 227 nm	9
HPLC	Fruit juices	Sample filtered through 0.45 µm filter	µ-Bondapak CN	2 % acetic acid/MeOH (95:5), flow rate 1.5 mL/min at room temperature	UV at 240 and 254 nm	10
HPLC	Foods	Extracted by shaking with methanol, centrifuging and filtering	Kromasil 100-5C18	Citric acid/sodium citrate buffer:acetonitrile, programmed	UV at 223 nm	11
HPLC	Labaneh (concentrated set yogurt)	Proteins were precipitated, methanol added and filtered	ODS C18 (150 mm × 4.6 mm, 5 µm)	Phosphate–methanol (90:10), flow rate 1.2 mL/min at room temperature	UV at 227 nm	12
HPLC	Quince jam	2 extraction methods were needed, one including XAD-2 cleaning step. Both include final extract in methanol	Spherisorb ODS-2 (25 cm × 0.46 cm, 5 µm)	Gradient of water–formic acid (19:1) [A] and methanol [B] at 0.9 mL/min	DAD at 280 nm	13

HPLC	Foods	Liquid samples dilute 10 fold in acetonitrile/ammonium acetate buffer solution. Solid samples blended with same buffer solution 1:5 followed by dilution as for liquid samples	Supelcosil LC-18 (250 mm × 4.6 mm, 5 µm)	90 % ammonium acetate buffer with 10 % acetonitrile	UV at 225 nm	14
HPLC	Quince jam	Extraction with methanol	Spherisorb ODS-2 (25 cm × 0.46 cm, 5 µm)	Gradient of water–formic acid (19:1) [A] and methanol [B] at 0.9 mL/min	DAD at 280 nm	15
HPLC	Cranberry juice	SPE and hydrolysed by acid before HPLC analysis	Eclipse XDR-C18 reversed-phase (150 mm × 4.6 mm, 5 µm)	Gradient of water–acetic acid (97:3) [A] and methanol [B] at 1.0–0.9 mL/min	DAD at 280 nm	16
HPLC	Pharm-aceutical syrup	Sample diluted with mobile phase and filtered through 0.45 µm filter	Symmetry Shield RPC8 (250 mm × 4.6 mm, 5 µm)	Methanol/(H_3PO_4 8.5 mM/ triethylamine pH = 2.8) 40:60 v/v	UV at 247 nm	17
HPLC	Yogurt, non-alcoholic beverages and fruit juices	Yogurt samples treated with potassium ferricyanide (III) and zinc sulphate. Non-alcoholic beverages and fruit juices diluted and filtered	Divinyl benzene–styrene copolymer (DVB-H)	0.01 N H_2SO_4:CH_3CN (75:25)	UV at 220 nm	18

Table 7.1 cont'd
(c)

Method	Matrix	Sample preparation	Method conditions	Reference
Micellar electrokinetic chromatography (MECC)	Cola beverages and jams	Butyl paraben was used as an internal marker	Additives were separated using a 20 mM berate buffer with 35 mM sodium cholate, 15 mM sodium dodecyl sulphate and 10 % methanol added at pH 9.3	20
Time resolved lanthanide-sensitised luminescence	Soft drinks	Sample (2.5 mL) was degassed, neutralised with 2 M sodium hydroxide and diluted to 10 mL with distilled water. 1 ml was used.	The method involved the formation of the corresponding ternary chelates with terbium (III) and trioctylphosphine oxide (TOPO) in the presence of Triton X-100 and the measurement of the initial rate and equilibrium signal of this system	21
Spectrophotometric	Soy sauce and pickles	Liquid samples used directly; samples with a high insoluble solid content homogenised with 4 times their volume of water and filtered	Benzoic acid is measured enzymatically through its reaction with benzoate 4-hydroxylase coupled with NADPH and O_2	22
HPTLC	Beverages	No extraction or clean-up required	Aliquots of samples and standards are chromatographed on preadsorbent silica gel of C18 bonded silica gel plates containing fluorescent indicator and the zones, which quench flurorescence, are compared by scanning densitometry	23
Potentiometric using a selective electrode	Beverages	Degassed then bubbled with O_2. Aliquot treated with aq nitric acid and extracted with chloroform. Evaporated to dryness, dissolved in NaOH solution and adjusted to pH 7 with $HClO_4$	An aliquot of 20 mL is employed for analysis with the benzoate-sensitive electrode. Electrode Pt\Hg\Hg-2(Bzt)(2)\graphite, where Bzt stands for benzoate ion. Electrode corresponds to Bzt with sensitivity of 57.7±1.0 mV/decade over the range 5×10^{-4} to 1×10^{-1} mol/L at pH 6.0–8.0	24

Table 7.2 Summary of statistical parameters for benzoic acid in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
GC	Foods	Full collaborative trial	see Table 7.3	1
HPLC	Orange juice	Full collaborative trial	see Table 7.4	8
HPLC	Foods	Full collaborative trial	see Table 7.5	11
GC–MS (SIM)	Foods	Precision of method established and applied to real samples (n=249)	Detection limit 100–200 pg. Mean recovery 97.2 % for cheese spiked at levels 50–500 mg/kg (n=3) for each level. Method applied to 249 samples of foods and beverages on sale in markets in Rome	2
HPLC	Foods	Precision of method established and applied to real samples (n=65)	Linear range 2.5–100 mg/L. Detection limit 10 mg/L in a juice matrix. Samples spiked at 0.10 and 0.05 % gave recoveries of 82–96 %	14
GC	Foods	Precision of method established and applied to real samples (n=37)	Detection limit lower than 0.5 ppm. Recoveries: Spiked vinegar at 200 µL 94.9 % CV 6.7 % (n=3). Spiked soy sauce at 200 µL 104.9 % CV 5.9 % (n=3). Method applied to 37 liquid food samples	7
GC	Foods	Precision of method established and applied to real samples (n=36)	Detection limit 2 ppm. Recovery studies performed on various foods spiked with benzoic acid at levels 200–1000 µg. Recoveries 93.2–102.2 %, CV <8 %	3
HPLC	Labaneh (concentrated set yogurt)	Precision of method established and applied to real samples (n=25)	Linear range 32–300 mg/L Recoveries added at 31.8 and 63.6 mg/100 g to labaneh, averaged 90.3 and 90.6 % with CV of 0.5 % and 0.2 %, respectively	12

Table 7.2 cont'd

Method	Matrix	Extent of validation	Statistical parameters	Reference
GC	Fatty foods	Precision of method established and applied to real samples	Linear range (µg/mL) 0.4–100 (FID), 1.5–500 (MS) (n=3) LOD (µg/mL) 0.15 (FID), 0.8 (MS) RSD (%) 3.1 (FID), 3.5 (MS) Checked with real samples for 5 samples analysed in triplicate by SPE–GC–FID. Plain mayonnaise 175 mg/kg (RSD 5.7 % n=15) Garlic mayonnaise 625 mg/kg (RSD 4.8 % n=15)	5
HPLC	Beverages and jams	Precision of method established and applied to real samples	Recovery data for 4 spiked beverage samples ranged from 98.1–104.2 %. Linear range 0 to 100 mg/L. Results of jam samples using this method compared favourably with NKLM method	9
HPLC	Quince jam	Precision of method established and applied to commercial samples	Detection limit for sodium benzoate was 0.5 µg/mL. The method was precise (SD=0.003; CV %=2.76; n=6). Recovery of sodium benzoate from spiked samples between 94.7 and 100 %	15
GC	Foods	Precision of method established and applied to real samples	Linear range 0.2–25 mg/L. Detection limit 0.07 mg/L RSD 2.9 % The method was applied to non-fatty foods i.e. soft drinks, jams, and sauces	6
Time resolved lanthanide-sensitised luminescence	Soft drinks	Precision of method established and applied to the direct analysis of several soft drinks (n=9)	The dynamic ranges of the calibration graphs obtained by using kinetic and equilibrium measurements were 0.2–36 µg/mL and 0.15–30 µg/mL and detection limits were 0.07 and 0.04 µg/mL RSD, ranged between 2.3 and 3.0 %. Analytical recoveries ranged from 89.3 to 108.5 %. Benzoic acid was determined using a system of 2 equations which were resolved by using the calibration data obtained individually and by multiple linear regression	21
Spectro-photometric	Soy sauce and pickles	Precision of method established and applied to commercial samples	Recovery at 30 and 300 µg/g in soy sauce and pickles ranged 95.9–99.7 %. Within-laboratory variability RSD 1.7 % and 1.1 % at 5 and 15 µg/mL levels (n=5). Limit of determination 5 µg/g.The method compared favourably with an HPLC method on 5 positive commercial samples	22

HPTLC	Beverages	Precision of method established and applied to commercial samples	Recoveries of benzoic acid from wine and juices spiked at 50–300 ppm averaged 98 % CV ranged from 2–5 %	23
GC–MS (SIM)	Foods	Precision of method established and applied to real samples	Recovery of spiked fruit vinegar (n=6) 76 % (RSD=8.0 %) Detection limit 0.1 ppb	4
Potentiometric using a selective electrode	Beverages	Precision of method established and applied to commercial samples	Detection limit 1.6×10^{-4} mol/L. The electrode shows easy construction, fast response time (10–30 s), low cost, excellent response stability and good selectivity for benzoate in the presence of several carboxylate and inorganic anions. Used to determine benzoate in beverages by means of standard additions method. The results using this electrode compared very favourably with those given by the official AOAC spectrophotometric method and by an HPLC procedure as well	24
HPLC	Syrup	Precision of method established	Linearity was verified by analysis in triplicate of 5 points in the range 0.10–0.30 mg/mL which corresponded to 50–150 % of the expected sample values. Recovery 100 %, RSD 1.38 %	17
HPLC	Yogurt, non-alcoholic beverages and fruit juices	Precision of method established	The method showed good precision and accuracy without interferences with other components of the samples	18
HPLC	Quince jam	Method applied to commercial samples in Portugal (n=6)	Samples analysed in triplicate for sodium benzoate: range 0.01–12.5 g/100 g, mean 0.17 g/100 g SD=0.337	13
HPLC	Fruit juices	Method applied to commercial samples in Indonesia (n=16)	Absorbance ratio (254/240 nm) for benzoic acid = 0.196±0.005	10
Micellar electrokinetic chromatography (MECC)	Cola beverages and jams	Method applied to cola samples (n=2)	Cola drink 1: Benzoic acid 39.3 µg/mL RSD 1.5 % (n=3) Cola drink 2: Benzoic acid 287 µg/mL RSD 1.2 % (n=3)	20
HPLC	Cranberry juice	Method applied to commercial samples	Canned cranberry juice contained 34 mg/L benzoic acid Fresh cranberry juice contained 41 mg/L	16

Table 7.3 Performance characteristics for benzoic acid in almond paste, fish homogenate and apple juice[1] (*JAOAC* (1983) **66**, 775, NMLK collaborative study)

Samples	Almond paste		Fish homogenate		Apple juice	
No. of laboratories	8	8	8	8	6	8
Units	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Mean value	982	1987	501	2044	41	1001
S_r	36	62	14	40	2.6	9.4
RSD_r	3.7 %	3.2 %	2.8 %	2.0 %	6.1 %	2.7 %
S_R	33	83	27	76	5.8	32
RSD_R	4.7 %	5.3 %	6.1 %	4.3 %	14.7 %	3.5 %
Ho_R	0.83	1.04	0.97	0.85	1.62	0.61
Av recovery	100.4 %	98.8 %	100.1 %	98.3 %	105.9 %	94.4 %

Key

Mean 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).

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 the reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

Table 7.4 Performance characteristics for benzoic acid in orange juice[8] (*Journal of AOAC International* (1995) **78** 80)

Sample (spike level)	0.5	1	3	4	10
No. of laboratories	9	9	9	9	9
Units	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
Mean	0.57	1.01	3.01	3.78	9.61
Mean recovery %	114.0	101.0	100.3	94.5	96.1
S_r	0.113	0.084	0.159	0.184	0.461
RSD_r	19.91 %	8.27 %	5.28 %	4.87 %	4.79 %
r	0.316	0.235	0.445	0.515	0.714
S_R	0.159	0.161	0.276	0.255	0.665
RSD_R	27.90 %	15.97 %	9.16 %	6.74 %	6.92 %
Ho_R	1.60	1.00	0.68	0.52	0.61
R	0.445	0.451	0.773	0.714	1.862

Key

Mean 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).

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 the reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

Table 7.5 Performance characteristics for benzoic acid in orange squash, cola drinks, beetroot and pie filling[11]

Sample	Orange squash		Cola drink	
No. of laboratories	8		7	7
Units	mg/kg		mg/kg	mg/kg
Mean	455.4	471.4	752.9	1234.4
Mean recovery %	100	99	95	89
S_r	7.06		7.63	6.71
RSD_r	2 %		1 %	1 %
r	20		21	19
S_R	18.79		30.73	89.48
RSD_R	4 %		4 %	7 %
R	53		86	251
Ho_R	0.6		0.7	1.3
Sample	Beetroot		Pie filling	
No. of laboratories	9		9	
Units	mg/kg		mg/kg	
Mean	1977.6	1752.0	1565.3	1773.2
Mean recovery %	94	93	95	97
S_r	118.47		42.80	
RSD_r	6 %		3 %	
r	331		120	
S_R	225.25		196.5	
RSD_R	12 %		12 %	
R	631		550	
Ho_R	2.3		2.3	

Key

Mean 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).

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 the reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

8

E220–8: Sulphites

8.1 Introduction

The major food groups contributing to dietary intake of sulphites consist of a wide variety: 15–500 mg/kg (dried fruit 600–2000 mg/kg; liquid pectin, horseradish pulp 800 mg/kg) and soft drinks 20 mg/kg (lime and lemon juice, 'barley water' 350 mg/kg; concentrated grape juice 2000 mg/kg; beer and cider 20–200 mg/kg; wines 200–260 mg/kg). The acceptable daily intake (ADI) for sulphites (expressed as sulphur dioxide) is 0.7 mg/kg body weight.

8.2 Methods of analysis

There are numerous methods published for the determination of sulphites in foodstuffs. The majority of these methods are for the determination of sulphur dioxide in foodstuffs. Quantitative methods for the determination of sulphur dioxide fall into two groups, direct and indirect methods. The former include titrimetric, polarographic, electrometric and colorimetric procedures, the latter generally involve separation by distillation in an inert atmosphere followed by absorption of the sulphur dioxide in an oxidising agent, typically iodine or hydrogen peroxide. The estimation is completed by volumetric, gravimetric, colorimetric or electrochemical procedures. Both types of methods may be used to measure free and total sulphur dioxide.[1]

Methods that have been developed for sulphites in foodstuffs include the Monier–Williams method,[2–5] enzymatic,[6,7] differential pulse polarography,[8,9] ion exclusion chromatography (IEC),[10,11] flow injection analysis (FIA),[12–15,20] sequential injection analysis (SIA),[16] HPLC,[17] capillary electrophoresis methods[18,19] and

vapour phase Fourier transform infrared spectrometry (FTIR).[21] Recently a comparison of five methods has been published for the analysis of total SO_2 in grape juice.[22] Classical methods, i.e. gravimetric[23] and titration[24–26] are still used by public analysts. A summary of these is given in Table 8.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available they have been summarised in Table 8.2. Many of these methods are AOAC Official Methods of Analysis and have been accepted as European Standards.

The optimised Monier–Williams method for the analysis of sulphites in foods is applicable to the determination of ≥ 10 ppm (μg/g) sulphites in foods and has been collaboratively tested.[2] The method measures free sulphite plus reproducible portion of bound sulphites (such as carbonyl addition products) in foods. The test portion is heated with a refluxing solution of hydrochloric acid to convert sulphite to sulphur dioxide. A stream of nitrogen is introduced below the surface of the refluxing solution to sweep sulphur dioxide through a water-cooled condenser and, via a bubbler attached to the condenser, into the hydrogen peroxide solution, where sulphur dioxide is oxidised to sulphuric acid. The generated sulphuric acid is titrated with standardised sodium hydroxide solution. The sulphite content is directly related to the generated sulphuric acid.[2–5] A summary of the procedure for this method is given in the Appendix and the performance characteristics are given in Table 8.3.

The enzymatic method for the determination of sulphite content, expressed as sulphur dioxide, in foodstuffs has been collaboratively tested.[7] Sulphite in liquid foods or extracts of solid foods is analysed according to the following principle, where NADH is reduced to nicotinamide adenine dinucleotide:

$$SO_3^{2-} + O_2 + H_2O \xrightarrow{\text{sulphite oxidase}} SO_4^{2-} + H_2O_2 \qquad [8.1]$$

$$H_2O_2 + NADH + H^+ \xrightarrow{\text{NADH peroxidase}} 2H_2O + NAD^+ \qquad [8.2]$$

Decrease in NADH is measured spectrophotometrically and is proportional to the concentration of sulphite. A summary of the procedure for this method is given in the Appendix with a summary of the statistical parameters being given in Table 8.4.

A differential pulse polarographic method for the analysis of sulphites in foods, applicable to the determination of ≥10 μg total SO_2/g in shrimp, orange juice, peas, dried apricots and dehydrated potatoes has been collaboratively tested.[9] The method measures sulphur dioxide which is purged with N_2 from acidified test samples, collected in electrolyte-trapping solution and then determined by differential pulse polarography.[8,9] A summary of the procedure for this method is given in the Appendix and a summary of the statistical parameters in Table 8.5.

An ion exclusion chromatographic (IEC) method for the analysis of sulphites in foods and beverages, applicable to the determination of SO_2 at ≥10 μg/g, has been collaboratively tested.[11] The method measures sulphur dioxide which is released by direct alkali extraction. Diluted test portions of liquids or diluted filtrates of

solid test portions are injected into LC or anion exclusion chromatographic system equipped with anion exclusion column and electrochemical detector.[10,11] A summary of the procedure for this method is given in the Appendix and a summary of the statistical parameters in Table 8.6.

A flow injection analysis (FIA) method for the analysis of sulphite (total) in foods and beverages, applicable to the determination of ≥5 µg/g total SO_2 in shrimp, potatoes, pineapple and white wine has been collaboratively tested.[13] The method measures sulphur dioxide by FIA using reaction with malachite green. In the FIA system, test solution is reacted with NaOH to liberate aldehyde-bound sulphite. Then, the test stream is acidified to produce SO_2 gas, which diffuses across a Teflon membrane in the gas diffusion cell into a flowing stream of malachite green, which is discoloured. The degree of discolouration of malachite green is proportional to the amount of sulphite in test solution.[12,13] A summary of the procedure for this method is given in the Appendix and a summary of the statistical parameters is given in Table 8.7.

A flow injection analysis (FIA) method for the analysis of sulphite (free) in wines, applicable to the determination of free sulphite and bound sulphite that is labile at pH 2.2 has been collaboratively tested.[15] The method measures sulphur dioxide by FIA using reaction with malachite green. Injected test portion aliquot is mixed with concentrated citric acid solution (pH about 2), which forms SO_2 gas from free sulphite and from the portion of bound sulphite that is labile under these conditions. SO_2 gas diffuses across a Teflon membrane in the gas diffusion cell into a flowing stream of malachite green solution. Malachite green is discolourised in proportion to the amount of SO_2 gas that diffuses across the membrane. The degree of discolouration of malachite green is measured spectrophotometrically.[14,15] A summary of the procedure for this method is given in the Appendix and a summary of the statistical parameters is given in Table 8.8.

An extensive survey of sulphite containing foods (211 samples of foods and beverages) has been carried out in Italy.[27] The determination of sulphite was performed on all samples according to AOAC 990.28[3] with the exception of wine for which the determination was performed according to EU official method (EC 1108/82). Analysis of the samples was performed in duplicate; the coefficient of variation was always below 10 %.

8.3 Recommendations

There are many methods available for the analysis of sulphites in foods and the decision as to what method should be used depends on the expected level of sulphite and the matrix to be analysed. Further method development is required to establish methods for determining sulphite in cabbage, dried garlic, dried onions, leeks and soy proteins as two of the standard methods[2,6] are not applicable to these matrices.

8.4 References

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8.5 Appendix: method procedure summaries

Analysis of foodstuffs with sulphite content ≥10 mg/kg using optimised Monier–Williams method[2]

Scope
Distillation method for determination of sulphite content, expressed as sulphur dioxide. The method is applicable in the presence of other volatile sulphur compounds. It is not applicable to cabbage, dried garlic, dried onions, ginger, leeks and soy proteins.

Principle
Free sulphite plus reproducible portion of bound sulphites (such as carbonyl addition products) in foods are measured. The test portion is heated with a refluxing solution of hydrochloric acid to convert sulphite to sulphur dioxide. A stream of nitrogen is introduced below the surface of the refluxing solution to sweep sulphur dioxide through water-cooled condenser and via a bubbler attached to the condenser, into the hydrogen peroxide solution, where sulphur dioxide is oxidised to sulphuric acid. The generated sulphuric acid is titrated with standardised sodium hydroxide solution. The sulphite content is directly related to the generated sulphuric acid (see[3,4]).

Procedure
Sample preparation and analysis should be carried out as quickly as possible to avoid loss of labile forms of sulphite.

(Note: to become familiar and proficient with the method before routine use, it is recommended that food test portions containing known amounts of sulphite are analysed. The analysis should be performed in a manner that precludes any loss of

sulphite by oxidation or reaction with components in food. Since sulphites are reactive with air and food matrices and often lack stability, portions are fortified with a stable source of sulphite, not sodium sulphite or similar salts. Sodium hydroxymethylsulphonate (HMS), which is a bisulphite addition product of formaldehyde and which is structurally similar to some combined forms of sulphite in foods, is useful for preparing stable fortified test materials.)

For analysis, 50 g of prepared sample of sulphite-free food are transferred to the flask (Fig. 1[2]). An aliquot portion of aqueous solution of HMS sodium salt is added. The solution is analysed immediately.

HMS recoveries of more than 80 % from food matrices fortified at 10 mg/kg are recommended to ensure accurate analytical data.

Sample preparation

Solid sample:
Transfer 50 g of food, or a quantity that contains 500 µg to 1500 µg of sulphur dioxide, to a food processor or blender. Add 100 ml of the ethanol/water mixture and briefly grind the mixture. Continue the grinding or blending only until the food is chopped into pieces small enough to pass through the ground glass joint of the flask.

Liquid samples:
Mix 50 g of test sample, or quantity that contains 500 µg of sulphur dioxide, with 100 mL of the ethanol/water mixture.

System preparation
Use the distillation apparatus assembled as shown in Fig. 1,[2] put the flask in the heating mantle and add 400 mL of water to the flask. Close the stopcock of the funnel and add 90 mL of hydrochloric acid solution to the funnel. Begin nitrogen flow at 200 mL/min ± 10 mL/min and initiate the condenser coolant flow. Add 30 mL of 3 % hydrogen peroxide solution to the vessel. After 15 min, the apparatus and water will be thoroughly deoxygenated and the prepared test portion may be introduced into the apparatus.

Sample introduction and distillation
Remove the dropping funnel and quantitatively transfer the test portion in aqueous ethanol to the flask. Wipe the tapered joint clean with a laboratory tissue, quickly apply stopcock grease to the outer joint of the funnel, and return the funnel to the flask. Examine each joint to be sure that it is sealed.

Use a rubber bulb equipped with a valve to apply head pressure above the hydrochloric acid solution in the funnel. Open the stopcock of the funnel and let hydrochloric acid solution flow into the flask. Continue to maintain sufficient pressure above the hydrochloric acid solution to force the solution into the flask. The stopcock may be closed, if necessary, to pump up pressure above the acid, and then opened again. Close the stopcock before the last 2 to 3 mL drain out of the funnel to guard against the escape of sulphur dioxide into the funnel.

Apply power to the heating mantle. Use a power setting that causes 80–90 drops/min of condensate to return to the flask from the condenser. Let the contents of the flask boil for 105 min, and then remove the vessel.

Determination and calculation

Titration
Immediately titrate (burette) the contents of the vessel with sodium hydroxide standard solution to a yellow end-point that persists longer than 20 s. Calculate the mass fraction, W, of sulphite, round the result to a whole number and express the sulphite content as sulphur dioxide in milligrams per kilogram, using equation [8.3]:

$$W = \frac{32.03 \times V \times 1000 \times N}{m} \qquad [8.3]$$

where
32.03 is the milliequivalent weight of SO_2 in grams per mole
N is the molarity of the sodium hydroxide standard solution, in mole per litre
V is the volume of sodium hydroxide standard solution with N = 0.010 mol/L required to reach the end point in millilitres
1000 is the factor to convert milliequivalents to microequivalents
m is the amount of test portion introduced into the round-bottomed flask in grams

Blank determination
Determine blank on reagents by titration and, if necessary, correct results accordingly. (For further details see BS EN 1988–1:1998.)[2]

Analysis of foodstuffs using enzymatic method[6]

Scope
Enzymatic method for determination of sulphite content, expressed as sulphur dioxide. Other sulphur-containing substances such as sulphate, sulphide or thiosulphate do not interfere with the determination. Carbonyl-sulphite complexes react as free sulphites. Isothiocyanates, occurring in, for example, mustard, interfere with the determination. The method is not applicable to cabbages, dried garlic, dried onions, ginger, leeks and soy proteins.

Principle
Sulphite oxidised to sulphate in the presence of sulphite oxidase with the liberation of hydrogen peroxide at the same time. Reduction of hydrogen peroxide and conversion of NADH to NAD^+ in the presence of NADH peroxidase. Conversion of $NADH^+$ to NAD^+ is determined spectrometrically at 340 nm and is proportional to the concentration of sulphite.

Procedure

Preparation of the sample test solutions

- *General*
  Remove high concentrations of ascorbic acid of more than 100 mg/kg or 100 mg/L of sample (see fruit juices). If the concentration of sulphite in the sample test solution is higher than 0.3 g/L, dilute the sample test solution prior to the determination or take a smaller sample volume.

Liquid samples

- *White wine, brandy and beer*
  Analyse white wine and brandy directly. Beer should be filtered to remove carbon dioxide. It may be necessary to decolourise beer. For the decolourisation, add not more than about 0.7 g of bentonite to 10 mL of filtered beer in a 50 mL glass beaker, stir the mixture for 2 min using a magnetic stirrer and then filter the solution into another 50 mL glass beaker.
  For the enzymatic determination take 100 µL to 200 µL of wine or 500 µL of brandy or beer respectively.
- *Red wines*
  Adjust 25 mL of red wine to pH 7.5 to 8.0 with the sodium hydroxide solution in a beaker. Transfer the solution into a 50 mL volumetric flask and dilute to the mark with water and mix. It is often necessary to decolourise red wine. This can be done as described for fruit juices.
- *Fruit juices*
  Centrifuge cloudy juices at approximately 2000 g. Add 5 mL of juice into a beaker and adjust the pH to 5 to 6 with the sodium hydroxide solution. Remove ascorbic acid by adding approximately 40 units of ascorbate oxidase in solution to the juice and leave the sample for 10 min, or remove the ascorbic units by stirring for 3 min with an ascorbate oxidase spatula. Then adjust the pH to 7.5 to 8.0 with the sodium hydroxide solution. In the case of coloured juices, add approximately 0.25 g of polyvinylpolypyrrolidone and stir the mixture for 1 min. Transfer the mixture to a 10 mL volumetric flask and dilute with water. Filter the solution and take 200 µL for enzymatic determination.

Solid foodstuffs

Homogenise the sample thoroughly and extract with water at 60 °C for 5 min. Shake occasionally. Cool the sample to ambient temperature before analysing. Vary the sample size depending on the amount of sulphite. In the case of e.g. potato flakes, take 5.0 g of homogenised material into a 50 mL volumetric flask. Add 40 mL of water. Close the flask and extract in a water bath at 60 °C for 5 min. Shake occasionally. Cool the volumetric flask, either by letting it stand for at least 15 min, to ambient temperature, or in a water bath of 20 °C, and dilute to the mark with water (V_3 = 50 mL). If necessary centifruge the solution.

The following sample quantities of some other foods are suggested:

- Dried fruit 1.0 g of sample/50 mL of water
- Jam 5.0 g of sample/50 mL of water
- Spices 0.1 g of sample/50 mL of water
- Dried potato products 2.0 g of sample/50 mL of water

Take 100 µL to 500 µL of these solutions for enzymatic determination.

Determination
Perform the determination according to Table 8.1 at a temperature of 20 °C to 25 °C in a quartz cell usually with a sample volume of 100 µL. If the sample volume is different from 100 µL adjust the volume of added water so that the final volume of water and sample is 2.00 mL. If the reaction has not stopped, continue to read the absorbance at intervals of 2 min until the change in absorbance is constant. If the absorbance decreases constantly, extrapolate the absorbance back to the time of addition of the sulphite oxidase suspension to estimate the A_2 to be used. (For further details see BS EN 1988–2:1998.)[6]

Analysis of foodstuffs with sulphite (total) content ≥10 mg/kg using differential pulse polarographic method[8]

Scope
Differential pulse polarographic method for determination of ≥10 µg total SO_2/g in shrimp, orange juice, peas, dried apricots and dehydrated potatoes.

Principle
SO_2 is purged with N_2 from acidified test sample, collected in electrolyte-trapping solution and then determined by differential pulse polarography.
(Notes:
1 Analyst must construct purge-trap apparatus and ensure proper operation by analysing aqueous SO_2 solutions before analysing test samples.
2 System must be purged with N_2 between test samples to remove residual SO_2.
3 Analysis must be completed without undue delay; aqueous solutions of sulphites are unstable.)

Procedure

Test sample preparation
Use open-pan balance (Mettler P1200 [replaced by PM2000], sensitivity 10 mg per division, or equivalent) to weigh representative test sample (≥10.00 g) into 200 mL Erlenmeyer. Add *c.* 0.5 g antifoam and then add 5 % alcohol so total weight of mixture is 100.00 g. Stopper and shake Erlenmeyer, or if necessary homogenise to obtain fine suspension. Complete test sample preparation quickly to minimise oxidation of SO_2 by atmosphere O_2.

Determination

Shake prepared test sample and immediately weigh aliquot of suspension (≤10 g) containing ≤60 μg SO_2 into 25 × 200 mm tube and, if necessary, add 5 % alcohol so total weight of mixture is *c.* 10 g. Add 10.0 mL electrolyte-trapping solution to dry polarographic cell and assemble apparatus as in Fig. 987.04.[8]

(a) Total SO_2 – Add to test sample tube 0.5 mL 2M ammonium acetate buffer and 0.2 mL H_2SO_4 (1 + 1) to adjust pH to *c.* 1.5. Purge with O_2-free N_2 for 10 min at 1 L/min. Stop N_2 flow, add 2 mL H_2SO_4 (1 + 1) to test sample tube and place tube in block heater preheated to 100 ºC. Purge with N_2 for 10 min at 1 L/min. Stop N_2 flow and lift exit tubing from electrolyte-trapping solution in polarographic cell (to prevent solution from backing up). To verify complete transfer of SO_2 from test sample, reinsert exit tubing into electrolyte-trapping solution, purge again for 5 min, and obtain polarogram. Repeat if necessary.
(b) Reagent blank – Prepare reagent blank in same manner as for test sample, and carry through analysis.

Calibration curve

Prepare calibration curve at time of test sample analysis as follows: Pipette 10.0 mL electrolyte-trapping solution into dry polarographic cell and add 50 μL 200 μg/mL standard solution. Bubble N_2 through solution for 4 min and obtain polarogram as for test sample. Repeat 5 times with additional 50 μL aliquots of standard, bubbling N_2 for 30 s after each addition. Construct calibration curve, representing 10, 20, 30, 40, 50, 60 μg SO_2 in cell.

Calculation

Obtain amount total SO_2, μg, in cell from calibration curve by using highest peak currents produced by test sample. Calculate SO_2 in test sample, μg/g, correcting for reagent blank if necessary.

Alternative trapping technique

Add 10.0 mL electrolyte-trapping solution to 25 mL graduated cylinder and insert exit tubing (Fig. 987.04, items 2 and 7). Add to test sample tube 0.5 mL 2 M ammonium acetate buffer and 0.2 mL H_2SO_4 (1 + 1) to adjust pH to *c* 1.5. Purge with O_2-free N_2 for 10 min at 1 L/min. Stop N_2 flow, add 2 mL H_2SO_4 (1 + 1) to test sample tube and place tube in block heater preheated to 100 ºC. Purge with N_2 for 15 min at 1 L/min. Stop N_2 flow and transfer solution to polarographic cell, deaerate, and obtain polarogram. Dilute aliquot with electrolyte-trapping solution if too concentrated. Calculate total SO_2 by using calibration curve. Purging time given should be sufficient to completely transfer SO_2 from most test portions. Verify complete transfer by additional purging, e.g., 5 min, using 10.0 mL fresh electrolyte-trapping solution. Allow longer purging time if required. (For further details see AOAC Official Method 987.04.)[8]

Analysis of foods and beverages with sulphite content ≥10 mg/kg using ion exclusion chromatographic method[10]

Scope

Ion exclusion chromatographic method for determination of SO_2 at ≥10 µg/g. Not applicable to dark-coloured foods or to ingredients where SO_2 is strongly bound, e.g. caramel colour. Method does not detect naturally occurring suphfite. Results of the interlaboratory study supporting the acceptance of the method:

$$S_r = 4.7;\ S_R = 8.9;\ RSD_r = 12.3\ \%;\ RSD_R = 21.2\ \%$$

Principle

SO_2 is released by direct alkali extraction. Diluted test portions of liquids or diluted filtrates of solid test portions are injected into LC or anion exclusion chromatographic system equipped with anion exclusion column and electrochemical detector.

Procedure

Determination

Dilute liquid test portion with pH 9 buffer so that height of sulphite peak from test portion is similar to that of 0.60 µg/mL standard within 50 %. For solid test portions, homogenise 0.2–1.0 g test portion in 10–100-fold excess pH 9 buffer for 1 min with homogeniser and filter (0.2–0.45 µm). Dilute filtrate as necessary, comparing signal intensity with that of 0.60 µg/mL working standard solution. For acidic test portions such as lemon juice, if pH of diluted test portion is <8, adjust to pH between 8 and 9 with dilute NaOH solution or perform extraction with 100 mM Na_2HPO_4, 10 mM D-mannitol solution. Inject 0.60 µg/mL standard solution, and then inject prepared, diluted test portion.

Extraction, filtration, dilution and injection should be done within 10 min because sulphite concentration in extract tends to decrease gradually. Errors due to gradual decrease in detector sensitivity during multiple test portion injections can be minimised by injecting standard solution alternatively with test portion injections. Cleaning electrode at beginning of each chromatographic run may alleviate decrease in sensitivity. To clean electrode, apply –1.0 V for several min followed by +1.8 V for several more min and then equilibrate at +0.6 V. Alternatively, short train of electrode cleaning voltages can be applied automatically after each injection.

Calculations

Calculate µg/g SO_2 in test portion as follows:

$$SO_2,\ \mu g/g = 0.60 \times (PH/PH') \times \text{dilution factor} \qquad [8.4]$$

Where PH and PH′ = peak height from test portion and standard, respectively, and dilution factor takes into account initial dilution for extraction and any subsequent dilution. (For further details see AOAC Official Method 990.31.)[10]

Analysis of foods and beverages with sulphite (total) content ≥5 mg/kg using flow injection analysis method[12]

Scope

Flow injection analysis method for determination of ≥5 µg/g total SO_2 in shrimp, potatoes, pineapple and white wine.

Results of the interlaboratory study supporting the acceptance of the method:

$$S_r = 23.5;\ S_R = 35.1;\ RSD_r = 7.5\ \%;\ RSD_R = 14.0\ \%$$

Principle

Sulphite is determined by flow injection analysis (FIA) using reaction with malachite green. In the FIA system, test solution is first reacted with NaOH to liberate aldehyde-bound sulphite. Then test stream is acidified to produce SO_2 gas, which diffuses across Teflon membrane in gas diffusion cell into flowing stream of malachite green, which is discoloured. Degree of discolouration of malachite green is proportional to amount of sulphite in test solution.

Procedure

Test portion preparation

(a) Solid foods – weigh and transfer to blender representative test portion. Add weight of sodium tetrachloromercurate (TCM) solution, at 3–30 times that of test portion. Use amount of reagent that will result in SO_2 concentration in final extract that is within range of standards and also result in semiliquid slurry in blender. Blend until slurry is homogeneous (*c.* 2 min), and centrifuge.
(b) White wine – weigh representative test portion (1–2 g) into tared 50 mL disposable tube. Add TCM solution at level 19 times weight of test portion, cap tube and mix well. Centrifuge if solution is turbid.

Determination

(a) System start-up – Begin pumping 2 donor reagents, sulphuric acid and sodium hydroxide, and recipient reagents, malachite green solution(2) and phosphate buffer reagent, through pump tubes specified in Fig. 990.29.[12] Once flow is established and system has equilibrated (*c.* 10–20 min), zero detector and monitor A at 615 nm with detector set at 0.1 AUFS and recorder at *c.* 0.5 cm/min. Base-line noise (peak to peak) should be <3 mm; if it is not, determine source of excessive noise and make necessary corrections. (Note: Some detectors are prone to trapping air bubbles that must be purged from flow cell.) If detector has variable electronic filter, this can be adjusted to *c.* 1–5 s to reduce baseline noise. Check flow rates of donor and recipient reagents to be sure that they meet specifications in Fig. 990.29.
(b) Test sample analysis – Repeatedly inject 10 µg/mL sulphite working standard condition, until peak height is stable and recorder deflection is *c.* 60–90 % full

scale. It may be necessary to adjust recorder span to achieve desired peak height. Following each injection of either test solution or standard into FIA system, wash out sample valve and first reaction coil by making injection of 1 M H_2SO_4 to remove any mercuric oxide that can precipitate inside reaction coil. Following is typical timing pattern that can be used to ensure adequate washing of valve and coil with no overlap of sample and 1 M H_2SO_4:

Repeated injections of 10 µg/mL standard should yield series of peaks with CV for peak height ≥5 % (n = 5). If this precision is not achieved, determine and correct source of problem.

Once system has stabilised, inject series of sulphite standard solutions once each. Inject prepared test solutions, taking care to inject only clear (or slightly turbid) supernate. Inject all test solutions in duplicate and be sure to include 1 M H_2SO_4 washing procedure between each injection. After *c.* 10 test solution injections (5 test solutions in duplicate), repeat series of standards. For any test solution with peak height that exceeds peak height of 10 µg/mL SO_2 standard, dilute test solution with TCM solution into analytical range (1–10 µg/mL).

After all test solutions and standards have been analysed, thoroughly rinse FIA manifold by pumping water through all 4 lines for 15 min, followed by FIA rinsing reagent, for 15 min. Manifold can be stored with this reagent. Release tension on pump rollers to prolong tubing life.

(c) Data reduction – Determine peak height of each test and standard solution injection to ±0.5 mm. If blank injection produced peak, subtract this peak height from all test and standard solutions. Average peak heights of each injection for individual standard concentrations and plot µg/mL vs peak height.

Determine SO_2 concentration in test extracts from standard curve and calculate concentration in test portion as follows:

$$SO_2\ \mu g/g = C \times (F/S) \qquad [8.5]$$

where
C = concentration in test extract
F = final total weight of test portion slurry
S = initial weight of test portion taken.

(For further details see AOAC Official Method 990.29.)[12]

Analysis of wine for determination of sulphite (free) using flow injection analysis method[14]

Scope

Flow injection analysis method for determination of free sulphite and bound sulphite that is labile at pH 2.2.

Results of the interlaboratory study supporting the acceptance of the method:

$S_r = 0.55$; $S_R = 1.18$; $RSD_r = 8.77$ %; $RSD_R = 19.27$ %

Principle

Injected test portion aliquot is mixed with concentrated citric acid solution (pH about 2), which forms SO_2 gas from free sulphite and from portion of bound sulphite that is labile under these conditions. SO_2 gas diffuses across Teflon membrane in gas diffusion cell into flowing stream of malachite green solution. Malachite green is discolourised in proportion to amount of SO_2 gas that diffuses across membrane. Degree of discolouration is measured spectrophotometrically.

Procedure

Determination

(a) System start-up – Begin pumping citric acid donor reagent and 2 recipient reagents, malachite green solutions and phosphate buffer reagent through pump tubes specified in Fig. 990.29[12] and carry out start-up procedure as in 990.29E(a) (see[12]).

(b) Test sample analysis – Repeatedly inject 15 µL of 40 µg/mL sulphite working condition solution until peak height is stable and recorder deflection is 60–90 % full scale. It may be necessary to adjust recorder span to achieve desired peak height.

Five injections of 40 µg/mL standard should yield series of 5 peaks with CV for peak heights ≥5 %. If this precision is not achieved, determine and correct source of problem. Once system has stabilised, inject 15 µL portions of sulphite working standard solutions, injecting each standard once. No test portion pretreatment is required. Thoroughly mix undiluted wine and inject 15 µL portion directly into FIA system. To prevent loss of free SO_2, protect test portion from atmosphere until just before injection. Inject all test portions in duplicate. After about 10 test portion injections, repeat series of standards.

For wines with free SO_2 levels <3 µg/mL, increase sensitivity either by injecting >15 µL portion or by decreasing full scale response on detector. Prepare standards in range of test samples (e.g. 2 µg/mL, 1 µg/g) and repeat determination of low level test portions using standards of 0–3 µg/mL for calibration.

After all test portions have been analysed, thoroughly rinse FIA manifold by pumping water through all 4 lines for 15 min, followed by FIA rinsing reagent for 15 min. Manifold can be stored with this reagent. Release tension on pump rollers to prolong tubing life.

(c) Data reduction – Determine peak height for each test portion and standard injection to ±0.5 mm. If blank injection produced peak, subtract this peak height from all test portions and standards. Average peak heights of each injection for individual standard concentrations and plot peak height versus µg/mL SO_2.

Determine SO_2 concentration in wine test portion directly from standard curve.

(For further details see AOAC Official Method 990.30.)[14]

Table 8.1 Summary of methods for sulphites in foods

(a)

Method	Matrix	Sample preparation	Method conditions	Reference
Optimised Monier–Williams	Foods .	To 50 g samples add 100 mL 5 % ethanol:water mixture	Reflux with HCl in distillation apparatus. Sweep SO_2 through a condenser with nitrogen into hydrogen peroxide solution. SO_2 oxidised to sulphuric acid which is titrated with standardised NaOH solution	2–5
Enzymatic	Foods	Liquid samples: adjusted to pH 7.5–8.0 with NaOH (2 mol/L). Solid samples: homogenise thoroughly and extract with water 5 min at 60 ºC	Sulphite oxidised to sulphate in the presence of sulphite oxidase with the liberation of hydrogen peroxide at the same time. Reduction of hydrogen peroxide and conversion of NADH to NAD^+ in the presence of NADH peroxidase. Conversion of $NADH^+$ to NAD^+ is determined spectrometrically at 340 nm and is proportional to the concentration of sulphite	6,7
Differential pulse polarographic	Foods	10 g sample + 0.5 g antifoam + 5 % alcohol to 100 g. Homogenise if necessary	Weigh 10 g sample solution into tube. Add 10 mL electrolyte-trapping soln to dry polarographic cell and assemble (as 8,9). SO_2 purged with N_2 from acidified sample, collected in electrolyte-trapping solution and then detected by differential pulse polarography	8,9
FIA	Foods and beverages	Solid foods: Add 3–30 times TCM solution to test portion, blend until homogeneous and centrifuge White wine: Add 19 times TCM solution to 1–2 g.	Test solution reacted with NaOH, then test stream acidified to produce SO_2 gas which diffuses across Teflon membrane in gas diffusion cell into flowing stream of malachite green, which is discoloured. Detector set at 615 nm	12,13

Table 8.1 cont'd

Method	Matrix	Sample preparation	Method conditions	Reference
FIA	Wines	Thoroughly mix undiluted wine	15 µL injected directly into FIA system, mixed with conc citric acid solution (pH ~2) which forms SO_2 gas from free sulphite and from portions of bound sulphite that is labile under these conditions. SO_2 gas diffuses across Teflon membrane in gas diffusion cell into flowing stream of malachite green, which is discoloured and measured spectrophotometrically	14,15
Gas diffusion sequential injection system (GDSIS) with spectrometric detection	Wines	Thoroughly mix undiluted wine	Based on the formation of a coloured product from the reaction of SO_2, formaldehyde and pararosaniline. An acid was added to sample prior to passage through gas diffusion unit which was incorporated into manifold to prevent interference in spectrophotometric measurement. SO_2 gas from free sulphite and from portions of bound sulphite are determined	16
Capillary electrophoresis	Foods and beverages	Sulphite in samples converted to sulphur dioxide and finally to sulphate using a Monier–Williams distillation	Sulphate determined by CE using 75 µm fused capillary column with a buffer consisting of 5 mM sodium chromate and 0.5 mM OFM anion-BT reagent, pH 8.0, with indirect UV detection at 254 nm	18
Capillary electrophoresis	Grape skin extract and elderberry colour	Sulphite in samples converted to sulphur dioxide using a Rankine distillation. SO_2 was trapped in 0.6 % hydrogen peroxide solution as sulphuric acid	The sulphate ion determined by CE using 80.5 cm fused silica capillary column with a buffer of organic acid pH 5.6, with indirect UV detection at 240 nm against the reference at 200 nm	19
FIA	Wines	FIA method for spectrometric determination of CO_2 and SO_2 simultaneously. Analytes isolated from sample matrix using gas-diffusion units	The SO_2 is based on the decolouration of malachite green by sulphur dioxide. Two FIA manifolds are presented; one for the determination of CO_2 and another for simultaneous determination of CO_2 and SO_2, similar to[16]	20

FTIR	Musts and wines	To 1 mL sample, previously treated with 0.5 mL 1 M KOH at 30 ºC, 0.5 mL 3.4 M H_2SO_4 was added	The SO_2 evolved was swept by a stream of nitrogen to a gas infrared cell. The flow injection (FI) recordings were registered and the analytical variable was the area of the co-added spectra obtained, in wave number range 1429 and 1300 cm^{-1}, at a resolution of 16 cm^{-1} for a time of 1.5 mins from the first SO_2 spectrum. External calibration with $Na_2S_2O_5$ solutions in 10 % ethanol, treated in the same way, was employed	21
Comparison of 5 methods including distillation, iodimetric and enzyme-based	Grape juice	Attention focused on total SO_2 legal limit of 10 mg/L fixed in Europe for grape juice	Total SO_2 was measured by EC1108/82 and EC2676/90 distillation methods, the modified Monier–Williams distillation method,[3] the Ripper-Schmitt iodimetric titration method and an enzyme-based method	22

(b)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
Ion exclusion chromatographic IEC–EC	Foods and beverages	Diluted portions of liquid samples or diluted filtrates of solid samples	Sulphonated polystyrene/ divinylbenzene	0.60 µg/ml standard sodium sulphite solution	Electrochemical (amperometric) at +0.6 v	10,11
HPLC	Foods	Blended with buffered formaldehyde solution. SPE clean-up on RP C18 columns	Whatman C8	34 g 40 % aqueous tetrabutylammonium hydroxide and 3 g acetic acid in 700 mL water	Fluorescence ex 400 nm em 470 nm	17

Table 8.2 Summary of statistical parameters for sulphites in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
Optimised Monier–Williams	Foods	Full collaborative trial	see Table 8.3	2–5
Enzymatic	Foods	Full collaborative trial	see Table 8.4	6,7
Differential pulse polarographic	Foods	Full collaborative trial	see Table 8.5	8,9
Ion exclusion chromatographic	Foods and beverages	Full collaborative trial	see Table 8.6	10,11
Flow injection analysis	Foods and beverages	Full collaborative trial	see Table 8.7	12,13
Flow injection analysis	Wines	Full collaborative trial	see Table 8.8	14,15
RP-IP-HPLC	Foods	Precision of method established with standards (n=45) and validated for food samples	Post reagent spiking, average recovery 100 % SD 5.2 % (n=45) at levels of 5, 10 and 20 ppm by weight as SO_2. Recovery for reversibly bound sulphite spike was 95 %	17
GDSIS	Wines	Precision of method established with standards (n=10)	Two second-order calibration curves were established: 2–40 mg/L for free SO_2 and 25–250 mg/L for total SO_2 RSD (n=10) <1.2 % for free SO_2 and <2.3 % for total SO_2	16

Capillary electrophoresis	Foods and beverages	Precision of method established and applied to real samples	The instrument repeatability of the CE procedure was satisfactory CV % 1.4–8.5 for 5–50 µg/mL standard. Level of detection was 5 mg/kg	18
Capillary electrophoresis	Grape skin extract and elderberry colour	Precision of method established and applied to real samples	Recoveries of SO_2 25–1000 µg/g added to grape skin extract were 86–104 %. Quantitative limit was 15 µg/g. Method applied to 10 commercial colours. SO_2 detected in 5 grape skin extracts at levels of 38.4–944 µg/g. Values determined by CE and colorimetry in good agreement	19
FIA	Wines	Precision of method established and applied to real samples	RSD lower than 4.5 % for SO_2 determination were found. Simultaneous determination manifold is applicable in the concentration range of 0.05–0.3 g/L of SO_2	20
FTIR	Musts and wines	Precision of method established and applied to real samples	Limit of detection = 34 ppm SO_2. RSD 1.1 % (n=3) for 1 mL must sample. Results obtained for natural wine and must compared well with those obtained using an iodimetric reference method	21
Comparison of 5 methods	Grape juice	Established methods applied to grape juice samples	Analysis of variance disclosed a significant difference among the total SO_2 content in grape juices determined by five methods. Each method showed limits in relation of their ability to release combined SO_2	22

Table 8.3 Performance characteristics for sulphites in hominy, fruit juice and seafood[2]

Identified in interlaboratory test conducted for FDA[4]			
Sample	Hominy	Fruit juice	Seafood
Analyte	Sulphite	Sulphite	Sulphite
No. of laboratories	18	21	20
Units	mg/kg	mg/L	mg/kg
Mean value	9.17	8.05	10.41
S_r	1.33	1.36	1.47
RSD_r	14.49 %	16.9 %	14.13 %
r	3.72	3.81	4.12
S_R	1.42	1.62	2.77
RSD_R	15.5 %	20.14 %	26.62 %
R	3.98	4.54	7.76

Key

Mean 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).

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).

Table 8.4 Performance characteristics for sulphites in wine, dried apples, lemon juice, potato flakes, sultanas and beer[6]

Interlaboratory test conducted by the Max von Pettenkofer Institute of the Federal Health Office, Food Chemistry Department, Berlin, BRD		
Sample	Sultanas	Beer
Analyte	Sulphite	Sulphite
No. of laboratories	13	14
Units	mg/kg	mg/L
Mean value	260	4.9
S_r	16	0.3
RSD_r	6 %	5.8 %
r	45	0.8
S_R	46	0.6
RSD_R	18 %	11.6 %
R	129	1.6

Table 8.4 cont'd

Interlaboratory test carried out by Swedish National Food Administration[7]

Sample	Wine	Dried apples	
Analyte	Sulphite	Sulphite	Sulphite
No. of laboratories	6	10	10
Units	mg/L	mg/kg	mg/kg
Mean value	75	800	960
S_r	3	106	128
RSD_r	4 %	13 %	13 %
r	8	298	358
S_R	6	111	133
RSD_R	8 %	14 %	14 %
R	16	311	374

Sample	Lemon juice	Potato flakes	
Analyte	Sulphite	Sulphite	Sulphite
No. of laboratories	10	10	10
Units	mg/L	mg/kg	mg/kg
Mean value	270	28.3	110
S_r	13		
RSD_r	5 %		
r	37		
S_R	28	13	15
RSD_R	10 %	45 %	13 %
R	79	36	42

Key

Mean 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).

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).

Table 8.5 Performance characteristics for total sulphite in shrimp, orange juice, dried apricots, dehydrated potato flakes and peas[8,9]

Interlaboratory test carried out by FDA[9]

Sample	Shrimp			Orange juice	
Spike	0	12	80	10	35
No. of laboratories	14	14	14	14	12
Units	µg/g	µg/g	µg/g	µg/g	µg/g
Mean value	1.8	6.8	60.6	8.0	30
S_r	0.33	1.5	5.2	1.4	4.0
RSD_r	18.3 %	22.1 %	8.5 %	17.5 %	13.3 %
S_R	1.1	2.5	8.1	1.6	4.8
RSD_R	61.1 %	36.8 %	13.4 %	20.0 %	16.0 %

Sample	Dried apricots		Dehydrated potato		Peas	
Spike	40	1100	20	400	10	25
No. of laboratories	12	14	10	12	14	16
Units	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
Mean value	22.0	975.6	4.6	289.6	5.7	15.8
S_r	3.9	61.4	0.28	34.2	1.0	1.5
RSD_r	17.7 %	6.3 %	6.1 %	11.8 %	17.5 %	9.5 %
S_R	4.1	94.1	1.6	34.1	1.8	3.4
RSD_R	18.6 %	9.6 %	34.8 %	11.8 %	31.6 %	21.5 %

Key

Mean 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).

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).

Table 8.6 Performance characteristics for total sulphite in starch, lemon juice, wine cooler, dehydrated seafood and instant mashed potatoes[10,11]

Interlaboratory test carried out by US Army Natick research[11]

Sample	Starch			Diluted lemon juice		
Spike	0	10	30	0	10	30
No. of laboratories	16	16	16	12	16	14
Units	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
Mean value[a]	0	7.7	27.3	21.2	11.2	30.3
S_r	0	0.7	2.4	2.1	1.9	5.2
RSD_r	0	9.5 %	8.8 %	9.8 %	17.0 %	17.2 %
S_R	0	1.3	3.9	2.1	2.7	5.2
RSD_R	0	16.3 %	14.1 %	9.8 %	24.1 %	17.2 %

Sample	Wine cooler			Dehydrated seafood		
Spike	0	10	30	0	40	80
No. of laboratories	16	18	16	18	18	18
Units	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
Mean value[a]	21.8	11.8	32.9	35.4	28.8	63.2
S_r	1.0	2.4	1.8	4.7	5.3	3.8
RSD_r	4.4 %	20.4 %	5.4 %	13.2 %	18.5 %	6.0 %
S_R	3.7	4.6	4.3	9.1	11.2	18.9
RSD_R	17.1 %	39.3 %	13.0 %	25.8 %	38.9 %	29.9 %

Sample	Instant mashed potato		
Spike	0	80	400
No of laboratories	18	14	18
Units	µg/g	µg/g	µg/g
Mean value[a]	384	92.8	422
S_r	19.5	10.2	109.8
RSD_r	5.1 %	11.0 %	26.0 %
S_R	32.5	16.3	109.8
RSD_R	8.5 %	17.5 %	26.0 %

[a]Mean initial sulphite level of unspiked samples. Mean recovery of added sulphite for spiked samples

Key

Mean 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).

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).

Table 8.7 Performance characteristics for total sulphite in shrimp, potatoes, pineapple and wine[13]

Interlaboratory test carried out by FDA[13]

Sample	Shrimp		Potatoes		Pineapple	
No. of laboratories	6	6	7	7	7	7
Units	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
Mean value	8.6	57	10.4	416	213	278
S_r	0.9	4.4	1.5	40.2	15.5	15.5
RSD_r	10.7 %	7.7 %	14.3 %	9.7 %	7.2 %	5.6 %
S_R	1.3	8.1	3.6	95.8	16.1	15.5
RSD_R	14.6 %	14.2 %	34.8 %	23.0 %	7.6 %	5.6 %

Sample	White wine		Red wine	
No. of laboratories	7	7	6	7
Units	µg/g	µg/g	µg/g	µg/g
Mean value	62	119	17	41
S_r	1.9	2.4	7.1	1.0
RSD_r	3.0 %	2.0 %	42 %	2.4 %
S_R	3.9	7.30	11.5	13.7
RSD_R	6.3 %	6.2 %	68 %	34 %

Key

Mean 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).

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).

Table 8.8 Performance characteristics for free sulphite in wine[14,15]

Interlaboratory test carried out by FDA[15]						
Sample	White wine			Red wine		
No. of laboratories	7	7	7	7	7	7
Units	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
Mean value	19.1	10.9	6.6	1.8	4.4	3.1
S_r	0.8	0.4	0.5	0.2	0.8	0.3
RSD_r	4.0 %	4.1 %	7.4 %	10.1 %	18.7 %	8.3 %
S_R	1.8	1.5	0.9	0.6	1.2	0.5
RSD_R	9.4 %	13.5 %	13.4 %	35.8 %	26.5 %	17.0 %

Key

Mean 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).

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).

9

E249–50: Nitrites

9.1 Introduction

The major food groups contributing to dietary intake of nitrites are cured, dried meat products 50 mg/kg (various meat products 100 mg/kg; cured bacon 175 mg/kg) – residual amounts. The acceptable daily intake (ADI) for nitrites, expressed as sodium nitrite, is 0.1 mg/kg body weight.

9.2 Methods of analysis

Many of the traditional methods for the determination of nitrite rely on variations of the Greiss diazotisation procedure, in which an azo dye is produced by coupling a diazonium salt with an aromatic amine or phenol. The diazo compound is usually formed with sulphanilic acid or sulphanilamide and the coupling agent is N-1-naphthylethylene diamine (NED).[1] These methods involve colorimetric determination.[2–11] Methods that have been developed more recently include spectroscopic determination after enzymatic reduction,[12–14] ion-exchange chromatography (IC),[15–18] flow analysis (FA),[19–20] differential pulse voltammetry (DPV)[21] and capillary electrophoresis (CE).[22] A summary of these methods is given in Table 9.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 9.2. Some of these methods are AOAC Official Methods of Analysis,[3–5] two methods have been accepted as a European Standard[10,14] and three as International Standards.[2,19,20]

There are three AOAC Official Methods for the determination of nitrite in foods:

1 Nitrates and nitrites in meat, xylenol method.[3]
2 Nitrites in cured meat, colorimetric method,[4] and
3 Nitrate and nitrite in cheese, modified Jones reduction method.[5]

These methods were developed in the early 1980s and there are no performance characteristics available for them.

The European Standard specifies two methods for the determination of nitrite in meat products:

1 Spectrometric determination:[10] Treatment in an aqueous extract of the analytical sample with sulphanilamide and N-(1-naphthyl)ethylenediammonium dichloride to produce a red compound with spectrometric measurement of the colour intensity of this red compound at 540 nm.
2 An ion-exchange chromatographic (IC) method:[15] The extraction of nitrate and nitrite is carried out with hot water. Any interfering substance is removed by clarification with acetonitrile and subsequent filtration. The determination is carried out by ion-exchange high performance liquid chromatography (IC) and ultraviolet (UV) detection at a wavelength of 205 nm.

A summary of the procedure for these methods is given in Appendix 1 and the performance characteristics are given in Table 9.3. NMKL have carried out collaborative studies which show that the European Standard ENV 12014-4 is well suited for the determination of nitrite and nitrate in different foods (e.g., meat products, vegetables, baby food and cheese).[16] The performance characteristics are given in Table 9.4.

The International Standard specifies three methods for the determination of nitrite in milk and milk products.

1 Cadmium reduction and spectrometric determination:[2] Test portion dispersed in warm water, followed by precipitation of the fat and proteins, and filtration. Reduction of the nitrate to nitrite in a portion of the filtrate by means of copperised cadmium. Development of a red colour in portions of both unreduced filtrate and of the reduced solution, by addition of sulphanilamide and N-1-naphthyl ethylenediamine dihydrochloride, and spectrometric measurement at a wavelength of 538 nm. Calculation of the nitrite content of the sample and of the total nitrite content after reduction of nitrate, by comparing the measured absorbances with those of a set of sodium nitrite calibration solutions.
2 Segmented flow analysis method:[19] Test portion suspended in an ammonium- and sodium chloride solution. Transference of a part of this suspension to the analyser. Dialysis of this suspension. Determination of the nitrite by a spectrometric method. Determination of the standard nitrite solutions by the same procedure. Calculation of the nitrite content by comparing the reading obtained from the test portion with the readings of the standard solution.
3 Flow injection analysis with in-line dialysis:[20] Test sample suspended in warm extraction buffer. Separation of fat by centrifugation and rapid cooling. Analyses of small portions of the de-fatted suspension by flow injection

analysis (FIA). In-line dialysis to remove protein and remaining fat. Reduction of nitrate to nitrite by cadmium. Reaction of nitrite with sulphanilamide and N-(1-naphthyl)-ethylenediamine providing a red-coloured azo dye. Measuring of the colour in a flow cell at maximum absorption of the dye at 540 nm with reference to the absorption measured at 620 nm. Calculation of the nitrite and nitrate contents of the sample with reference to the measured absorbances for a series of standard solutions of nitrite and nitrate, respectively. If the nitrite content exceeds 0.5 mg per kg, or exceeds 10 % of the nitrate content, correction of the nitrate content by subtracting the nitrite content from the obtained nitrate results.

A summary of the procedure for these methods and precision data are given in Appendix 2.

9.3 Recommendations

There are numerous methods available for the analysis of nitrite in foods and the decision as to what method should be used depends on the matrix and the analytical instrumentation available. Methods may need to be adapted for different matrices.

9.4 References

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9.5 Appendix 1: method procedure summaries (meat – DD ENV 12014)

Spectrometric determination of nitrate and nitrite content of meat products after enzymatic reduction of nitrate to nitrite[10]

Preparation of the sample solution

Homogenise the laboratory sample with the appropriate equipment. Take care that the temperature of the sample material does not rise above 25 ºC. If a mincer is used, pass the sample at least twice through the equipment. Weigh, to the nearest 10 mg, 10 g of the homogenised sample into e.g. a wide neck conical flask, add about 50 mL of water and homogenise for 30 s to 60 s. Rinse the shaft of the homogeniser into the flask with 50 mL of hot water, then add 0.2 mL of bromothymol blue solution, titrate the extract with sodium hydroxide solution until the colour changes from yellow to bluish green/greenish grey and then heat for 15 min in a boiling water bath. During this process shake repeatedly and

dissolve any lumps formed with a homogeniser.

(Note: In order to become familiar with the various shades of colour in adjusting the pH while learning the method, titrate a few sample extracts using the indicator and then measure the pH. For uncooked meat products the pH should not exceed 8.5 since otherwise it may not be possible to clarify the solutions by filtering after adding the Carrez reagent. In the case of simmered or boiled meat products, the danger of this happening is much less and the pH may be allowed to rise to about 9.5. If a pH meter is used, adjust the pH value to between 8.0 and 8.5.)

In the case of boiled sausages, the (instantaneous) colour change to bluish-green when adjusting the pH is readily detected. On the other hand, this change is usually sluggish in the case of extracts of uncooked sausages and the shade of colour is greyish-green. In the case of boiled sausages containing blood, a similar shade of colour is usually observed. Even in the case of extracts having a strong natural colour, the colour change can be recognised very readily with a little practice despite the fact that the shade of colour is frequently not the theoretically expected one because the colour of the extract is superimposed on that of the indicator.

In the case of extracts of uncooked sausages, a reversal of the colour change is sometimes observed after a certain time. In such cases, readjust the pH by adding a little sodium hydroxide solution.

Cool to room temperature and transfer the contents of the flask quantitatively to a 200 mL volumetric flask and add 4 mL each of Carrez solutions No. 1 and No. 2 shaking after each addition. Then dilute to the mark with water, mix thoroughly and filter through a fluted filter paper, discarding the first 20 mL of the filtrate. The clear residual filtrate is used for the determination (sample solution).

Calibration graph for the nitrite content

Mix 2.0 mL of water for the blank and 2.0 mL of each of the standard sodium nitrite solutions with 1.0 mL of water and 3.0 mL of the colour reagent mixture in a test tube, shake and store the solution in the dark at room temperature. After 30 min, measure the absorbance values of each solution at wavelength of 540 nm in a spectrometer against water. Plot the absorbance values obtained for the four sodium nitrite solutions against the corresponding absolute amounts of nitrite ions (in 200 mL solution), respectively, in milligrams of nitrite ion.

Calibration graph for the nitrate content

Introduce 0.2 mL of the NADPH solution, 2.0 mL of water for the blank or 2.0 mL of each of the standard potassium nitrate solutions respectively and 0.8 mL of nitrate reductase buffer solution into a test tube, mix thoroughly and allow to stand for 1 h at room temperature. Then add 3.0 mL of the colour reagent mixture, shake and store the solution in the dark at room temperature. After 30 min, measure the absorbance values of each solution at a wavelength of 540 nm in a spectrometer against water. Plot the absorbance values obtained for the four potassium nitrate standard solutions against the corresponding absolute amounts of total nitrate/

nitrate, (in 200 mL solution), in milligrams of nitrite ion.

Determination of nitrite content
Mix 2.0 mL of the sample solution in a cell with 1.0 mL of water and 3.0 mL of the colour reagent mixture and allow to stand in the dark at room temperature. After 30 min, measure the absorption A_{NO_2} in a spectrometer at a wavelength of 540 nm against water.

Determination of the total nitrite/nitrate content
Introduce 0.2 mL of the NADPH solution, 2.0 mL of sample solution and 0.8 mL of nitrate reductase buffer solution into a test tube, mix thoroughly and allow to stand for 1 h at room temperature. Then add 3.0 mL of the colour reagent mixture, shake and store the solution in the dark at room temperature. After 30 min, measure the absorption $A_{NO_2+NO_3}$ at a wavelength of 540 nm in a spectrometer against water.

Calculation of the nitrite content
Read off the absolute amount of nitrite, X_{NO_2}, corresponding to the absorption value A_{NO_2} determining nitrite without reduction step from the nitrite calibration graph.

Calculate the mass fraction, W_{NO_2}, in milligrams per kilogram of nitrite ion in the sample using equation [9.1]:

$$W_{NO_2} = \frac{X_{NO_2} \times 1000}{m} \qquad [9.1]$$

where:

X_{NO_2} is the absolute amount of nitrite (in 200 mL solution) without reduction step, in milligrams, read off from the calibration graph

m is the mass of the test portion, in 200 mL of the sample test solution, in grams

Calculation of the total nitrite/nitrate content
Read off the amount of the absorption value $A_{NO_2+NO_3}$ determining total nitrite/nitrate with reduction step from the nitrate calibration graph as nitrite ion.

Calculate the mass fraction, $W_{NO_2+NO_3}$, in milligrams per kilogram, of total nitrite/nitrate as nitrite ion in the sample using equation [9.2]:

$$W_{NO_2+NO_3} = \frac{X_{NO_2+NO_3} \times 1000}{m} \qquad [9.2]$$

where:

$X_{NO_2+NO_3}$ is the absolute amount of total nitrite/nitrate (in 200 mL solution) expressed as nitrite ion after the reduction step, in milligrams, read off from the calibration graph

m is the mass of the test portion, in 200 mL of the sample test solution, in grams

Calculation of the nitrate content
Calculate the mass fraction of nitrate, W_{NO_3}, expressed as nitrite ion, in milligrams per kilogram, with equation [9.3]:

$$W_{NO_3} = W_{NO_2+NO_3} - W_{NO_2} \quad [9.3]$$

The conversion factor from nitrite to nitrate is 1.35.

Regression graph
Alternatively, the calculation may also be carried out mathematically using a regression graph. (For further information on this method see DD ENV 12014-3:1998.)

Ion-exchange chromatographic (IC) method for the determination of nitrate and nitrite content of meat products[15]

Sample preparation
Homogenise the laboratory sample with the appropriate equipment. Take care that the temperature of the sample material does not rise above 25 ºC. If a mincer is used, pass the sample at least twice through the equipment. Weigh, to the nearest 10 mg, 10 g of the homogenised sample into e.g. a 150 mL wide neck conical flask (test portion).

Extraction and clarification
The analysis should be performed in a single working day.

Add 50 mL of water at a temperature of 50 ºC to 60 ºC to the test portion in the wide neck flask. Mix thoroughly with the homogeniser. Rinse the homogeniser with water and add the washings to the flask. Quantitatively transfer this slurry into a 200 mL volumetric flask by rinsing the wide neck flask with water. Add 50 mL of acetonitrile. Mix gently. Allow to cool to room temperature. Dilute to the mark with water.

Filter first through the fluted filter paper and then through a membrane filter of pore size of approximately 0.45 µm. If this solution is clear, it may be injected. If the solution is still not clear, filter through a membrane filter of pore size of approximately 0.22 µm (sample test solution). Prepare a blank replacing the test portion by 10 mL of water.

Preparation of the calibration graph
To plot a calibration graph, inject equal volumes of the standard solutions first and then the blank solution under the conditions as described above.

Check the linearity of the calibration graph.

IC operating conditions
The column used must be found satisfactory to adopt the following parameters:

Mobile phase	Buffered acetonitrile at pH 6.5
UV	205 nm
Injection volume	40 µL (minimum)
Flow rate	1 mL/min

If other columns are used, adjust chromatographic conditions.

IC measurement

Inject the standard solutions first and then the blank and the sample test solutions under the conditions as described above. One of the standard solutions should be injected every five sample test solutions when performing a series of analyses. If the peak obtained for the sample falls outside the range of the calibration graph, dilute the sample test solution in the mobile phase and repeat the measurement step. Identify the nitrate or nitrite peak by comparing the retention times for the standard solutions and the sample test solutions. Read off the content of nitrate or nitrite of the sample test solution from the calibration graph. Check the blank value to ensure that there was no nitrate and/or nitrite contamination during the sample preparation.

Expression of results

Calculate the mass fraction of nitrite, $W_{NO_2^-}$, expressed in milligrams of ion per kilogram with equation [9.4]:

$$W_{NO_2^-} = \frac{200 \times A_{NO_2^-}}{m} \times F \qquad [9.4]$$

where:

$A_{NO_2^-}$ is the value for nitrite, read off the calibration graph, in milligrams per litre
200 is the volume of the diluted test portion, in millilitres
m is the initial mass of the test portion, in grams
F is the dilution factor

Round the results without any decimals.

Calculate the mass fraction of nitrate, $W_{NO_3^-}$ expressed in milligrams of ion per kilogram with equation [9.5]:

$$W_{NO_3^-} = \frac{200 \times A_{NO_3^-}}{m} \times F \qquad [9.5]$$

where:

$A_{NO_3^-}$ is the value for nitrate, read off the calibration graph, in milligrams per litre; 200, m, F, see equation [9.4].

Round the result to the nearest whole number.

Precision: general

Details of the interlaboratory test of the method are summarised in Table 9.3. The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and matrices other than those given in Table 9.3.

Repeatability
The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit r in not more than 5 % of the cases.

The values are:

Nitrite corned beef (NO_2^-) mean = 38.9 mg/kg r = 4.4 mg/kg

Reproducibility
The absolute difference between two single test results on identical test material reported by two laboratories will exceed the reproducibility limit R in not more than 5 % of the cases.

The values are:

Nitrite corned beef (NO_2^-) mean = 38.9 mg/kg r = 10.3 mg/kg

(For further information on this method see DD ENV 12014-4:1998.)

9.6 Appendix 2: method procedure summaries (milk and milk products – BS EN ISO 14673)

Method using cadmium reduction and spectrometry[2]

Preparation of test sample: dried milk and dried whey
Transfer the test sample to a sample container of capacity about twice the volume of the test sample. Close the container immediately. Mix the test sample thoroughly by repeatedly shaking and inverting the container.

Preparation of test sample: caseins and caseinates
Thoroughly mix the test sample, if necessary after transferring all of it to a sample container of suitable capacity, by repeatedly shaking and inverting the container. Transfer 50 g of the test sample to the test sieve. If the 50 g portion directly passes, or nearly completely passes the sieve, pass the whole mixed test sample through the sieve. If the test sample does not pass completely through the sieve, use the grinding device to achieve that condition. Immediately transfer all the sieved test sample to the sample container and mix thoroughly in the closed container. During these operations, take precautions to avoid any change in the water content of the product.

After the test sample has been prepared, proceed with the determination as soon as possible.

Preparation of test sample: cheese
Prior to analysis, remove the rind or mouldy surface layer of the test sample so as to provide a test sample representative of the cheese as it is usually consumed.

Grind the test sample by means of an appropriate device. Mix the ground mass quickly and, if possible, grind a second time and again mix thoroughly. If the sample cannot be ground, mix it thoroughly by intensive stirring and kneading. Transfer the test sample to an airtight sample container to await the determination which should be carried out soon after grinding. If a delay is unavoidable, take all precautions to ensure proper conservation of the test sample and to prevent condensation of moisture on the inside surface of the container. Ground cheese showing unwanted mould growth or beginning to deteriorate should, however, not be examined. Clean the device after grinding of each sample.

Preparation of test sample: whey cheese
Prepare the test sample as specified.

Preparation of the copperised cadmium column
Transfer an amount of cadmium granules of approximately between 40 g and 60 g for each column into a 250 mL conical flask. Add sufficient of hydrochloric acid working solution to cover the cadmium. Swirl for a few minutes. Decant the solution. Wash the cadmium in the conical flask with water, until it is free from chloride (i.e. until reaction with silver nitrate is negative).

Copperise the cadmium granules by adding the copper (II) sulphate solution of which about 2.5 mL copper solution per g of cadmium is needed and swirl for 1 min. Decant the solution and wash the copperised cadmium immediately with water, taking care that the cadmium is continuously covered with water. Terminate the washing when the wash water is free from precipitated copper.

Fit a glass wool plug to the bottom of the glass column intended to contain the copperised cadmium (see Fig. 1[2]). Fill the glass column with water. Transfer the copperised cadmium into the glass column with minimum exposure to air. The height of the copperised cadmium should be 15 cm to 20 cm. Avoid trapping air bubbles between the copperised cadmium granules. The level of the liquid should not fall below the top of the copperised cadmium.

Condition the newly prepared column by running through it a mixture of 750 mL of water, 225 mL of the standard potassium nitrate solution, 20 mL of the buffer solution and 20 mL of the EDTA solution at a flow rate not exceeding 6 mL/min. Wash the column with 50 mL of water.

Checking the reducing capacity of the column
Check the column at least twice a day, but also at the beginning and at the end of a series of determinations for its capacity.

Pipette 20 mL of the standard potassium nitrate solution into the reservoir on top of the column. Immediately add 5 mL of the buffer solution to the contents of the reservoir. Run the contents of the reservoir through the column at a flow rate not exceeding 6 mL/min. Collect the eluate in a 100 mL volumetric flask. When the reservoir of the reduction column is nearly empty, wash the walls of the reservoir with about 15 mL of water. Repeat the washing with another 15 mL of water after the water has run off. When the second washing has run into the

column, completely fill the reservoir with water. Run the complete content of the reservoir through the column at maximum flow rate. Collect nearly 100 mL of the eluate.

Remove the 100 mL volumetric flask. Dilute its contents to the 100 mL mark with water and mix well. Pipette 10 mL of the eluate in another 100 mL volumetric flask. Dilute with water to obtain a volume of about 60 mL and mix. Proceed as specified under 'Colour development and measurement' below. Calculate the percentage reducing capacity of the column (0.067 µg of NO_2^- per mL corresponds to 100 % reducing capacity) from the nitrite content obtained and that determined from the calibration graph. If the reducing capacity is less than 95 % regenerate the column as specified.

Regeneration of the column

Regenerate the column at the end of each day of using or, if the check indicates a loss of efficiency, more frequently. Add about 5 mL of the EDTA solution and 2 mL of hydrochloric acid working solution to 100 mL of water and mix. Run the thus obtained solution through the column at a flow rate of about 10 mL/min. When the reservoir is empty, wash the column successively with water, with hydrochloric acid working solution and with water again. If the efficiency of the column still is not satisfactory, repeat the procedure specified above.

Preparation of test portion

- *Dried milk*: Weigh, to the nearest 1 mg, approximately 10 g of the prepared test sample. Transfer the test portion quantitatively to a 500 mL conical flask.
- *Dried whey*: Weigh, to the nearest 0.1 mg, approximately 5 g of the prepared test sample. Transfer the test portion quantitatively to a 500 mL conical flask.
- *Caseins*: Weigh, to the nearest 0.01g, approximately 10 g of the prepared test sample. Transfer the test portion quantitatively to a 500 mL conical flask.
- *Caseinates*: Weigh, to the nearest 0.01 g, approximately 2 g of the prepared test sample. Transfer the test portion quantitatively to a 500 mL conical flask.
- *Cheese*: Weigh, to the nearest 0.1 mg, approximately 10 g of the prepared test sample. Transfer the test portion quantitatively to the glass container of the laboratory mixer or homogeniser.
- *Whey cheese*: Weigh, to the nearest 1 mg, approximately 5 g of the prepared test sample. Transfer the test portion quantitatively to the glass container of the laboratory mixer or homogeniser.

Extraction and deproteination: dried milk

Add progressively 136 mL of preheated water at 50–55 °C to the test portion. Disperse the test portion by stirring with a glass rod or by shaking the conical flask. Add in the following order, swirling thoroughly after each addition, 12 mL of zinc sulphate solution, 12 mL of potassium hexacyanoferrate (II) solution and 40 mL of buffer solution and mix. In order to obtain a clear filtrate, leave the mixture in the

conical flask for at least 15 min, but not longer than 1 h. Then filter through a filter paper, collecting the filtrate in a 250 mL conical flask (see notes under 'Extraction and deproteination: whey cheese').

Extraction and deproteination: dried whey
Add progressively 136 mL of preheated water at 50–55 °C to the test portion. Disperse the test portion by stirring with a glass rod or by shaking the conical flask. Cover the conical flask with aluminium foil or a watch glass and place it in the water bath with boiling water for 15 min. Remove the flask from the water bath and wait until the temperature has dropped to between 55 °C and 60°C. Add in the following order, swirling thoroughly after each addition, 12 mL of zinc sulphate solution, 12 mL of potassium hexacyanoferrate (II) solution and 40 mL of buffer solution and mix.

In order to obtain a clear filtrate, leave the mixture in the conical flask for at least 15 min, but not longer than 1 h. Then filter through a filter paper, collecting the filtrate in a 250 mL conical flask (see notes under 'Extraction and deproteination: whey cheese').

Extraction and deproteination: caseins and caseinates
Add progressively 136 mL of preheated water at 50–55 ºC and 10 mL of buffer solution to the test portion. Disperse the test portion by stirring, using the magnetic stirrer. Add in the following order, swirling thoroughly after each addition, 12 mL of zinc sulphate solution, 12 mL of potassium hexacyanoferrate (II) solution and 30 mL of buffer solution and mix. In order to obtain a clear filtrate, leave the mixture in the conical flask for at least 15 min, but not longer than 1 h. Then filter through a filter paper, collecting the filtrate in a 250 mL conical flask (see notes under 'Extraction and deproteination: whey cheese').

Extraction and deproteination: cheese
Add progressively 144 mL of preheated water at 50–55 ºC to the test portion. Mix in the mixer or homogeniser until the test portion is well suspended. Add in the following order, swirling thoroughly after each addition, 6 mL of zinc sulphate solution, 6 mL of potassium hexacyanoferrate (II) solution and 40 mL of buffer solution and mix. In order to obtain a clear filtrate, leave the mixture in the conical flask for 3 min. Then filter through a filter paper, collecting the filtrate in a 250 mL conical flask (see notes under 'Extraction and deproteination: whey cheese').

Extraction and deproteination: whey cheese
Add progressively 134 mL of preheated water at 50–55 ºC to the test portion. Mix in the mixer or homogeniser until the test portion is well suspended. Add in the following order, swirling thoroughly after each addition, 12 mL of zinc sulphate solution, 12 mL of potassium hexacyanoferrate (II) solution and 40 mL of buffer solution and mix. In order to obtain a clear filtrate, leave the mixture in the conical flask for at least 15 min, but not longer than 1 h. Then filter through a filter paper, collecting the filtrate in a 250 mL conical flask.

(Note 1: It is essential to obtain a clear filtrate within the time specified. For this purpose, it may be necessary to use a larger volume of each precipitation reagent (for example, if well-matured cheeses are analysed). Reduce the volume of the preheated water accordingly to maintain the volume of filtrate at about 200 mL.

Note 2: The total volume of filtrate approximates to about 200 mL. This is regarded as such in the calculation.)

Reduction of nitrate to nitrite

Pipette 20 mL of the filtrate into the reservoir on top of the prepared reduction column. Add 5 mL of buffer solution to the contents of the reservoir. Mix by stirring with a small glass rod. Pass the contents of the reservoir through the column at a flow rate not exceeding 6 mL/min. Collect the eluate in a 100 mL volumetric flask. When the reservoir of the reduction column is nearly empty, wash the walls of the reservoir with about 15 mL of water. After the water has run off, repeat the same washing with another 15 mL of water. After the second washing has run into the column, completely fill the reservoir with water. Pass the complete content of the reservoir through the column at maximum flow rate. Collect nearly 100 mL of the eluate in the 100 mL volumetric flask. Remove the 100 mL volumetric flask. Dilute its contents to the 100 mL mark with water and mix well.

Colour development and measurement

Pipette equal volumes (for example 25 mL) of the filtrate obtained (from extraction and deproteination) and of the eluate into separate 100 mL volumetric flasks. Add water to each of the flasks to obtain a volume of about 60 mL and mix. Add to the solution 6 mL of solution I (450 mL concentrated hydrochloric acid diluted to 1000 ml with water) and then 5 mL of solution II (0.5 g of sulphanilamide dissolved in 75 mL water and 5 mL concentrated hydrochloric acid in a 100 mL volumetric flask with heating on a water bath. Cool to room temperature and dilute to the 100 mL mark with water and mix. The solution is filtered if necessary). Mix carefully and leave the solution protected from direct sunlight at room temperature for 5 min. Add to the solution 2 mL of solution III (0.1 g N-1-naphthyl ethylenediamine dihydrochloride dissolved in water in a 100 mL volumetric flask and diluted to the 100 mL mark with water and mixed. The solution is filtered if necessary). Mix carefully and leave the solution protected from direct sunlight at room temperature for 5 min. Dilute its contents to the 100 mL mark with water and mix. Measure the absorbance of the solution III. Mix carefully and leave the solution protected from direct sunlight at room temperature for 5 min. Dilute its contents to the 100 mL mark with water and mix. Measure the absorbance of the solution obtained above against that of the blank test at a wavelength of 538 nm within 15 min.

Blank test

Carry out a blank test in parallel with the determination but omitting the test portion.

Preparation of calibration graph
Pipette 0 mL (zero member), then 2, 4, 6, 8, 12 and 20 mL of the sodium nitrite standard solution into separate 100 mL volumetric flasks. Add water to each of the flasks to obtain volumes of about 60 mL.

Proceed as specified under 'Colour development and measurement'.

Measure the absorbances of the blank test solutions against that of the zero member at a wavelength of 538 nm within 15 min. Plot the absorbances obtained against the nitrite concentrations, in micrograms per millilitre, calculated from the amounts of standard sodium nitrite solution added.

Calculation of the nitrite content
Calculate the nitrite content of the sample, W_{Ni}, using the following equation:

$$W_{Ni} = \frac{20\,000 \times c_1}{m \times V} \qquad [9.6]$$

where:
W_{Ni} is the nitrite content of the sample, in milligrams of NO_2 per kilogram
c_1 is the numerical value of the concentration read from the calibration graph, corresponding to the measured absorbance of the test portion solution in micrograms of nitrite ion per millilitre
m is the mass of the test portion in grams
V is the volume of the aliquot portion taken from the filtrate, in millilitres

Expression of results
Express the results to 1 decimal place.

Repeatability
The absolute difference between two single test results, obtained out simultaneously or in rapid succession by the same operator under the same conditions on identical test material, will in not more than 95 % of cases be greater than 1.0 mg/kg.
(For further information on this method see BS EN ISO 14673-1:2001.)

Method using segmented flow analyses[18]

Preparation of test sample: cheese
Prior to analysis, remove the rind or mouldy surface layer of the test sample so as to obtain a sample representative of the cheese as it is usually consumed. Grind the test sample by means of an appropriate grinding device. Mix the ground mass quickly and, if possible, grind it a second time and again mix thoroughly. Clean the device after grinding each sample. If the sample cannot be ground, mix thoroughly by intensive kneading. Transfer the test sample as soon as possible after grinding into an airtight sample container. Close the container until the time of analysis. If a delay after grinding is unavoidable, take every precaution to ensure proper

preservation of the sample and to prevent condensation of moisture on the inside surface of the container.

Preparation of test sample: dried milk products
Transfer the test sample into an airtight sample container of capacity about twice the volume of the test sample. Close the container immediately. Mix the test sample thoroughly by repeatedly shaking and inverting the container.

Preparation of test sample: liquid milk products
When testing a sample from non-skimmed milk products adjust the temperature of the test sample to between 35 and 40 ºC in the water bath. Mix the test sample thoroughly, but gently, by repeatedly inverting the sample bottle without causing frothing or churning, and cool quickly to approximately 20 ºC.

Preparation of the reduction column
Weigh about 10 g cadmium granules in a glass beaker. Add about 25 mL of hydrochloric acid and wash the cadmium granules by swirling the glass beaker for several minutes. Decant the hydrochloric acid and subsequently wash the cadmium granules with water until the water is pH neutral. Check the pH with indicator paper. Transfer about 50 mL copper sulphate solution to the washed cadmium granules in the glass beaker. Allow the obtained mixture to stand for 5 min while regularly swirling the beaker. Decant the copper sulphate solution and wash the cadmium granules 10 times with water. Keep the cadmium granules immersed in water.

Connect the glass funnels with the tube, fold in a U-form and fill almost completely with water. By means of a spatula transfer small portions of the cadmium granules through the funnel into the tube. Tap the tube to pack the column. After the column has been filled almost completely, transfer a glass wool plug with a length of about 1 cm into both ends of the tube. Before use, run the nitrate solution with the highest nitrate concentration through the column for 1 h.

Checking the reducing capacity of the column
Using the analyser measure subsequently the absorbance of two cups with the sodium nitrate standard solution of 1.2 mg NO_3^- per mL, two cups with water and two cups with a sodium nitrate standard solution of 0.89 mg NO_3^- per mL.

$$\text{The rate } \frac{\text{average absorption of the nitrate standard solution}}{\text{average absorption of the nitrite standard solution}} \text{ must be } 0.95$$

When the ratio is lower than 0.95, a new column should be prepared.

Preparation of test portion

- *Cheese*: Weigh, to the nearest 0.005 g, 2.500 g of test sample in a glass beaker. Add 50 mL of ammonium and sodium solution III at about 50 ºC and suspend

the sample with the aid of the suspension apparatus. Immediately cool the glass beaker with the test portion. Fill a cup of the sample exchanger with the test portion, in such a way that the separated fat remains in the glass beaker.

- *Dried milk products*: Weigh, to the nearest 0.005 g, 2.500 g of the test sample in a glass beaker. Add 50 mL of ammonium and sodium solution III and suspend the test sample with the aid of the suspension apparatus. Fill a cup of the sample exchanger with the test portion.
- *Liquid milk products*: Weigh, to the nearest 0.01 g, 10.00 g of the test sample in a 50 mL volumetric flask. Make up to the 50 mL mark with ammonium and sodium solution III and mix. Fill a cup of the sample exchanger with the test portion.

Determination

Start the pump, the spectrometer, the recorder and the data processing equipment of the analyser. Run the reagents through the analyser according to the diagram (Fig. 2, BS EN ISO 14673-2:2001) for 15 min. Fill the sample exchanger of the analyser in the following order: start with the 5 tubes filled with sodium nitrate standard solutions placed in order of increasing nitrate content followed by the tubes with the test portion. Start the sample exchanger of the analyser. Activate the data processing equipment according to the directions for use. When the determination is finished flush the system with an alkaline detergent for at least 15 min and subsequently with water for at least 15 min.

Dilutions

If the nitrite content of the test portion is higher than the nitrite content of the highest sodium nitrate standard solution, repeat the analysis in duplicate by again preparing two new test portions according to 'Preparation of test portion'. Dilute these new test portions in such a way that the expected results given by the recorder will be lying between the result of the lowest but one sodium nitrate standard solution and the highest standard solution. Then transfer the thus diluted test portion into a cup.

Reference sample

Examine the reference test sample at least in duplicate, according to 'Determination'.

Calibration curve

Calculate the most suitable calibration curve and the correlation coefficient for both the sodium nitrate and the sodium nitrite standard solutions. The obtained correlation coefficient should be >0.9985.

Checking the drift of standard solutions

Check regularly the drift using the appropriate standard solution. The drift path between two successive (drift) standards may be max 10 %. The drift path between the highest and lowest results obtained on one day may be max 20 %. When this value is exceeded check the cause and repeat the examinations.

Calculation of nitrate or nitrite content

Convert the result given by the recorder for the test portion to μg nitrate or nitrite per mL read from the calibration curve of the standard solutions.

Calculate the nitrate or nitrite contents of the sample by using one of the following equations:

$$\text{For dried milk products: } W_n = W_t \times d \times f_d \quad [9.7]$$

$$\text{For liquid milk products: } W_n = W_t \times 5 \times f_l \quad [9.8]$$

$$\text{For cheese: } W_n = W_t \times 20.5 \times f_c \quad [9.9]$$

where:

W_n is the numerical value of the nitrate or nitrite content of the test sample, in micrograms per kilograms

W_t is the numerical value of the amount of the nitrate or nitrite content of the test portion read from the calibration curve, in micrograms per mL

d is the numerical value of the dilution factor

f_d is the multiplying factor for dried milk obtained by the following formula ($f_d = 20.6$)

$$f_d = \frac{50 \times 1.03 \times 400}{1000}$$

f_l is the multiplying factor for liquid milk obtained by the following formula ($f_l = 5$):

$$f_l = \frac{50 \times 100}{1000}$$

f_c is the multiplying factor for cheese obtained by the following formula ($f_c = 20.5$):

$$f_c = \frac{50 \times 1.025 \times 400}{1000}$$

where:

50 is the conversion value to change the expression from mg per mL to mg per 50 mL

1.03 is the ratio of the volume of the test portion (2.5 g + 50 mL) and the volume of the standard solutions (50 mL)

1.025 is the correction value for the moisture content of cheese (average moisture content: 50 %)

400 is the conversion value to change the expression from 2.5 g to 1 kg

1000 is the conversion value to change the expression from μg to mg

Expression of results

Express the results for nitrite to 1 decimal place.

Repeatability
The absolute difference between two single test results, carried out simultaneously or in rapid succession by the same operator under the same conditions on identical test material, will be greater than 0.5 mg/kg in not more than 95 % cases.

Reproducibility
The absolute difference between two single and independent results obtained by two operators working in different laboratories on identical test material, will be greater than 1.0 mg/kg in not more than 95 % of cases.

(For further information on this method see BS EN ISO 14673-2:2001.)

Method using flow injection analyses with in-line dialysis[19]

Preparation of test sample: cheese
Prior to analysis, remove the rind or mouldy surface layer of the cheese, in such a way as to provide a sample representative of the cheese as it is usually consumed. Grind the test sample by means of an appropriate device; mix the ground mass quickly, and if possible grind a second time and again mix thoroughly. Transfer the test sample to an air-tight container to await analysis, which should be carried out as soon as possible after grinding. If a delay is unavoidable, take all precautions to ensure proper preservation of the sample and to prevent condensation of moisture on the inside surface of the container. Ground cheese showing unwanted mould growth or beginning to deteriorate should not be analysed. Clean the device after grinding each sample.

Checking the reducing capacity of the cadmium column
Carry out this check at least twice a day at the beginning and at the end of a series of determinations. Prepare the FIA system for operation as described above ('Determination'). Fill a cup with nitrite reference solution. Fill a cup with the nitrate calibration standard solution. Analyse the nitrate calibration standard solution and the nitrite reference solution. Divide the peak height found for nitrate by the peak height for nitrite and multiply by 100 to obtain the percentage reducing capacity of the column. If the reducing capacity is less than 95 %, the column should be regenerated (see 'Regeneration of the cadmium column').

Regeneration of the cadmium column
Regenerate the column at the end of each day after use, or more frequently if the check indicates a loss of efficiency. Disable pump flows for carrier solution CS and reagents R1 and R2. Unscrew the tubing at the inlet of the dialysis module connecting the injector to the dialysis module. Run water through the injector used for carrier solution C1 until the system is filled. Connect the cadmium column to the outlet of the injector. Start the pump again and make 3 to 5 injections of reagent solution R2 followed by nitrate standard solution. Wash the column by passage of carrier solution.

Test portion
Weigh, to the nearest 0.1 mg, 2.5 g of the test sample into a centrifuge tube of 50 mL.

Extraction
Add 24 mL of extraction buffer, preheated to a temperature of 50–55 ºC, to the test portion. Mix with the homogeniser for about 3 min until the test portion is well suspended. Centrifuge at *c.* 1500 g for 5 min. Place the centrifuge tube in a mixture of water and ice for 15 min. Use a pipette to withdraw the de-fatted suspension from underneath the fat layer in the centrifuge tube and to fill the cups of the FIA, for both the nitrite and nitrate determination, with the obtained suspension.

Determination of nitrite
Install the manifold according to the scheme in Fig. 1 (see BS EN ISO 14673-3:2001). Use the pump tubings for the determination of nitrite while leaving out the cadmium reduction column. Connect the bottles with both reagent solutions R1 and R2 and carrier solution. Start the pumps to flush the system for 5 min to 10 min. Load the FIA-program for nitrite. Run the nitrite standard solutions to calibrate the system, followed by the test portion suspensions. Check the calibration, both at the end of a series and after each group of 10 samples, by analysing the nitrite calibration standard solution of 0.10 mg nitrite ion per litre.

Calculation of the nitrite content
Calculate the nitrite content of the sample W_{NI}, using the following equation:

$$W_{NI} = \frac{25}{M} \times C_{Ni} \qquad [9.10]$$

where:
W_{NI} is the nitrite content of the sample, in milligrams of nitrite ion (NO_2^-) per kilogram
C_{Ni} is the numerical value of the concentration read from the calibration graph, corresponding to the measured absorbance of the test portion suspension, in micrograms of nitrite ion per litre
m is the mass of the test portion, in grams

Expression of results
Express the results to 1 decimal place.

Repeatability
The absolute difference between two single test results, carried out simultaneously or in rapid succession by the same operator under the same conditions on identical test material, will be greater than 0.5 mg/kg in not more than 95 % of cases.

Reproducibility
The absolute difference between two single and independent results obtained by two operators working in different laboratories on identical test material, will be greater than 1.0 mg/kg in not more than 95 % of cases.

(For further information on this method see BS EN ISO 14673-3:2001.)

Table 9.1 Summary of methods for nitrites in foods

(a)

Method	Matrix	Sample preparation	Method conditions	Reference
Spectrometry	Milk and milk products	Dispersed in warm water, fat and proteins precipitated, filtered	Aqueous extract is treated with sulphanilamide and N-1(-naphthyl)ethylenediamine dihydrochloride. Red compound produced is measured at 538 nm	2
Distillation/ spectrometric	Meat	Mixed in warm water on steam bath, transferred to volumetric flask	Nitrate using *m*-xylenol. After nitration distil into receiver containing NaOH. Transfer distillate to vol flask and dilute with water. Measure colour at 450 nm	3
Colorimetric	Meat	Mixed with hot water, placed on steam bath for 2 h. Cooled, diluted to volume. Filter	Add sulphanilamide reagent to an aliquot, after 5 min add NED reagent, mix, let colour develop for 15 min. Transfer to photocell and determine *A* at 540 nm against blank	4
Reduction/ colorimetric	Cheese	Clarification of aqueous extract with zinc hydroxide + NaOH. Placed on water bath at 50 ºC for 10 min. Cooled. Made to volume	Diazotisation of sulphanilic acid with the nitrite, and coupling with 1-naphthylamine hydrochloride to form a pink azo dye whose absorbance is measured at 522 nm and compared with standard curve	5
Colorimetric	Cheese		Griess method	6
Colorimetric	Meat	Addition of 40 g sand in extraction step, collection of 1st 40 mL filtrate and use of excess of NED	Modified Norwitz–Keliher method	7
Spectro-photometric	Water and fruits	Nitrite diazotised with SAM pH 2.0–5.0, SM pH 1.8–5.6 and SD pH 1.8–4.0 in a HCl medium to form water-soluble colourless diazonium cations	Cations coupled with sodium 1-naphthol-4-sulphonate (NS) pH 9.0–12.0 for the SAM–NS system, pH 8.6–12.0 for SM–NS system and pH 9.4–12.0 for SD–NS system to be retained on naphthalenetetradecyldimethyl-benzylammonium (TDBA)-iodide(I) adsorbent packed in a column. Dissolved out of column with DMF and *A* measured at 543 nm for SAM–NS, 537 nm for SM–NS and 530 nm for SD-NS	8

Spectro-photometric	Water	Sulphanilic acid and N-1(-naphthyl)-ethylenediamine solutions were added to form the azo dye	After 30 min the solution was passed through a sodium dodecyl sulphate coated alumina column and eluted with 7 mL HCl–CH_3COOH (2:3). *A* of effluent measured at 553 nm	9
Spectrometric	Meat	Homogenised, proteins precipitated, filtered	Aqueous extract is treated with sulphanilamide and N-1(-naphthyl)ethylenediamine dihydrochloride. Red compound produced is measured at 540 nm	10
Enzymatic spectrometric	Beer	Degassed and diluted with 0.2 M phosphate buffer pH 7.6. 0.5 mM methyl-viologen, NiR and 14.5 mM $Na_2S_2O_4$ added	Sample incubated at 37 ºC for 15 min; 250 µL 1 N H_2SO_4 added. NiR catalyses the reduction of nitrite to ammonia. The ammonia produced determined by spectrometric method. NiR immobilisation allowed use of a continuous monitoring flow reactor	12
Enzymic spectro-photometric	Meat and fish products	Homogenised with 6x volume of buffer solution adjusted to pH 7 and diluted to volume with water. Ultrafiltrate	1 mL filtrate in test tube containing 3 mL buffer 100 µL of each NADH and FAD solution. 20 mL of enzyme solution injected to start reaction. After incubating the mixture for 10 min at 30 ºC, the decrease in absorbance (at 340 nm) was measured against a blank	13
Enzymic spectro-photometric and spectrometric	Turkish sucuk (fermented sausage)	Dispersed in warm water, fat and proteins precipitated, filtered. Either treated through a Cd Reduction column or by means of enzymatic reduction with nitrate reductase	Cd Column method: aqueous extract is treated with sulphanilamide and N-1(-naphthyl)ethylenediamine dihydrochloride. Red compound produced is measured at 525 nm. Enzymatic reduction method: *A* measured at 540 nm after addition of colour solutions	14
Segmented flow analyses	Milk and milk products	Suspension in water. Transfer part of suspension to analyser	Dialysis of suspension. Conversion of nitrate to nitrite. Determination of nitrite by a spectrometric method	19

Table 9.1 cont'd

Method	Matrix	Sample preparation	Method conditions	Reference
Flow injection analyses with in-line dialysis	Milk and milk products	Suspension in warm extraction buffer. Separation of fat by centrifugation and rapid cooling. Analyse small part of suspension by FIA	In-line dialysis of suspension. Conversion of nitrate to nitrite by Cd. Reaction of nitrite with sulphanilamide and N-1(-naphthyl)ethylenediamine providing red azo dye. Measure red colour in flow cell at 540 nm with reference to absorption measured at 620 nm	20
Differential pulse voltammetry (DPV)	Food	Juice and beer, no sample preparation. Tomato, 2 g homogenised	DPV with 3 electrodes i.e. porphyrinic microelectrode–nitrite microsensor working electrode, SCE and a Pt wire as counter electrode. Potential range +0.4 to +1.2 V vs SCE with amplitude 25 mV, pulse width 50 ms and scan rate 20 mV/s	21
Capillary electrophoresis (CE)	Vegetables	Nitrite extracted from vegetables by mixing and diluting samples with water at a moderate temperature	CE method is a low-concentration method. Before injection, samples filtered through 0.45 μm filter. Injection carried out by electromigration for 10 s at –10 kV. Runs were also carried out at –15 kV	22

(b)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
IC	Meat, vegetables and baby foods	Extracted from test sample with hot water. Treated with acetonitrile, filtered	Anion exchange, 4.6 × 150 mm, 10 µm; IC-PAK A HC (high capacity) – Waters	Aqueous buffered acetonitrile at pH 6.5, flow rate 1.0 mL/min, injection volume 100 µL	UV at 205 nm	15,16
HPLC/IC	Human plasma and urine	Deproteinised with acetonitrile, lyophilised and reconstituted in buffer	Anion exchange column	20 mM NaCl with 1 mM NaH_2PO_4 at pH 7 isocratic, flow rate 1.0 mL/min, injection volume 20–100 µL	UV at 210 nm. DAD 205–300 nm	17
IC	Spinach	Addition of borax to pH 8.6. Hot water extraction, cleaned and filtered	IC anion PRP-X100 column (125 × 4 mm, 5 µm)	2 mM phthalic acid–10 % acetone (pH 5.0), flow-rate 1.0 mL/min, injection volume 20 µL	Coulometric, oxidation potential 700 mV	18

Key:
SAM = suphanilamide
SM = sulphamethizole
SD = sulphadimidine

Table 9.2 Summary of statistical parameters for nitrites in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
Ion-exchange chromatographic (IC)	Meat products, vegetables, baby foods and cheese	Full collaborative trial	see Table 9.4 Detection limit for nitrite ion is 1 mg/kg. Recoveries for residual nitrite/nitrate ranges from 96 to 108 % and repeatability and reproducibility were satisfactory	16
Spectrometric	Meat products	Full collaborative trial	see Table 9.3	10
Ion-exchange chromatographic (IC)	Meat products	Full collaborative trial	see Table 9.3	15
Capillary electrophoresis (CE)	Vegetables	Precision of method established and applied to real samples (n=15)	Linear calibration curve range 0.1–2.5 μg/mL LOD = 0.034 μg/mL LOQ = 0.1 μg/mL [RSD = 4.21 %, (n=6)] Recovery 99.2 %, RSD 6.1 % for 1.0 μg/mL spike in blank vegetable	22
Spectrometric	Turkish sucuk (fermented sausage)	Internationally accepted methods applied to real samples (n=7)	Good agreement was achieved between both methods i.e. Cd or enzymatic reduction. Samples A–G: Enzymatic reduction: Mean 4–17 ppm SD ±2 ppm Cd reduction: Mean 3–20 ppm SD ±2 ppm	14

Enzymic spectrometric	Meat and fish products	Precision of method established and applied to real samples (n=5)	Linear in range 0.1–10 µg/mL. RSD at 5 µg/g 1.7 % (n=5). Comparison study showed method was superior to GC method for samples containing large amounts of reducing substances. Good agreement achieved between both methods for other foods. Cod roe 2.73±0.11 µg/g (n=3) Salmon roe 2.00±0.10 µg/g (n=3) Pork ham 25.9±0.10 µg/g (n=3) Fish sausage 19.0±0.10 µg/g (n=3) Meat sausage 15.0±0.15 µg/g (n=3)	13
Differential pulse voltammetry	Food (DPV)	Precision of method established and applied to real samples (n=5)	Linear calibration curve r = 0.9824 Detection limit 2.2 µg/mL for beer Apple juice 4.1±0.3 µg/mL (n=6) SD 0.29 µg/mL Exotic juice 2.5±0.6 µg/mL (n=6) SD 0.57 µg/mL Multivitamin juice 3.4±0.7 µg/mL (n=6) SD 0.67 µg/mL Beer 29.6±0.4 µg/mL (n=6) SD 0.38 µg/mL Tomatoes 18.7±0.7 µg/g (n=6) SD 0.65 µg/g	21

Table 9.2 cont'd

Method	Matrix	Extent of validation	Statistical parameters	Reference
Spectro-photometric	Water and fruit	Precision of method established and applied to real samples (n=3)	Linear calibration curve 2–40 ng NO_2-N/mL. Detection Limits: 1.4 ngNO_2-N/mL for SAM–NS, 1.2 ngNO_2-N/mL for SM–NS and 1.0 ngNO_2–N/mL for SD–NS. Concentration factor is 8 for SAM–NS and SM–NS and 12 for SD–NS. Method successfully applied to the determination of low levels of nitrite in water and some fruit samples. Apple NO_2-N 0.41 ±0.02 µg/g (n=3) Pear NO_2-N 0.18 ±0.01 µg/g (n=3) Persimmon NO_2-N 0.30 ±0.01 µg/g (n=3)	8
Spectro-photometric	Water	Precision of method established and applied to real samples (n=3)	Linear range 0.4–24 ng/mL. Preconcentration factor 70. Detection limit: (3σ) for 500 mL sample 0.173 ng/mL. RSD 0.36 % for (n=6) of 10 ng/mL nitrite. Rain water A 0.023 mg/L Rain water B 0.017 mg/L River water 0.108 mg/L	9
Segmented flow analyses	Milk and milk products	Repeatability and reproducibility	see Appendix 2	19
Flow injection analyses with in-line dialysis	Milk and milk products	Repeatability and reproducibility	see Appendix 2	20
Spectrometry	Milk and milk products	Repeatability	see Appendix 2	2

IC	Spinach	Method applied to real samples (n=3)	Detection limit 0.1 µg/mL, RSD 2–16 %	18
Colorimetric	Meat	Precision of method established	93 % recovery of nitrite. CV~8 %. Calibration curve linear between 0.2 and 1.0 ppm of $NaNO_2$ concentration in final solution	7
Enzymatic spectrometric	Beer	Precision of method established	Using enzyme solution: Nitrite assay linear 10^{-8}–10^{-2} M with LOD of 10^{-8} M and recovery 90–107 %. Imprecision 4–10 % on entire calibration curve. With NiR immobilised: linear range 10^{-5}–10^{-2} M and LOD 10^{-5} M Enzymatic assay in good agreement with results obtained using commercial nitrite determination kits	12
HPLC	Human plasma and urine	Precision of method established	Sensitivity 0.01 µmol/L with recovery of 99.6 % RSD_r 1.6–6.0 % for (n=6) of 31.2 µg/mL nitrite RSD_R 8.15 % for (n=6) of 15.6 µg/mL nitrite	17

Key:
SAM = suphanilamide
SM = sulphamethizole
SD = sulphadimidine

Table 9.3 Performance characteristics for nitrite in meat products[10,15]

Method	Part 3, page 9	Part 4, page 8	
Sample	Sausage	Corned beef	
No. of laboratories	19	14*	14
Units	mg/kg	mg/kg	mg/kg
Mean value	37	7	38.9
S_r	2	1.2	1.5
RSD_r	5.8 %	17.0 %	4.0 %
r	6	3.3	4.4
S_R	3	2.3	3.7
RSD_R	7.7 %	2.9 %	9.4
R	8	6.5	10.3

* Data for information only

Key

Mean 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).

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)

Table 9.4 Performance characteristics for nitrite in foods[16]

Sample	Sausage		Baby food	Sausage
No. of laboratories	11	11	11	11
Units	mg/kg	mg/kg	mg/kg	mg/kg
Mean	47	160	58	161
S_r		6.1		6.1
S_R	4.1	9.3	5.0	12.5
RSD_R	8.7 %	5.8 %	8.6 %	7.8 %
Horrat value	1.0	0.8	1.0	1.0
Sample	Salami	Pâté	Salami	
No. of laboratories	11	14	13	11
Units	mg/kg	mg/kg	mg/kg	mg/kg
Mean	7	65	52	9
S_r	2.2			2.2
S_R	2.0	11.0	4.7	1.5
RSD_R	27.7 %	17.1 %	8.9 %	17.0 %
Horrat value	2.3	2.0	1.0	1.5

Key

Mean	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.
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)
Horrat value	The observed RSR_R value divided by the RSD_R value calculated from the Horwitz equation at the concentration of interest ($RSD_R = 2^{1-0.5\log C}$)

10

E297: Fumaric acid and its salts

10.1 Introduction

The major food groups contributing to dietary intake of fumaric acid and its salts are desserts and sugar confectionery with the maximum permitted level of 4000 mg/kg being allowed in dry powdered dessert mixes, gel-like desserts and fruit flavoured desserts. The ADI for fumaric acid + its salts is 6 mg/kg body weight/day.

10.2 Methods of analysis

There are numerous methods published for the determination of fumaric acid in foodstuffs. The majority of these methods are applicable to fruit juices, fruits, wines and beverages and are HPLC,[1–21] GC,[22] GC/MS,[23,24] polarographic,[25] capillary isotachophoresis (cITP)[26] and cITP-CZE[27]. A summary of them is given in Table 10.1, together with the matrices to which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 10.2. Methods need to be developed for the determination of fumaric acid in desserts and sugar confectionery as these are the major food groups contributing to dietary intake of fumaric acid and its salts.

A suitable method for the analysis of fumaric acid in apple juices was collaboratively trialled.[1] The method consisted of dilution of the apple juice with an equal volume of water and filtering the mixture through a 0.45 μm filter before HPLC analysis. The fumaric acid was analysed using a C18 reversed phase column, phosphate buffer gradient elution HPLC with UV detection at 210 nm. The procedure for this method is given in the Appendix and the performance characteristics are given in Table 10.3.

A suitable method for the analysis of organic acids, including fumaric acid, in beer was carried out on four Italian lager beer samples.[13] The method consisted of degassing and decolourising the beer before filtering and neutralising prior to the sample being placed on an anionic resin column. The organic acids were eluted from the column with 0.1 N HCl, diluted with water and filtered prior to HPLC analysis. The fumaric acid was analysed by HPLC using an Alltima C18 column with methanol–water–phosphoric acid mobile phase with refractive index detection. The procedure for this method is given in the Appendix and the performance characteristics are given in Table 10.4.

10.3 Recommendations

Methods are available for fumaric acid in fruits and fruit juices but no methods are available for desserts or sugar confectionery, therefore these methods need to be developed.

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10.5 Appendix: method procedure summaries

Analysis of apple juice[1]

Fumaric acid was determined using C18 reverse phase column (25 or 30 cm long) packed with 5 μm spherical particles, under the following conditions: ambient temperature, mobile phase of 0.01 M KH_2PO_4 in 0.75 % phosphoric acid at 0.8 mL/min, and a UV detector at 214 (or 210) nm. Samples were analysed by diluting 5.00 g juice with 5.00 g water, mixing thoroughly, filtering through a 0.45 μm filter and injecting 20 μL (or standard loop).

Analysis of lager beers[13]

Organic acid determination by HPLC

Sample preparation

Lager beers were degassed in a rotary evaporator at a temperature below 30 ºC for 30 min. Samples were decolourised with activated carbon (2 g/100 mL of beer) and percolated through filter paper (Whatman 1; 11 μm). Propionic acid (5 mL) was added to the filtered solution (internal standard solution 5 mg/mL H_2O) and then neutralised with 1 N NaOH to pH values of about 7.50–8.00.

Anionic resin adsorption

A chromatographic column (1 cm i.d. × 25 cm) filled with Amberlite Resin IRA 400 (anionic) or Dower 1X2 (*c.* 10 mL) was wet with 20 mL of 0.1 N NaOH and washed with distilled water to pH 7.0. After neutralisation, the sample was slowly filtered through this column in order to exchange all the organic acids. Afterwards, the sugar fractions were removed by rinsing with distilled water (*c.* 25 mL) and the organic acids were released from the anionic resin *c*. 25 mL of 0.1 N HCl and washed with distilled water until they reached a volume of 50 mL. The treated sample was filtered at 0.4 μm and was ready for HPLC analysis.

HPLC conditions

Column	Alltima C18 Alltech (4.6 mm i.d. × 250 mm) with Hamilton C18 precolumn
Mobile phase	H_2O–H_3PO_4–MeOH (94:50:1)
Flow rate	0.8 mL/min at ambient temperature
Injection volume	100 μL
Detection	Refractive index detector (RI-3 varian differential)

Table 10.1 Summary of methods for fumaric acid in foods
(a)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
HPLC	Apple juice	Diluted with equal volume of water, filtered through 0.45 µm filter	C18 reverse phase	0.01 M KH_2PO_4 in 0.75 % phosphoric acid at 0.8 mL/min, 20 µL injection	UV at 214 nm (or 210 nm)	1
LC	Apple juice	Diluted to 3–5º Brix, filtered through 0.45 µm filter	Bio-Rad HPX-87 Aminex ion exclusion at 80 ºC	0.006 N H_2SO_4, 0.6 mL/min, 5 µL injections	207 nm	2
HPLC	Orange juice	8 mL sample + 1 mL 2.5 % meta-phosphoric acid +1 mL 2 % propionic acid centrifuged. 1 mL of centrifugate passed through SCX (benzenesulphonyl-propyl) extraction cartridge (pre-treated with 1 mL MeOH and 10 mL H_2O). Cartridge washed with 2 mL H_2O and eluate was diluted to 4 mL with mobile phase and filtered through 0.45 µm filter	YMC-Pack ODS-AQ	20 mM KH_2PO_4 at pH 2.8, 0.7 mL/min, 20 µL injection	214 nm	3
Ion exchange chromatography	Fruits, juices		Shim-pack ion-exchange at 60 ºC	5 mM H_2SO_4, 1 mL/min	210 nm	4
HPLC	White wine, grapes, potato salad		Shimpack IE at 60 ºC	5 mM H_2SO_4, 1 mL/min	210 nm	5
HPLC	Apple and pear juices					6

HPLC	Apple drinks	100 µL sample	TSK-gel ODS-120A at 25 ºC	0.1 M $NaHClO_4$– 0.01 M NaH_2PO_4 (pH 2.6), 8 mL/min	210 nm	7
HPLC	Fruit juices		Spherisorb ODS-2	0.01 M KH_2PO_4– 0.6 mM tetrabutyl-ammonium phosphate (adjusted to pH 2.5 with H_3PO_4), 1 mL/min	210 nm	8
HPLC	Green beans	Homogenised beans were stirred with 4.5 % metaphosphoric acid solution. Filtered and diluted with acid and filtered	Spherisorb ODS-2 C18 with pre-column Newguard RP-18	Water (adjusted to pH 2.2 with H_2SO_4) at 0.5 mL/min, 20 µL injection	215 nm	9
HPLC	Red raspberry juice	Juice eluted through disposable cartridge and filtered	Spherisorb ODS-2 and Spherisorb ODS-1 with pre-column ODS-10	0.2 M phosphate buffer, pH 2.4	AOAC official method 986.13 [11]	10
HPLC	Cranberry juice cocktail, apple juice	Juice eluted through Sep-Pak C18 disposable cartridge and filtered. Analysed by HPLC with columns in tandem	Supelcosil LC-18, Radial-Pak C18 with pre-column ODS-10	0.2 M phosphate buffer, pH 2.4, 0.8 mL/min, 5 µL injection	214 nm	11
HPLC	Apricot cultivars	Purée diluted with water and clarified by centrifugation and the extract was filtered through 0.45 µm filter	Aminex HPX-87H at 65 ºC	4 mM H_2SO_4, 0.6 mL/min, 20 µL injection	210 nm	12

Table 10.1 cont'd

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
HPLC	Lager beers	Beer degassed in a rotary evaporator. Decoloured with activated carbon and percolated through filter paper. Propionic acid was added and neutralised with 1 N NaOH to pH 7.5–8.0. Sample filtered through Amberlite resin column. Sugar fractions removed with water and the organic acids released using 0.1 N HCl and washed with distilled water. Treated sample filtered at 0.4 μm	Alltima C18 Alltech with Hamilton C18 precolumn	H_2O/H_3PO_4–MeOH (94:50:1), 0.8 mL/min, 100 μL injection	Refractive index	13
HPLC	Honey	Aq honey solution was percolated through a Bond Elut SAX cartridge, previously treated with 1 M NaOH followed by washing with water and further treatment with acetic acid of pH 2.05 and pH 4.5. After washing with water the cartridge was dried with a stream of compressed air and the analytes were eluted with 0.5 M H_2SO_4	2 Spherisorb ODS-1 columns in series	Aq H_2SO_4 (pH 2.45), 0.7 mL/min, 100 μL injection	210 nm	14
HPLC	Apples				Dolenc & Stampar 1997 method[21]	15
Ion chromatography	Pears and peach nectar, apple juice	Diluted with water, filtered through paper and passed through a 0.45 μm filter	IonPac ICE-AS6	0.5 mM hepta-fluorobutyric acid, 1 mL/min, 5 mM tetrabutylammonium hydroxide, 5 mL/min, 25 μL injection	Suppressed conductivity, UV spectro-photometry (207 nm)	16

RP-IPC (Reverse phase ion-pair chromatography)	Beverages	Ethanol containing 3-methyl-glutaric acid as IS was added to sample solution plus 2-nitrophenylhydrazine hydrochloride solution and 1-ethyl-3-(-3-dimethyl-aminopropyl) carbodiimide hydrochloride solution. Leave the mixture at 80 ºC for 5 min add 10 % KOH soln, heat for further 5 min, cool for 5 min	J'sphere ODS-M 80 with a BBC-5-C8 guard column at 35 ºC	Phosphate buffer-acetonitrile-methanol containing tetra-ethylammonium bromide, 2 mL/min, 5–10 µL injection	UV–vis	17
Ion chromatography	Grape juice	Diluted 20 fold with water and passed through a 0.45 µm filter	Dionex AS11 with AG11 pre-column	NaOH gradient in water–MeOH–EtOH (74:13:13, v/v/v), 2 mL/min, 25 µL injection	Electro-chemical, suppressed conductivity	18
HPLC	Wine	Wine adjusted to pH 8 with 0.1 M NaOH and applied to a Bond Elut SAX cartridge previously washed with MeOH and water. Organic acids eluted with 0.05 M H_2SO_4 (final vol >5 mL)	Aminex HPX 87H at 60 ºC	0.05 mM H_2SO_4 at 0.8 mL/min, 10 µL injection	220 nm	19
HPLC	Grape musts and wine	Direct injection of wine diluted (1:20)	Aminex HPX 87H at 45 ºC	6 % acetonitrile in 0.045 N H_2SO_4 at 0.5 mL/min, 20 µL injection	UV at 210 nm	20
HPLC	Cherry cultivars	Fruit purée (10 g) was diluted with water (30 mL), centrifuged at 6000 g for 15 min and filtered through 0.45 µm filter	Aminex HPX 87H at 65 ºC	4 mM H_2SO_4, 20 µL injection	UV at 210 nm	21

Table 10.1 cont'd

(b)

Method	Matrix	Sample preparation	Method conditions	Detection	Reference
GC–MS	Alcoholic beverages	Simple pre-concentration based on solid phase (anion exchange) disk extraction, and in-vial elution and silylation	The derivatised extract injected into GC–MS system	Mass selective	23
GC	Soft drinks and jams	Dissolved in NH_3–NH_4Cl buffer soln of pH 9. Aliquot passed through an anion-exchange column packed with QAE-Sephadex A25. Column washed with water and eluted with 0.1 M HCl Lower layer derivatised with bis(trimethylsilyl)acetamide and chlorotrimethylsilane (TMCS)	Glass column packed with 3 % SE-30 on Chromosorb W AW-DMCS and temp-programmed from 140 to 200 ºC at 2 ºC/min with N_2 carrier gas at 40 mL/min, 5 µL injection	FID	22
GC–MS	Strawberry tree fruits	Powdered fruits were defatted then extracted 2x with 80 % EtOH + 4x with 70 % EtOH. Extracts combined and centrifuged. Half of extract was used to determine non-volatile acids. TMS derivatives were formed by the addition of BSTFA and TMCS and heating for 20 min. After reaction anhydrous sodium sulphate added to ensure dryness and cooled	HP-1 capillary column (25 m × 0.32 mm i.d., 0.17 mm film thickness) initially at 100 ºC and then 8 ºC/min to 290 ºC. Hydrogen carrier gas at 55 cm/s, 0.5–1.0 µL injection	FID and MS	24

Polarographic	Foods	Make a test solution in methanol. Dilute test solution (5 mL) to 25 mL with electrolyte solution	Prepare a standard curve by plotting numerical values of solutions against µg fumaric acid/mL	Voltammetric or polarographic instrument scanning up to 3.0 volts	25
Two-dimensional cITP	Feed additives	Sample (100 mg) was dissolved in 0.1 M NaOH (500 mL)	Preseparation was run in first capillary at pH 6.1 of leading electrolyte (LE). The pH of (LE) in second capillary 2.5, with β-cyclodextrin added. The terminating electrolyte (TE) was 5 mM caproic acid	Conductivity and UV	26
cITP–CZE	Apple juice	Sample (5 µL) injected directly or after dilution (10x) via sampling valve	LE: 10 mM HCl+β-alanine+5 mMβ-cyclodextrin+0.05 % hydroxypropylmethylcelullose (HPMC) pH 3 TE: 10 mM citric acid BE: 20 mM citric acid+β-alanine+5 nMβ-cyclodextrin+0.1 % HPMC, pH 3.3	UV	27

Table 10.2 Summary of statistical parameters for fumaric acid in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
LC	Cranberry juice cocktail and apple juice	AOAC Official Method 986.13	Ref: *JAOAC* (1986) **69**, 594	11
Polarographic	Foods	AOAC Official Method 965.28	Ref: *JAOAC* (1996) **49**, 701, *JAOAC* (1968) **51**, 533	25
HPLC	Apple juice	Full collaborative trial	Details given in Table 10.3. RSD_R values are 20 % or less at the 1 mg/100 g level	1
HPLC	Honey	Performance of method established and applied to 4 samples (n=3)	Recovery 97.9–99 % at 1 ppm level (n=3). Detection limit 0.004 ppm Multifloral honey 2.6±1.3 mg/kg (n=3) Strawberry tree honey 1.3±0.3 mg/kg (n=3) Asphodel honey 0.5±0.2 mg/kg (n=3) Red gum honey 1.6±0.4 mg/kg (n=3)	14
cITP–CZE	Apple juice	Method parameters established and tested on 7 real samples (n=3)	Precision: RSD 1.07 % (n=6) Accuracy: 95.4±3.5 % (n=3) Linearity: 0–1000 ng/mL Detection limit 10 ng/mL	27
cITP	Feed additives	Method parameters established and tested on 2 real samples (n=3)	Accuracy, determined by spiked samples, typical recovery 94 to 98 %. Precision obtained from 10 repetitive determinations RSD = 3.3 % Real sample: 42.9±1.5 g/100 g (n=3)	26

GC	Soft drinks, jams	Performance of method established and applied to commercial samples	Detection limit 0.005 % Recoveries from soft drinks samples 80.6–93.3 % (n=5) jam samples 88.9–88.1 % (n=5)	22
RP–IPC	Beverages	Performance of method established and applied to beverage samples	Calibration graph linear. Recovery in red wine 103.9 %±3.2 (n=9) CV 3.1 % at 50 nmol 99.5 %±2.8 (n=9) CV 2.8 % at 200 nmol	17
LC	Green beans	Performance of method established and used for survey of 1992 harvest	Calibration graph linear 0.05–0.4 mg/100 mL Detection limit 1×10^{-4} mg/mL Recovery 99.2 % Method precision (coefficient of variation) 1.5 % (n=10) Measurement precision (coefficient of variation) 1.01 %	9
IC	Pears, peach nectar, apple juice	Performance of method established and applied to commercial samples	RSD 4.5 % (n=5) for peak area on standard solution 2 mg/L Detection limit 0.5 mg/L	16
IC	Grape juice	Performance of method established and applied to grape juice samples	Detection limit 76 µg/L RSD 0.94 %	18
GC–MS	Alcoholic beverages	Performance of method established and applied to commercial samples	Upper concentration limit 3 mg/L Recovery 59.1 % (n=3) in standard	23

Table 10.2 cont'd

Method	Matrix	Extent of validation	Statistical parameters	Reference
HPLC	Orange juice	Performance of method established on spiked samples	RSD 1.1–6.4 % (n=6). Recoveries were >93 %.	3
HPLC	Lager beers	No performance data available. Method used for survey	See Table 10.4	13
LC	Apple juice	No performance data available. Method used for survey (30 samples)	30 apple juice samples analysed from various countries. Fumaric acid levels ranged from ND–4.3 mg/L	2
HPLC	Apricot cultivars	No performance data available. Method used for survey	RSD 0.13 % 15 samples analysed (n=3)	12
GC–MS	Strawberry tree fruits	No performance data available. Method applied to samples	Fumaric acid content 1.94±0.07 mg/g dry weight (n=3)	24
HPLC	Apple drinks	No details given	Recovery 98.2–104.5 %	7
IEC	Fruits, juices	No details given	Recovery 92 %	4

Table 10.3 Performance characteristics for fumaric acid in collaborative trial prepared apple juice samples[1]

Sample	A	B	C	D
Analyte	Fumaric acid	Fumaric acid	Fumaric acid	Fumaric acid
No. of laboratories	9	9	9	9
Units	mg/100g	mg/100g	mg/100g	g/100g
Mean value	0.856	1.368	0.384	0.879
SD	0.175	0.219	0.079	0.161
Coefficient of Variation CV	20.5 %	16.0 %	20.5 %	18.4 %
Intralaboratory SD (estimate)		0.084		
Intralaboratory CV (estimate)		9.7 %		

Table 10.4 Performance characteristics for fumaric acid in lager beers[13]

Sample	A	B	C	D
Analyte	Fumaric acid	Fumaric acid	Fumaric acid	Fumaric acid
No. of determinations	6	6	6	6
Units	mg/L	mg/L	mg/L	mg/L
Mean value	42	41	63	29
±SD	3	4	4	4

11

E310–12: Gallates

11.1 Introduction

The major food groups contributing to dietary intake of gallates are snacks, sauces, fats and oils with the maximum permitted level of 400 mg/kg being allowed in dietary supplements and chewing gum. The ADI for gallates is 0.5 mg/kg body weight/day.

11.2 Methods of analysis

There are numerous methods published for the determination of gallates (propyl, octyl and dodecyl) in foodstuffs. The majority of these methods are applicable to foods and are HPLC,[1–10] micellar electrokinetic chromatography (MECC),[11,12] spectrophotometric,[13,14] voltammetric,[15] TLC[16] and colorimetric.[17] A summary of these is given in Table 11.1, together with the matrices to which the methods apply. If statistical parameters for these methods were available these have been summarised in Table 11.2.

Two of these methods[1,17] have been adopted as AOAC official methods. The liquid chromatographic method for the analysis of gallates in oils, fats and butter oil was collaboratively trialled.[1,2] The method consists of phenolic antioxidants being extracted into acetonitrile. The extract is concentrated and diluted with 2-propanol. Antioxidants are separated by liquid chromatography and measured by UV detection at 280 nm. The procedure for this method is given in the Appendix and the performance characteristics are given in Table 11.3.

11.3 Recommendations

There are many methods available for the analysis of gallates in fatty foods and the

decision as to what method should be used depends on the matrix to be analysed. The majority of them are for liquids i.e. oils and further method development may be required to adapt them to be applicable for all matrices.

11.4 References

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propyl gallate and *tert*-butylhydroquinone by use of chemometric approaches', Ni Y, Wang L, Kokot S. *Analytica Chimica Acta* (2000) **412**, 185–193.
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11.5 Appendix: method procedure summaries

Analysis of oils, fats and butter oil[1, 2]

AOAC official method 983.15, phenolic antioxidants in oils, fats and butter oil, liquid chromatographic method, IUPAC–AOAC method

Principle
Antioxidants are extracted into acetonitrile. Extract is concentrated and diluted with 2-propanol. Antioxidants are separated by liquid chromatograph and measured by ultraviolet detection at 280 nm.

Determination

(a) *Extraction* – Accurately weigh to nearest 0.01 g 50 mL beaker containing *c*. 5.5 g liquid or butter oil or *c*. 3.0 g lard or shortening (liquefied in bulk using 60 °C water bath or oven, and swirled or shaken to ensure homogeneity). Decant as much test portion as possible into 125 mL separatory funnel containing 20 mL (22.5 mL for lard or shortening) saturated hexane. Reweigh beaker to determine test portion weight. Swirl to mix test portion with hexane, and extract with three 50 mL portions of saturated acetonitrile. If emulsions form, hold separatory funnel under hot tap water 5–10 s. Collect extracts in 250 mL separatory funnel and let combined extracts drain slowly into 250 or 500 mL round-bottom flask to aid removal of hexane-oil droplets. (Note: at this point, 150 mL acetonitrile extract may be stored overnight, refrigerated.)

Evaporate to 3–4 mL, using flash evaporator with ≤40 °C water bath, within 10 min. (Note: (1) Prolonged evaporation time may cause TBHQ losses. To decrease evaporation time, use efficient vacuum source and water-ice condenser cooling. (2) Use 500 mL flask to reduce 'bumping' losses. Take care to ensure quantitative transfer of extract after evaporation.) Using disposable pipette, transfer acetonitrile-oil droplet mixture to 10 mL glass-stoppered graduated cylinder. Rinse flask with small portions non-saturated acetonitrile. As rinse pools in flask bottom, pipette rinse to cylinder until 5 mL is collected. Rinse pipette through top and continue to rinse flask with small portions 2-propanol, transferring rinses to cylinder until 10 mL is collected. Mix cylinder contents. (Note: delay in analysing extracted test portion may cause TBHQ loss.)

(b) *Chromatography* – Using sample loop injection valve, inject 10 µL sample extract and elute with solvent gradient programme for test extracts. Before and after every 3–4 test injections, or more frequently if differences between standard peak heights are found to be >5 %, inject 10 µL antioxidant working standard solution (10 µL/mL) and elute with solvent gradient programme for standards. For analyte peaks off scale or >3x standard, quantitatively dilute test extract with 2-propanol-acetonitrile (1 + 1) and reinject. Identify peaks by comparison with retention times of standard.

For reagent blank determination, take 25 mL saturated hexane and follow extraction (a), from '. . .extract with three 50 mL portions of saturated acetonitrile.' Inject 10 µL reagent blank extract and elute with solvent gradient program for samples. The reagent blank should have no peaks interfering with antioxidant determination.

Use electronically determined peak height, or measure peak height to 0.1mm, using blank gradient chromatogram as guide to follow baseline. Determine antioxidant peak heights and average antioxidant standard peak heights (from duplicate injections before and after test injection, corrected for gradient blank).

Calculation

Calculate concentration of antioxidant as follows:

$$\text{Antioxidant, } \mu g/g = (R_x/R_s) \times (C_s/W_x) \times D \qquad [11.1]$$

where:

R_x and R_s are peak heights from test portion and standard, respectively
C_s is concentration standard, µg/mL
W_x is test portion weight, g/mL, in undiluted 10 mL test extract
D is dilution factor, if solution injected is diluted

(For further information see AOAC official method 983.15.)

Table 11.1 Summary of methods for gallates in foods

(a)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
HPLC	Oils, fats and butter oil	Antioxidants extracted into acetonitrile. Extract is concentrated and diluted with 2-propanol. 10 µL injection	C18 bonded spherical (preferred) silica or equivalent	5 % acetic acid in H_2O (A) acetonitrile–methanol (1:1) (B) Gradient. Flow rate: 2.0 mL/min	UV at 280 nm	1, 2
RP-HPLC	Pharmaceutical formulations	Sample filtered through 0.45 µm filter	LiChrosorb RP-18 (250 × 4.6 mm, 7 µm)	MeOH–H_2O + 1 % acetic acid, flow rate 1 mL/min, injection 10 µL	UV at 230 nm	3
HPLC	Olive oil	Dissolved in petroleum ether. Extracted with 3 × 72 % EtOH. Extracts combined and filtered through 0.45 µm filter. Made to volume with EtOH. 20 µL injection	LiChrosorb RP-18 (150 × 3.9 mm, 10 µm)	0.1 M sodium dodecyl sulphate (SDS) / 0.01 M H_3PO_4 /30 % PrOH	UV at 290 nm	4
HPLC	Bakery products	10 g minced sample with 2,4,6-trimethylphenol as IS was added and homogenised with acetonitrile–isopropanol (1:1). Liquid phase separated and re-extracted x2. Combined and rotary evaporated and then made up to volume. 10 µL injected	Extrasil ODS2 (25 × 0.46 cm, 5 µm) and precolumn Kromasil ODS2	Gradient at 1.5 mL/min A, acetic acid–MeOH (5:95) and B, acetic acid–water (5:95)	UV at 280 nm	5

HPLC	Foods	Extracted with acetonitrile–2-propanol–ethanol (2:1:1). Extract in freezer for 1 h then filtered. Filtrate concentrated under vacuum. 10 µL injected	CrestPac C18S (4.6 × 150 mm)	Gradient elution system of 5 % acetic acid and MeOH–acetonitrile (50:50). Flow rate 1 mL/min	UV at 280 nm	6
LC	Foods and drugs	MeOH added to sample and sonicated and re-extracted. Extracts filtered on a SepPak silica cartridge connected to syringe. 0.2 µL injected	Kromasil C18 (300 mm × 1 mm, 5 µm)	Water–MeOH (10:90), 0.01 M $LiClO_4$, pH 5.5 at 50 µL/min	Electrochemical oxidation potential +0.8 V versus Ag–AgCl	7
LC	Edible oils	Microemulsion of oil prepared by mixing 5 % oil with 95 % water–SDS–*n*-pentanol (37.5:12.5:50). 20 µL injected	Spherisorb ODS-2 (125 × 4 mm, 5 µm) and guard column (35 × 4.6 mm, 10 µm)	0.1 M SDS, 2.5 % *n*-propanol and 10 mM phosphate at pH 3. Flow rate 1 mL/min	UV at 284 nm	8
LC	Dairy products and dietetic supplements	Samples prepared in mobile phase at following concentrations: liquid milk 20 %; powdered milk and cream 1 %; dietetic supplement 0.5 %. Solutions filtered through 0.45 µm filter. 20 µL injected	Spherisorb ODS-2 (125 × 4 mm, 5 µm) and guard column (35 × 4.6 mm, 10 µm)	0.090 M SDS, 6.6 % *n*-propanol and 10 mM phosphate at pH 3. Flow rate 1 mL/min	UV at 284 nm	9
HPLC-ECD	Foods	Samples prepared in mobile phase. Solutions filtered through 0.45 µm filter. 20 µL injected	Lichrocart RP18 (250 × 4 mm, 5 µm)	Isocratic CH_3CN–THF–H_2O (60:25:15). Flow rate 0.9 mL/min	3 electrodes, reference electrode at 0.90 V, sensitivity is 50 nA	10

Table 11.1 cont'd

(b)

Method	Matrix	Sample preparation	Method conditions	Reference
Micellar electrokinetic chromatography (MECC)	Cola beverages and jams	Butyl paraben was used as an internal marker	Additives were separated using a 20 mM berate buffer with 35 mM sodium cholate, 15 mM sodium dodecyl sulphate and 10 % methanol added at pH 9.3	11
MECC vs HPLC	Antioxidants		4 antioxidants were separated completely with excellent resolution and efficiency within 6 min and picomole amounts of antioxidants were detectable using UV absorption. RP–HPLC separation not as efficient and requires larger sample amounts and longer separation time	12
Stopped flow mixing and a T-format luminescence spectrometer	Foods	Sample dissolved in petroleum ether and extracted 3 × with 72 % EtOH, filtered and diluted to volume. 0.7 mL used	2 solutions prepared to fill syringes in stopped flow module. One based on lanthanide chelate with terbium in presence of Triton X-100 and tri-*n*-octylphosphine oxide (for PG) and the other based on a reaction between the oxidised form of Nile Blue and BHA. In each run 150 µL of each solution is mixed in the mixing chamber. The excitation wavelength used is 310 nm. Emission 545 nm for PG and 665 nm for BHA	13
Stopped flow mixing and diode-array	Foods	Sample dissolved in petroleum ether and extracted 3 × with 72 % EtOH, filtered and diluted to volume. 0.7 mL used	Based on kinetic behaviour of PG and BHA when reacted with 3-methylbenzothiazolin-2-one hydrazone in the presence of cerium (IV) and on the joint use of the stopped-flow mixing technique and a diode-array detector, which allows kinetic data to be obtained at 2 wavelengths simultaneously	14

Voltammetric	Foods	For vegetable oil samples: equal volume of MeOH added, shaken, centrifuged. MeOH extracts made to volume, 1.0 mL aliquot used. For solid samples: ground to powder, shaken with petroleum ether and extracted as for oil samples	1.0 mL of 1.0 M perchloric acid and 0.1 mL MeOH added to aliquot and transferred to electrochemical cell and diluted to 10 mL with water. Stirred for 60 s after 2 s pause, a linear potential scan taken from 1–1300 mV at the glass carbon electrode (vs Ag–AgCl) with scan rate of 75 mV/s. The obtained linear sweep voltammograms (LSV) showed well-defined oxidation waves with a peak potential of 599 mV	15
UV, TLC–UV, colorimetry, TLC–colorimetry	Hydrogenated vegetable fat	100 mg/g BHA, BHT and PG added in fat	UV methods not appropriate for products containing 2 or more antioxidants and/or interfering substances. TLC–UV and TLC–colorimetry are more adequate; the colorimetric method can also be used, depending on the interfering substances and the colorimetric specificity. Colorimetric method is more economical and rapid than methods using TLC	16
Colorimetric	Food	Dissolve in petroleum ether and extract with aqueous ammonium acetate solution. Filter ammonium acetate extract and transfer aliquot to conical flask	Dilute to 20 mL with ammonium acetate solution. Add exactly 4 mL H_2O and pipette 1 mL ferrous tartrate reagent into flask. Mix well and measure *A* at 540 nm against reagent blank. Calculate amount of PG from standard curve	17

PG = propyl gallate
OG = octyl gallate
DG = dodecyl gallate

Table 11.2 Summary of statistical parameters for gallates in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
LC	Oils, fats and butter oil	AOAC Official Method 983.15 Full collaborative trial	Ref: *Journal of AOAC International* (1993) **76**, 765 Details given in Table 11.3	1,2
HPLC	Bakery products	Precision of method established and applied to 15 commercial samples	Linear calibration curve in range 2–100 µg/mL. Recovery calculated for the IS was 94.6 % (n=10). Cake spiked with 16 µg/g. CV % was 5.3 % for PG, 5.7 % for OG and 5.9 % DG. Of 15 samples analysed none contained gallates	5
Stopped flow mixing and a T-format luminescence spectrometer	Foods	Precision of method established and applied to 10 commercial samples	Calibration graphs linear over range 0.09–3.5 µg/mL. The relative standard deviation was 2.3 %. LOD was 0.03 µg/mL for PG. The method was applied to the determination of PG and BHA in several commercial foods with recoveries ranging between 94.1 and 102.1 % for PG	13
Stopped flow mixing and diode-array	Foods	Precision of method established and applied to 8 commercial samples	Calibration graphs linear over range 1.6–27.5 µg/mL. The relative standard deviations for both systems are close to 2 %. LOD = 0.5 µg/mL for PG. The method was applied to the determination of PG and BHA in several commercial foods with recoveries ranging between 97.2 and 103.5 %	14
Voltammetric	Foods	Precision of method established and applied to 8 commercial samples	Linear calibration graph obtained for PG in range 1.0–15.0 mg/L. LOD 0.54 mg/L. Recovery ranged from 85–118 % for PG in spiked food samples	15

LC	Dairy products and dietetic supplements	Precision of method established	Linear calibration curve. LOD 0.05 ng, repeatability 0.7 % (n=6) for microemulsion containing 5 µg/g PG. Recovery ranged from 96 to 103 % (n=3) for 5 foods spiked at 2 µg/g PG (i.e. powdered milk, cream, milk and 2 dietetic supplements). Linear calibration curve. LOD 0.2 ng, repeatability 0.5 % (n=6) for microemulsion containing 5 µg/g OG. Recovery ranged from 99 to 103 % (n=3) for 5 foods spiked at 2 µg/g OG (i.e. powdered milk, cream, milk and 2 dietetic supplements)	9
RP-HPLC	Pharmaceutical formulations	Precision of method established	PG: Linear calibration curve in range 0.00006–5.1 µg/mL. LOD = 0.14 pg Mean recovery of 99 % from olive oil spiked at 3 levels 29.7, 212.2 and 424.4 mg/kg PG (n=5), range 95.3–100.9 %. OG: Linear calibration curve in range 0.028–14.1 µg/mL. LOD = 0.31 pg. Mean recovery of 99 % from olive oil spiked at 3 levels 56.5, 197.6 and 395.3 mg/kg OG (n=5), range 92.5–105.0 %. DG: Linear calibration curve in range 0.034–20.3 µg/mL. LOD = 0.33 pg. Mean recovery of 99 % from olive oil spiked at 3 levels 67.7, 203.1 and 473.8 mg/kg DG (n=5), range 95.7–105.7 %	4
HPLC	Foods	Precision of method established	Recovery ranged from 84.9 to 96.5 % (n=3) for 5 foods spiked at 100 µg/g PG (i.e. corn oil, butter oil, butter, niboshi, frozen shrimp). Recovery ranged from 87.4 to 96.2 % (n=3) for 5 foods spiked at 100 µg/g OG. Recovery ranged from 87.8 to 91.9 % (n=3) for 5 foods spiked at 100 µg/g DG	6
LC	Edible oils	Precision of method established	Linear calibration curve. LOD 2.5 ng, repeatability 2.5 % (n=5) for microemulsion containing 10 µg/g PG. Linear calibration curve. LOD 5.9 ng, repeatability 1.9 % (n=5) for microemulsion containing 10 µg/g OG	8
LC	Foods and drugs	Precision of method established	Linear calibration curve. LOD = 0.9 ppb. RSD (n=5) repeatability <3 % for PG	7

Table 11.3 Performance characteristics for gallates in oils, lard and butter oil[1,2]

Sample	Oils		Lard	
Analyte	Propyl gallate	Propyl gallate	Propyl gallate	Propyl gallate
No. of laboratories	7	7	7	7
Units	mg/g	mg/g	mg/g	mg/g
Spike value	193.7	96.7	19.4	96.9
Mean value	184	93.8	17.6	90.1
S_r	16.0	4.50	2.01	3.18
RSD_r %	8.66	4.80	11.5	3.53
S_R	16.0	4.50	2.52	3.18
RSD_R %	8.66	4.80	14.3	3.53
Recovery	95.2 %	96.9 %	90.9 %	93.0 %

Sample	Lard		Butter oil	
Analyte	Propyl gallate	Propyl gallate	Propyl gallate	Propyl gallate
No. of laboratories	7	7	7	7
Units	mg/g	mg/g	mg/g	mg/g
Spike value	38.7	92.1	46.0	9.20
Mean value	34.6	89.3	46.9	9.53
S_r	1.55	4.76	3.86	0.450
RSD_r %	4.48	5.33	8.23	4.72
S_R	1.55	6.08	4.54	0.875
RSD_R %	4.48	6.81	9.67	9.17
Recovery	89.4 %	97.0 %	102 %	104 %

Sample	Butter oil		
Analyte	Octyl gallate	Octyl gallate	Octyl gallate
No. of laboratories	7	7	7
Units	mg/g	mg/g	mg/g
Spike value	89.2	43.7	8.76
Mean value	86.3	42.0	8.19
S_r	3.80	2.89	1.69
RSD_r %	4.40	6.87	20.6
S_R	4.37	2.89	1.69
RSD_R %	5.06	6.87	20.6
Recovery	96.8 %	96.2 %	93.5 %

Sample	Butter oil		
Analyte	Dodecyl gallate	Dodecyl gallate	Dodecyl gallate
No. of laboratories	7	7	7
Units	mg/g	mg/g	mg/g
Spike value	101.1	50.6	10.1
Mean value	96.7	48.8	9.76
S_r	4.02	2.98	0.468
RSD_r %	4.16	6.12	4.80
S_R	7.94	3.05	0.742
RSD_R %	8.21	6.24	7.61
Recovery	95.7 %	96.5 %	96.4 %

12

E320: BHA

12.1 Introduction

The major food groups contributing to dietary intake of BHA are cakes, cookies and pies, other fine bakeryware and emulsified sauces with the maximum permitted level of 400 mg/kg being allowed in dietary supplements and chewing gum. The ADI for BHA is 0.5 mg/kg body weight/day.

12.2 Methods of analysis

There are numerous methods published for the determination of BHA in foodstuffs. The majority of these are applicable to foods and are GC,[1–4] HPLC,[5–14] micellar electrokinetic chromatography (MECC),[15,16] spectrophotometric,[17–20] voltammetric[21] and TLC[22] methods. A summary of these methods is given in Table 12.1, together with the matrices to which they are applicable. If statistical parameters for these methods were available they have been summarised in Table 12.2.

Two of these methods[1,5] have been adopted as AOAC official methods. The liquid chromatographic method for the analysis of BHA in oils, fats and butter oil was collaboratively trialled.[5,6] The method consists of phenolic antioxidants being extracted into acetonitrile. The extract is concentrated and diluted with 2-propanol. Antioxidants are separated by liquid chromatography and measured by UV detection at 280 nm. The procedure for this method is given in the Appendix and the performance characteristics are given in Table 12.3.

12.3 Recommendations

There are many methods available for the analysis of BHA in fatty foods and the decision as to what method should be used depends on the matrix to be analysed. The majority of these methods are for liquids i.e. oils and further method development may be required to adapt these methods to be applicable for all matrices.

12.4 References

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12.5 Appendix: method procedure summaries

Analysis of oils, fats and butter oil[5,6]

AOAC official method 983.15, phenolic antioxidants in oils, fats and butter oil, liquid chromatographic method, IUPAC–AOAC method

Principle

Antioxidants are extracted into acetonitrile. Extract is concentrated and diluted with 2-propanol. Antioxidants are separated by liquid chromatograph and measured by ultraviolet detection at 280 nm.

Determination

(a) *Extraction* – Accurately weigh to nearest 0.01 g 50 mL beaker containing *c*. 5.5 g liquid or butter oil or *c*. 3.0 g lard or shortening (liquefied in bulk using 60 °C water bath or oven, and swirled or shaken to ensure homogeneity). Decant as much test portion as possible into 125 mL separatory funnel containing 20 mL (22.5 mL for lard or shortening) saturated hexane. Reweigh beaker to determine test portion weight. Swirl to mix test portion with hexane, and extract with three 50 mL portions of saturated acetonitrile. If emulsions

form, hold separatory funnel under hot tap water 5–10 s. Collect extracts in 250 mL separatory funnel and let combined extracts drain slowly into 250 or 500 mL round-bottom flask to aid removal of hexane-oil droplets. (Note: at this point, 150 mL acetonitrile extract may be stored overnight, refrigerated.)

Evaporate to 3–4 mL, using flash evaporator with ≤40 °C water bath, within 10 min. (Note: (1) Prolonged evaporation time may cause TBHQ losses. To decrease evaporation time, use efficient vacuum source and water-ice condenser cooling. (2) Use 500 mL flask to reduce 'bumping' losses. Take care to ensure quantitative transfer of extract after evaporation.) Using disposable pipette, transfer acetonitrile-oil droplet mixture to 10 mL glass-stoppered graduated cylinder. Rinse flask with small portions non-saturated acetonitrile. As rinse pools in flask bottom, pipette rinse to cylinder until 5 mL is collected. Rinse pipette through top and continue to rinse flask with small portions 2-propanol, transferring rinses to cylinder until 10 mL is collected. Mix cylinder contents. (Note: Delay in analysing extracted test portion may cause TBHQ loss.)

(b) *Chromatography* – Using sample loop injection valve, inject 10 µL sample extract and elute with solvent gradient program for test extracts. Before and after every 3–4 test injections, or more frequently if differences between standard peak heights are found to be >5 %, inject 10 µL antioxidant working standard solution (10 µL/mL) and elute with solvent gradient program for standards. For analyte peaks off scale or >3x standard, quantitatively dilute test extract with 2-propanol-acetonitrile (1 + 1) and reinject. Identify peaks by comparison with retention times of standard.

For reagent blank determination, take 25 mL saturated hexane and follow extraction (a), from '. . . extract with three 50 mL portions of saturated acetonitrile'. Inject 10 µL reagent blank extract and elute with solvent gradient program for samples. The reagent blank should have no peaks interfering with antioxidant determination.

Use electronically determined peak height, or measure peak height to 0.1 mm, using blank gradient chromatogram as guide to follow baseline. Determine antioxidant peak heights and average antioxidant standard peak heights (from duplicate injections before and after test injection, corrected for gradient blank).

Calculation

Calculate concentration of antioxidant as follows:

$$\text{Antioxidant, } \mu g/g = (R_x/R_s) \times (C_s/W_x) \times D \qquad [12.1]$$

where:

R_x and R_s are peak heights from test portion and standard, respectively
C_s is concentration standard, µg/mL
W_x is test portion weight, g/mL, in undiluted 10 mL test extract
D is dilution factor, if injected solution is diluted

(For further information see AOAC official method 983.15.)

Table 12.1 Summary of methods for BHA in foods

(a)

Method	Matrix	Sample preparation/extraction	Column	Conditions	Detection	Reference
GC	Cereals	Ground sample placed in chromatographic column and eluted with CS_2, di-BHA internal standard added to eluate. Evaporate to <5.0 mL under N_2. Make up to 5.0 mL	2 columns: QF-1 and Apiezon L/gas-chrom Q at 160 ºC	Injection size 3.0–9.0 µL, flash heater 200 ºC, N_2 flow rate: 40 mL/min for Apiezon column and 25 mL/min for QF-1 column	FID at 210 ºC	1
GC	Fatty foods	Samples manually extracted with a mixture of solvents then subjected to continuous SPE system	Fused-silica capillary column HP-5 (30 m × 0.32 mm, 1 µm)	Oven temp: 125–315 ºC, 10 ºC/min; injection port 250 ºC, N_2 carrier 1 mL/min	FID at 310 ºC, ionisation energy 70 eV MS 50–500 m/z (105 m/z)	2
GC–MS (SIM)	Foods	Solid phase extraction (SPE) with a polymer-based cartridge and pH adjustment of sample (pH = 3.5) in pre-treatment	HP-INNOWax (30 m × 0.25 mm i.d. 0.25 µm)	Splitless GC. temp 220 ºC; splitless flow (helium) 11 psi. Oven temperature programmed 100–240 ºC	MS selected ion monitoring (SIM) mode. m/z 165	3
GC	Edible fats and oils	10 % tetramethylammonium hydroxide (TMAH) methanol solution added to 60–80 mg oil sample and vortex-mixed for 20 min methylation. Extracted with ether and 0.2 % tetradecanol added and mixed thoroughly. 0.3 µL ether layer injected	CP-SIL 8CB mega-bore capillary column (30 m × 0.53 mm, 1.5 µm)	Splitless injection at 270 ºC. Temperature programmed 140–300 ºC. Carrier gas N_2 at 4 mL/min	FID at 300 ºC	4

Table 12.1 contd
(b)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
HPLC	Oils, fats and butter oil	Antioxidants extracted into acetonitrile. Extract is concentrated and diluted with 2-propanol. 10 µL injection	C18 bonded spherical (preferred) silica or equivalent	5 % acetic acid in H_2O (A) Acetonitrile–methanol (1:1) (B). Gradient. Flow rate: 2.0 mL/min	UV at 280 nm	5, 6
HPLC	Oils, foods and biological fluids	Antioxidants extracted into acetonitrile. Resulting extract was filtered through 0.5 µm filter before injection. 10 µL injection	LiChrosorb RP-18 (4 mm × 250 mm, 5 µm)	Gradient; of H_2O–acetonitrile–acetic acid (66.5:28.5:5) and acetonitrile–acetic acid (95:5) at flow rate 1 mL/min	Fluorometric at 310 nm emission and 280 nm excitation	7
RP-HPLC	Pharmaceutical formulations	Sample filtered through 0.45 µm filter	LiChrosorb RP-18 (250 × 4.6 mm, 7 µm)	MeOH–H_2O + 1 % acetic acid, flow rate 1 mL/min, injection 10 µL	UV at 230 nm	8
HPLC	Olive oil	Dissolved in petroleum ether. Extracted with 3 × 72 % EtOH. Extracts combined and filtered through 0.45 µm filter. Made to volume with EtOH. 20 µL injection	LiChrosorb RP-18 (150 × 3.9 mm, 10 µm)	0.1 M sodium dodecyl sulphate (SDS) / 0.01 M H_3PO_4 /30 % PrOH	UV at 290 nm	9

HPLC	Bakery products	10 g minced sample with 2,4,6-trimethylphenol as is was added and homogenised with acetonitrile–isopropanol (1:1). Liquid phase separated and re-extracted x2. Combined and rotary evaporated and then made up to volume 10 µL injected	Extrasil ODS2 (25 × 0.46 cm, 5 µm) and precolumn Kromasil ODS2	Gradient at 1.5 mL/min A, acetic acid–MeOH (5:95) and B, acetic acid–water (5:95)	UV at 280 nm	10
HPLC	Foods	Extracted with acetonitrile–2propanol–ethanol (2:1:1). Extract in freezer for 1 h then filtered. Filtrate concentrated under vacuum. 10 µL injected	CrestPac C18S (4.6 × 150 mm)	Gradient elution system of 5 % acetic acid and MeOH–acetonitrile (50:50). Flow rate 1 mL/min	UV at 280 nm	11
LC	Foods and drugs	MeOH added to sample and sonicated and re-extracted. Extracts filtered on a SepPak silica cartridge connected to syringe. 0.2 µL injected	Kromasil C18 (300 mm × 1 mm, 5 µm)	Water–MeOH (10:90), 0.01 M $LiClO_4$, pH 5.5 at 50 µL/min	Electrochemical oxidation potential +0.8 V vs Ag–AgCl	12
LC	Edible oils	Microemulsion of oil prepared by mixing 5 % oil with 95 % water–SDS–*n*-pentanol (37.5:12.5:50). 20 µL injected	Spherisorb ODS-2 (125 × 4 mm, 5 µm) and guard column (35 × 4.6 mm, 10 µm)	0.1 M SDS, 2.5 % *n*-propanol and 10 mM phosphate at pH 3. Flow rate 1 mL/min	UV at 284 nm	13
LC	Dairy products and dietetic supplements	Samples prepared in mobile phase at following concentrations: liquid milk 20 %; powdered milk and cream 1 %; dietetic supplement 0.5 %. Solutions filtered through 0.45 µm filter. 20 µL injected	Spherisorb ODS-2 (125 × 4 mm, 5 µm) and guard column (35 × 4.6 mm, 10 µm)	0.090 M SDS, 6.6 % *n*-propanol and 10 mM phosphate at pH 3. Flow rate 1 mL/min	UV at 284 nm	14

Table 12.1 cont'd

(c)

Method	Matrix	Sample preparation	Method conditions	Reference
Micellar electrokinetic chromatography (MECC)	Cola beverages and jams	Butyl paraben was used as an internal marker	Additives were separated using a 20 mM berate buffer with 35 mM sodium cholate, 15 mM sodium dodecyl sulphate and 10 % methanol added at pH 9.3	15
MECC vs HPLC	Antioxidants		4 antioxidants were separated completely with excellent resolution and efficiency within 6 min and picomole amounts of antioxidants were detectable using UV absorption. RP–HPLC separation not as efficient and requires larger sample amounts and longer separation time	16
Stopped-flow mixing and a T-format luminescence spectrometer	Foods	Sample dissolved in petroleum ether and extracted 3 × with 72 % EtOH, filtered and diluted to volume. 0.7 mL used	2 solutions prepared to fill syringes in stopped flow module. One based on lanthanide chelate with terbium in presence of Triton X-100 and tri-*n*-octylphosphine oxide (for PG) and the other based on a reaction between the oxidised form of Nile Blue and BHA. In each run 150 µL of each solution is mixed in the mixing chamber. The excitation wavelength used is 310 nm. Emission 545 nm for PG and 665 nm for BHA	17
Stopped-flow mixing and diode-array	Foods	Sample dissolved in petroleum ether and extracted 3 × with 72 % EtOH, filtered and diluted to volume. 0.7 mL used	Based on kinetic behaviour of PG and BHA when reacted with 3-methylbenzothiazolin-2-one hydrazone in the presence of cerium (IV) and on the joint use of the stopped-flow mixing technique and a diode-array detector, which allows kinetic data to be obtained at 2 wavelengths simultaneously	18

Spectro-photometric	Oils	Dissolve 10 g oil in 100 mL petroleum ether. Extract with 4 × 20 mL acetonitrile. Combine and evaporate to dryness. Dissolve in aqueous methanol (1:1)	Method A: (Fe(III) – TPTZ). Ferric chloride and TPTZ were added successively to the sample. Diluted with water and heated in a boiling water bath, cooled phosphoric acid added. Made to volume. Absorbance measured at 590 nm. Method B: (TTC-NaOH). TTC solution added to sample in separating funnel. After 5 min add NaOH solutiona and *n*-butanol. Absorbance of *n*-butanol layer measured at 480 nm	19
Derivative spectrometry	Lard	10 g sample dissolved in 25 mL hexane and extracted with 25 mL DMSO. DMSO extract centrifuged and filtered	Absorption spectrum recorded between 250 nm and 400 nm with a scan speed of 100 nm/min. BHA content determined by measuring a second-derivative signal at 311.5 and comparing value with calibration graph	20
Voltammetric	Foods	For vegetable oil samples equal volume of MeOH added, shaken, centrifuged. MeOH extracts made to volume, 1.0 mL aliquot used. For solid samples: ground to powder, shaken with petroleum ether and extracted as for oil samples	1.0 mL of 1.0 M perchloric acid and 0.1 mL MeOH added to aliquot and transferred to electrochemical cell and diluted to 10 mL with water. Stirred for 60 s. After 2 s pause, a linear potential scan taken from 1–1300 mV at the glass carbon electrode (vs Ag–AgCl) with scan rate of 75 mV/s. The obtained linear sweep voltammograms (LSV) showed well-defined oxidation waves with a peak potential of 629 mV	21
UV, TLC–UV, colorimetry, TLC–colorimetry	Hydrogenated vegetable fat	100 mg/g BHA, BHT and PG added in fat	UV methods not appropriate for products containing 2 or more antioxidants and/or interfering substances. TLC–UV and TLC–colorimetry are more adequate; the colorimetric method can also be used, depending on the interfering substances and the colorimetric specificity. Colorimetric method is more economical and rapid than methods using TLC	22

TPTZ = 2,4,6-tripyridyl-S-triazine
TTC = triphenyl tetrazolium chloride
BHA = butylated hydroxyanisole
BHT = butylated hydroxytoluene
PG = propyl gallate

Table 12.2 Summary of statistical parameters for BHA in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
LC	Oils, fats and butter oil	AOAC Official Method 983.15 Full collaborative trial	Ref: *J.AOAC International* (1993) **76**, 765 Details given in Table 12.3	5,6
GC	Edible fats and oils	Precision of method established and applied to 18 commercial samples	Recovery of spiked blended oil at 26 and 52 µg/0.1 g (n=3) 106.2 % and 96.9 % (RSD= 5.8 % and 8.4 %). Compared to AOAC method.[1] This method had a higher extraction effect and higher accuracy than the AOAC method. BHA found in 8 of the commercial samples i.e. soybean oil at 50.4 µg/g, olive oils 29.1, 34.5 and 43.8 µg/g, vegetable oil 40.0 µg/g and blended oils 24.8, 15.7 and 23.8 µg/g	4
HPLC	Bakery products	Precision of method established and applied to 15 commercial samples	Linear calibration curve in range 2–100 µg/mL. Recovery calculated for the IS was 94.6 % (n=10). Cake spiked with 16 µg/g CV % was 3.5. 9 of 15 samples analysed contained BHA from 17.5 to 55.6 µg/g fat	10
LC	Foods and drugs	Precision of method established and applied to 10 commercial samples	Linear calibration curve. LOD = 0.9 ppb. RSD (n=5) repeatability <3 % 3 of 10 samples analysed contained BHA Keratosane 11.3±0.4 mg/kg Chewing gum 152±2 mg/kg (86 % of packet quoted level) Dry potato flakes 9.9±0.3 mg/kg	12

Stopped-flow mixing and a T-format luminescence spectrometer	Foods	Precision of method established and applied to 10 commercial samples	Calibration graphs linear over range 0.3–15 µg/mL. The relative standard deviation was 1–2.1 %. The method was applied to the determination of PG and BHA in several commercial foods with recoveries ranging between 93.1 and 102.9 % for BHA	17
GC	Fatty foods	Precision of method established and applied to real samples (n=9)	Linear range (µg/mL) 0.4–100 (FID), 1.5–500 (MS) (n=3) LOD (µg/mL) 0.15 (FID), 0.8 (MS) RSD (%) 3.1 (FID), 3.2 (MS). Checked with real samples for 5 samples analysed in triplicate by SPE–GC–FID Plain mayonnaise 190 mg/kg (RSD 5.3 % n=15) Sunflower seed oil 134 mg/kg (RSD 3.7 % n=15) Sunflower margarine 170 mg/kg (RSD 4.1 % n=15)	2
Stopped-flow mixing and diode-array	Foods	Precision of method established and applied to 8 commercial samples	Calibration graphs linear over range 0.3–30 µg/mL. The relative standard deviations for both systems are close to 2 %. The method was applied to the determination of PG and BHA in several commercial foods with recoveries ranging between 97.2 and 103.5 %	18
Voltammetric	Foods	Precision of method established and applied to 8 commercial samples	Linear calibration graph obtained in range 0.5–15.0 mg/L. LOD 0.19 mg/L. Recovery ranged from 87–131 % for spiked food samples	21

Table 12.2 cont'd

Method	Matrix	Extent of validation	Statistical parameters	Reference
GC–MS (SIM)	Foods	Precision of method established and applied to real samples (n=4)	Recovery of spiked fruit vinegar at 3 ppb (n=6) 70.1 % (RSD=0.7 %). Detection limit 0.1 ppb. BHA found in samples of soy sauce at 190 ppb, wine at 2 ppb and vinegar at 3 ppb	3
HPLC	Oils, foods and biological fluids	Precision of method established and applied to 2 commercial samples	Linear calibration curve in range 0.01–50 µg/mL. LOD = 93 ng/mL. Recovery of 97.8±0.3 % from fish oil containing 0.5 µg/g BHA (n=3) Smoked sausage 22.4±1.8 mg/kg wet basis (n=3) Dried anchovy 32.9±1.9 mg/kg wet basis (n=3)	7
LC	Dairy products and dietetic supplements	Precision of method established	Linear calibration curve. LOD 0.3 ng, repeatability 0.8 % (n=6) for microemulsion containing 5 µg/g BHA. Recovery ranged from 92 % to 102 % (n=3) for 5 foods spiked at 2 µg/g BHA (i.e. powdered milk, cream, milk and 2 dietetic supplements)	14
RP-HPLC	Pharmaceutical formulations	Precision of method established	Linear calibration curve in range 0.036–27 µg/mL. LOD = 0.67 pg. Mean recovery of 102 % from olive oil spiked at 3 levels 54.1, 198.3 and 432.6 mg/kg BHA (n=5), range 96.2–105.7 %	9
HPLC	Foods	Precision of method established	Recovery ranged from 91.4 % to 94.8 % (n=3) for 5 foods spiked at 100 µg/g BHA (i.e. corn oil, butter oil, butter, niboshi, frozen shrimp)	11
LC	Edible oils	Precision of method established	Linear calibration curve. LOD 3.8 ng, repeatability 1.4 % (n=5) for microemulsion containing 10 µg/g BHA	13
Spectro-photometric	Oils	Precision of method established	Calibration graphs for both methods linear. RSD for Method A was 1.17 % and for method B was 1.58 %. Recoveries of BHA at 5 mg added to oils is as follows: 96.1–98.1 % for Method A and 96.2–97.3 % for Method B	19
Derivative spectrometry	Lard	Precision of method established	Recovery of spiked lard at levels from 24.8–97.9 µg/g were 99.5–109.3 %	20
GC	Cereals	AOAC Official Method 968.17	Ref: *JAOAC* (1967) **50**, 880; (1968) **51**, 943; (1970) **53**, 39. No details available	1

Table 12.3 Performance characteristics for BHA in oils, lard and butter oil[5,6]

Sample		Oils		Lard
No. of laboratories	7	7	7	7
Units	mg/g	mg/g	mg/g	mg/g
Spike value	198.5	99.2	19.9	99.2
Mean value	197	99.7	19.5	97.4
S_r	6.54	5.43	0.43	2.49
RSD_r %	3.32	5.45	2.19	2.56
S_R	6.61	6.15	0.76	3.72
RSD_R %	3.36	6.17	3.92	3.82
Recovery	99.1 %	101 %	98 %	98.2 %
Sample	Lard		Butter oil	
No. of laboratories	7	7	7	7
Units	mg/g	mg/g	mg/g	mg/g
Spike value	39.7	101.3	50.6	10.1
Mean value	38.3	96.3	48.8	10.2
S_r	1.90	8.49	2.29	0.515
RSD_r %	4.97	8.81	4.70	5.06
S_R	1.90	8.49	2.50	0.597
RSD_R %	4.97	8.81	5.12	5.87
Recovery	96.6 %	95.2 %	96.5 %	101 %

13

E334–7, E354: L-tartaric acid and its salts

13.1 Introduction

The major food groups contributing to dietary intake of L-tartaric acid and its salts are fresh pasta, various fruit and vegetable preparations, beer and grape juice. The acceptable daily intake (ADI) for L-tartaric acid is 30 mg/kg body weight.

13.2 Methods of analysis

There are several methods published for the determination of L-tartaric acids in foodstuffs. Methods that have been developed more recently include the HPLC[1–3] and capillary electrophoresis methods[4–6] as well as the traditional methods of titration.[7–9] A summary of these methods is given in Table 13.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 13.2. Some of these methods are AOAC Official Methods of Analysis[7–9] and one has been accepted as a European Standard.[1]

The European Standard[1] specifies a method for the determination of tartaric acid in grape juices by high performance liquid chromatography (HPLC). The content of tartaric acid in grape juices is determined by HPLC (using UV detection). The separation takes place on an ion-exclusion column. A summary of the procedure for this method is given in the Appendix and the performance characteristics are given in Table 13.3.

13.3 Recommendations

There are several methods available for the analysis of L-tartaric acid in foods but none specifically for pasta or beer. Further method development is required to establish methods for these matrices.

13.4 References

1 'Fruit and vegetable juices – Determination of tartaric acid in grape juices – method by high performance liquid chromatography', BS 12137: 1998.
2 'Tartaric acid in frozen musts and wines. Optimization of Rebelein's method and validation by HPLC', Almela L, Lázaro I, López-Roca J M, Fernández-López J A. *Food Chemistry* (1993) **47**, 357–361.
3 'Determination of organic acids in wines: a review', Alonso E V, de Torres A G, Molina A R, Pavon J M C. *Quimica Analitica* (1998) **17**(4), 167–175.
4 'Capillary electrophoresis for evaluating orange juice authenticity: a study on Spanish oranges', Saavedra L, Ruperez F J, Barbas C. *Journal of Agricultural and Food Chemistry* (2001) **49**(1), 9–13.
5 'Simultaneous determination of 8 kinds of organic acids in formula feed by capillary electrophoresis', Ishikuro E, Hibino H, Soga T, Yanai H, Sawada H. *Journal of the Food Hygienic Society of Japan*. (2000) **41**(4), 261–267.
6 'Simultaneous determination of organic acids in wine samples by capillary electrophoresis and UV detection: Optimization with five different background electrolytes', Castineira A, Pena R M, Herrero C, Garcia-Martin S. *HRC-Journal of High Resolution Chromatography* (2000) **23**(11), 647–652.
7 'AOAC Official Method 920.69. Tartaric acid (total) in wines, titration method', *AOAC Official Method of Analysis* (2000) 28.1.34 p 9.
8 'AOAC Official Method 928.05. Tartaric acid in cheese, quantitative method', *AOAC Official Method of Analysis* (2000) 33.7.20 p 75.
9 'AOAC Official Method 910.03. Tartaric acid (total) in fruits and fruit products, bitartrate method', *AOAC Official Method of Analysis* (2000) 37.1.39 p 11.

13.5 Appendix: method procedure summaries

Fruit and vegetable juices – determination of tartaric acid in grape juices – method by high performance liquid chromatography [1]

Preparation of the test sample

Normally products should not be pre-treated. However, dilution may be necessary and their analysis by this method should be on a volumetric basis, results being expressed per litre of sample. The analysis of concentrated products may also be carried out on a volumetric basis, after dilution to a known relative density. In this case the relative density should be indicated. Based on a weighed sample and taking the dilution factor for analysis into account, the results may also be expressed per kilogram of product. In products with a high viscosity and/or a very high content of cells (for example pulp), a determination on the basis of a weighed test sample is the usual procedure.

Dilute grape juices at 1 to 20 volumes (concentrates 1 to 100) and directly use them for HPLC analysis after filtration through a 0.45 µm membrane filter. (When frozen samples are used, ensure that there is no sediment in the sample before dilution).

Preparation of calibration solutions

Prepare the calibration solutions in the range of 100 mg/L to 500 mg/L of tartaric acid using suitable solutions of the standard tartaric acid solution. Use the solutions as described in HPLC analysis.

HPLC analysis

Inject the calibration solutions and the samples in an HPLC system with the following conditions:

Eluent	0.005 mol/L sulphuric acid
Column	Separation column, ion-exclusion column made of sulphonated divinyl benzene–styrene copolymer in hydrogen form, typical particle size 10 µm, (300 mm × 7.8 mm) with a cation H^+ precolumn
Flow	e.g. 0.6 mL/min (to avoid a high pressure the flow should be increased slowly from 0.2 mL/min to 0.6 mL/min during equilibration)
Wavelength	210 nm
Injection volume	typically 15 µL
Running time	20 min
Retention time	approximately 10 min for tartaric acid
Temperature	40 °C

Table 13.1 Summary of methods for L-tartaric acid in foods

(a)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
HPLC	Grape juices	Dilute 1 to 20 volumes	Ion-exclusion	0.005 M H_2SO_4	UV at 210 nm	1
HPLC	Frozen musts and wine	Samples filtered through 0.45 µm filter	Polymeric ION 300 (300 mm × 7.8 mm) at 70 ºC	0.008 N H_2SO_4 at 0.5 mL/min	UV/vis	2
HPLC	Wines		Aminex A-25	0.9 M Na formate (pH 7.5)	Refractometric or UV at 254 nm	3
			Aminex A6 or A8 or Beckman M-72	H_2O–MeOH (4:1)	Refractometric	
			Microcrystalline cellulose powder	ethyl acetate–propanol–water (121:49:30)	Conductiometric or refractometric	
			LiChrosorb RP-18	H_2O–MeOH–0–05 M phosphoric acid (69:1:30)	Absorbance at 210 nm	

Table 13.1 cont'd

(b)

Method	Matrix	Sample preparation	Method conditions	Reference
Rebelein's colorimetric	Frozen musts and wine	Thawed sample acidified to pH 1 with 14 N HNO_3	5 mL added to 10 mL Solution I (100 mM silver nitrate in aq acetic acid 30 %) and 10 mL Solution II (85 mM ammonium metavanadate, 150 mM NaOH and 656 mM sodium acetate). Stirred for 15 mins at room temperature and filtered. Colour absorbance measured at 530 nm	2
Capillary electrophoresis (CE)	Orange juice	Dilution 1:1 with water and centrifugation or filtration	The separation was performed on a capillary electrophoresis P/ACE with UV detection at 200±10 nm. Injection was by pressure for 5 s (20 psi). The neutral capillary was polyacrilamide coated 57 cm long by 50 µm i.d. and operated at –14 kV potential. The electrolyte used was phosphate buffer 200 mM, pH 7.50	4
Capillary electrophoresis (CE)	Formula feeds	Extracted with water, filtered, diluted with 0.02 M NaOH and deproteinised by an ultrafiltration filter (30 kDa cut-off)	Capillary: fused silica 50 µm i.d. × 112.5 cm. Buffer; 20 mM 2,6-pyridinedicarboxylic acid, 0.5 mM *n*-hexadecyltrimethylammonium hydroxide (pH 5.7). Wavelength: signal 350 nm, reference 275 nm. Applied voltage –30 kV. Injection: pressure of 50 kPa for 6 s	5
Capillary electrophoresis (CE)	Wines	Dilution 1:40 with water and filtered through 0.45 µm filter	Capillary: fused silica 75 µm i.d. × 60 cm. Buffer; 3 mM phosphate and 0.5 mM myristyltrimethylammonium bromide (MTAB) at pH 6.5. Direct UV detection at 185 nm. Applied voltage 20 kV. Hydrodynamic mode injection for 30 s	6

Titration	Wines	Neutralise with 1 M NaOH, add tartaric acid, acetic acid and KCl. Add alcohol, stir until precipitate forms and refrigerate for 15 H	Decant onto filter paper in Buchner funnel. Wash precipitate 3 times with alcoholic KOH solution. Transfer precipitate and filter paper to beaker, wash Buchner with hot water, heat to boiling point and titrate hot solution with 0.1 M NaOH	7
Titration	Cheese	Add hot water to sample shaking vigorously then 2 % $Na_2C_2O_4$ soln. Shake, add 2 % HCl and KCl. Stand for 10 min, filter and transfer filtrate to vol flask. Neutralise with 1 M NaOH using phenolphthalein and add 5.2 mL in excess. Dilute to volume with water. Stand and filter	Add tartaric acid solution followed by CH_3COOH and alcohol to filtrate. Cool on ice bath. Stand overnight in refrigerator. Filter through glass fibre filter with KCl wash solution. Place glass fibre filter and precipitate in beaker and wash with water. Heat solution to boiling point and titrate while hot with 0.1 M NaOH, using phenolphthalein	8
Titration	Fruits and fruit products	Bitartrate method		9

Table 13.2 Summary of statistical parameters for L-tartaric acid in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
HPLC	Grape juice	Full collaborative trial	see Table 13.3	1
Capillary electrophoresis (CE)	Formula feeds	Precision of method established and applied to commercial feed ingredients (n=20)	Recovery test was conducted with 3 kinds of formula feeds spiked with tartaric acid at 0.1 %. The mean recovery values 90.2 % (77 %–106 %), Collaborative study of 3 labs on spiked samples mean recovery 90.2 % RSD_r = 7.7 % and RSD_R = 16.3 %	5
Capillary electrophoresis (CE)	Wines	Precision of method established and applied to commercial red wines (n=2)	Linear range 0–50 mg/L. Detection limit = 0.04 mg/L. Recovery of 92 % established on a wine containing 1752 mg/L spiked with 400 mg/L	6
Capillary electrophoresis (CE)	Orange juice	Precision of method established with Spanish Navelina orange juice (n=63) and applied to commercial samples (n=9)	This evaluation was conducted to establish ranges of acid concentrations and to compare them with those found in commercial juices. Citrate/isocitrate ratio mean 113 (RSD 10 %). Only one of 9 commercial juices presented values within the range for Navelina orange juice and 3 of them had measurable tartrate values, which is not a natural component of orange juice, showing mixtures with cheaper fruits	4
HPLC cf colorimetric	Frozen musts and wine	Methods applied to real samples	0.19–3.84 g tartaric acid/L, recovery 95–105 %. Data obtained from both methods agreed	2

Table 13.3 Performance characteristics for L-tartaric acid in grape juices[1]

Sample	White grape juice	White grape juice	Red grape juice	Red grape juice
No. of laboratories	8	10	9	9
Units	g/L	g/L	g/L	g/L
Mean value	4.19	2.55	3.77	2.15
S_r	0.0621	0.0513	0.0566	0.026
RSD_r	1.48	2.01	1.50	1.21
r	0.17	0.14	0.16	0.07
S_R	0.1148	0.1253	0.1363	0.0931
RSD_R	2.74	4.91	3.62	4.33
R	0.32	0.35	0.38	0.26

Identified in interlaboratory test conducted by International Fruit Union, Paris, France.

Key

Mean 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).

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).

14

E355–7, E359: Adipic acid and its salts

14.1 Introduction

The major food groups contributing to dietary intake of adipic acid and its salts are desserts especially jellies with a maximum permitted level of 10 000 mg/kg, expressed as adipic acid, being allowed in powders for home preparation of drinks. The ADI for adipic acid and its salts is 5 mg/kg body weight/day.

Adipic acid is a dibasic carboxylic acid that occurs naturally in beet juice. This acid is widely used in the food industry for buffering and neutralising purposes. Since it is hygroscopic, it is useful for preparing baking powder and soft drink powders.[1]

14.2 Methods of analysis

Many references were available for adipate esters in packaging materials, the specific migration of di-(2-ethylhexyl)adipate DEHA from PVC films, and the determination of adipic acid content of acetylated di-starch adipates (modified starch). There are several methods published for the determination of adipic acid in foodstuffs. These are summarised in Table 14.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 14.2. The majority of these methods are applicable to wine, candy, soft drinks and ice lollies, and are HPLC,[2,3] GC,[1] and capillary zone electrophoresis[4] methods. Methods published for the determination of adipic acid esters in food packaging materials, food simulants and acetylated cross-linked starches are GC[5–8] methods and these are also summarised in Table 14.1. Adipic acid is widely used in the food industry and so it is surprising to find that there are only a limited number of published methods for it. Further

methods need developing for the determination of adipic acid and its salts in desserts as this is the major food group contributing to dietary intake of adipic acid.

A method published in 1979 for the analysis of adipic acid in orange soft drinks[1] was carried out on a concentrated orange soft drink known and verified to be free of adipic acid, which was contaminated with 0.125 g adipic acid/kg in diluted solution (1+4). The prepared dilution was sent to 6 laboratories in Japan for analysis. The method consisted of extraction and derivatisation before analysing by gas–liquid chromatography with flame ionisation detection. The procedure for this method is given in Appendix 1 and the performance characteristics are given in Table 14.3.

An improved GC determination of adipate in starch was internationally collaboratively trialled.[4] The method is summarised in Appendix 2 and the performance characteristics are given in Table 14.4.

14.3 Recommendations

The improved GC determination of adipate in starch has been fully validated but there are few published methods for adipic acid in foods. Some of these methods were published before 1980 and required extraction with diethyl ether before derivatisation and GC analysis.[1] Therefore new methods need to be developed that can be applied to foodstuffs especially desserts.

14.4 References

1 'Gas-liquid chromatographic determination of adipic acid in crackling candy and soft drinks', Ito Y, Ogawa S, Iwaida M. *J. Assoc. Off. Anal. Chem.* (1979) **62**(4), 937–938.

2 'Determination of organic acids in foods by ion-exclusion chromatography', Fujimura K, Tsuchiya M. *Bunseki Kagaku* (1998) **37**(10), 549–553. [Japanese]

3 'Measurements of urinary adipic acid and suberic acid using high-performance liquid chromatography', Yoshioka K, Shimojo N, Nakanishi T, Naka K, Okuda K. *Journal of Chromatography B* (1994), **655**, 189–193.

4 'Determination of organic acids in food samples by capillary zone electrophoresis', Klampfl C W, Buchberger W, Haddad P R. *Journal of Chromatography A* (2000) **881**(1–2), 357–364.

5 'Specific migration testing with alternative fatty food simulants', Cooper I, Goodson A, OBrien A. *Food Additives and Contaminants* (1998) **15**, 72–78.

6 'Determination of adipic acid esters potentially migrated from plastic films for food-wrapping by gas chromatography and gas chromatography combined with mass spectrometry', Katase T, Kim Y. *Bunseki Kagaku* (1999) **48**(6), 649–655. [Japanese]

7 'Phthalate and adipate esters in Australian packaging materials', Balafas D, Shaw K J, Whitfield F B. *Food Chemistry* (1999) **65**, 279–287.

8 'Statistical evaluation of an international collaborative study concerning the improved gas chromatographic determination of adipate in starch', Brunt K, Mitchell G A. *Starch/Stärke* (1997) **49**, 296–301.

9 'Improved method for the determination of total adipyl content in acetylated adipyl cross-linked starches', Sanders P, Brunt, K. *Starch/Stärke* (1994) **46**, 255–259.

10 'Gas chromatographic determination of free adipic acid in adipyl cross-linked starches', Sanders P, Brunt, K. *Starch/Stärke* (1996) **48**, 448–452.

14.5 Appendix 1: method procedure summaries (analysis of orange drinks[1])

GC–FID method

Reagents and apparatus

a *Diazomethane test solution*: 2 g of *N*-nitrosomethylurea was weighed into a 50 mL Nessler tube and 20 mL ether was added and mixed well. The solution was cooled with ice-cold water and 205 mL 20 % NaOH was carefully added, gently mixed and the Nessler tube was capped loosely. It was allowed to stand with occasional gentle mixing until the bubbling of gas ceased. The upper layer was transferred to a flask and dehydrated with a small amount of solid NaOH.

b *Adipic acid standard solution*: 1000 µg/mL. 100 mg adipic acid was dissolved in 100 mL acetone.

c *FID gas chromatograph*: Yanco G-80 with strip chart recorder. Operating conditions: temperatures (ºC) – column 100, detector 200, injection port 200; nitrogen carrier gas flow *c.* 25 mL/min.

d *GLC column*: Glass, 200 cm × 3 mm, packed with 5 % DEGS (diethylene glycol succinate) + 1 % H_3PO_4 on 80–100 mesh Chromosorb W.

Extraction and derivative formation

10 g sample was weighed, 20 mL water was added and the pH was adjusted to >10 with 1 N NaOH. If the sample was insoluble in alkali it was mixed in a Waring blender until it was well suspended. 50 mL of ethyl ether was added and shaken vigorously. The layers were allowed to separate; the upper layer was discarded and the lower layer was re-extracted with 50 mL ether and shaken vigorously. The pH was adjusted to <2 by adding 2 mL 1 N H_2SO_4. The solution was saturated with NaCl and the adipic acid was extracted by vigorous shaking with 3 × 50 mL portions of ether. The water layer was discarded and the ether was dried with anhydrous Na_2SO_4. The ether layer was filtered and concentrated to <3 mL in a vacuum at 35 ºC and diluted to 3 mL with acetone and the adipic acid was methylated by adding 2 mL diazomethane reagent to make a final volume of 5 mL. An aliquot was injected into the gas chromatograph.

Preparation of calibration graph

Standard solution was pipetted into separate tubes in quantities of 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL. Each was diluted to 3 mL with acetone and 2 mL of diazomethane test solution was added to prepare solutions containing 0, 500, 1000, 1500, 2000, 2500 and 3000 µg adipic acid/5 mL, respectively. Aliquots were injected into chromatograph and peak height (mm) was plotted against µg adipic acid/mL.

14.6 Appendix 2: method procedure summaries: analysis of starch[8]

GC–FID method (total adipate[9])

Sample preparation

50 mg of the acetylated adipic cross-linked starch sample was weighed accurately in a glass reaction tube, and 1.5 mL distilled water and 1.0 mL aqueous solution containing 0.05 mg pimelic acid/mL were added. The reaction tube was shaken to disperse the sample and 2.5 mL of 4 M NaOH were added. Agitation of the reaction tube continued in order to dissolve the starch sample. The reaction tube was closed and the adipyl–starch ester bond was saponified by continually rotating the tube for at least 5 min. 1.0 mL of conc HCl was added and the mixture was homogenised. 5 mL ethyl acetate were added, the tube was closed and shaken vigorously for 1 min to extract the adipic acid and pimelic acid into the ethyl acetate.*After phase separation the (upper) ethyl acetate layer was transferred with a glass Pasteur pipette into a clean reaction tube. The ethyl acetate extraction of the aqueous solution was repeated 3 times and the ethyl acetate fractions were collected. These collected fractions were evaporated to dryness with a stream of nitrogen in a Pierce Reacti-Vap evaporator at a temperature of 30 ºC in a water bath.

Silylation

After that 0.3 mL of acetonitrile were added to the dry residue and the reaction tube was placed in an ultrasonic bath for several minutes to dissolve the residue. 0.3 mL of BSTFA/1 % TMCS solution was added and the mixture was homogenised again in the ultrasonic bath for several minutes. (BSTFA = bis-(trimethylsilyl)-trifluoroacetamide, TMCS = trimethylchlorosilane.) After reaction time of at least 30 min in a water bath at a temperature of 30 ºC, 0.3 µL of the reaction mixture was injected into the capillary GC.

GC conditions

Column	WCOT-fused silica CP-sil 5CB, 50 m × 0.32 mm, 0.12 µm film
Carrier gas	Helium (pressure 0.7 bar)
Injection	Cold on-column
Oven temperature	Programmed: 130 ºC for 1 min, 5 ºC/min to 190 ºC, 25 ºC/min to 290 ºC for 5 min then cooled to 130 ºC
Detector	FID at 300 ºC (hydrogen pressure 0.5 bar, air pressure 1.0 bar)

GC–FID method (free adipic acid[10])

Aqueous extraction
500 mg of the acetylated adipic cross-linked starch sample was weighed accurately (1.0 mg) in a glass reaction tube, and 4.0 mL distilled water and 1.0 mL aqueous solution containing 0.05 mg pimelic acid/mL were added. The reaction tube was closed and the adipic acid was extracted by continually rotating the tube for 16 h. The aqueous suspensions were centrifuged for 10 min at 2900 g. The starch-free aqueous solution was transferred to a clean reaction tube. 100 µL of 6 M HCl and 5 mL ethyl acetate were added, the tube was closed and shaken vigorously for 1 min to extract the adipic acid and pimelic acid into the ethyl acetate. Then as for total adipate (see p. 177) from*.

Methanol–acetic acid extraction
500 mg of the acetylated adipic cross-linked starch sample was weighed accurately (1.0 mg) in a glass reaction tube, and 4.0 mL methanol, 1.0 mL aqueous solution containing 0.05 mg pimelic acid/mL and 100 µL acetic acid were added. The reaction tube was closed and the adipic acid was extracted by continually rotating the tube for 16 h. The aqueous suspensions were centrifuged for 10 min at 2900 g. The starch-free supernatant was transferred to a clean reaction tube and evaporated to dryness with a stream of nitrogen in a Pierce Reacti-Vap evaporator at a temperature of 30 ºC in a water bath.

Silylation
As for total adipate (see p. 177).

GC conditions
As for total adipate (see p. 177).

Calibration
Four 50 mg (total adipate) or five 500 mg (free adipic acid) samples of waxy corn starch were weighed into reaction tubes. 1.0 mL aqueous or methanolic pimelic acid containing 0.05 mg pimelic acid/mL was added into each tube followed by the addition of 0.00, 0.25, 0.50, 0.75 and 1.00 mL aqueous adipic acid solution, containing 0.05 mg adipic acid/mL into the respective tubes. The volume was adjusted to 2.50 mL (total adipate) with distilled water and the procedure as described in the sample preparation section was carried out on 5.0 mL (free adipic acid) with the extraction solvent, and the extraction procedure was carried out as described above.

Table 14.1 Summary of methods for adipic acid in foods

(a)

Method	Matrix	Sample preparation	Method conditions	Detection	Reference
GC	Crackling candy and soft drinks	An alkaline solution of sample was extracted with ethyl ether. H_2SO_4 was added to water layer (pH<2). The acidified layer was saturated with NaCl and then extracted with ether. After drying the ether layer was concentrated	The adipic acid in the concentrate was methylated using the diazomethane method	FID	1
Capillary zone electrophoresis (CZE)	Wine		7.5 mM 4-aminobenzoic acid, 10.5 mM trihydroxymethyl-aminomethane, 0.1 mM tetra-decyltrimethylammonium bromide (pH 7.0) with LiOH	Indirect UV absorbance and direct conductivity simultaneously	4
GC	Food simulants	Olive oil simulant diluted with *n*-heptane. Other simulants not diluted	Di(2-ethylhexyl)adipate (DEHA) Retention time 8.6 min. Column: BPX5 0.25 µm film. Temperature programmed 100–360 ºC. Carrier gas: helium. Cold on column injector	FID at 370 ºC	5
GC–MS	Food wrapping		Adipic acid esters migrated into *n*-heptane	GC–MS	6

Table 14.1 cont'd

Method	Matrix	Sample preparation	Method conditions	Detection	Reference
GC–MS	Packaging materials	1 g of packaging material was Soxhlet extracted with chloroform–methanol (2:1), 50 mL for 6 h. Extracts transferred to 50 mL vol flask and made up to volume with chloroform–methanol. 1 mL transferred to vials for GC–MS analysis	Di(2-ethylhexyl)adipate (DEHA) Column: HP-5MS 0.25 μm film. Temperature programmed 70–280 ºC. Carrier gas: helium. Split injector 1:25, 2 μL injection	FID at 370 ºC	7
GC	Starch	Total adipate: sample saponified with alkali and extracted with ethyl acetate, evaporated and silylated. Free adipate: aqueous extraction, then extracted 4 × with ethyl acetate, evaporated and silylated	Column: CP-sil 5CB (50 m × 0.32 mm, 0.12 μm film). Temperature programmed 130–290 ºC. Carrier gas: helium. 0.3 μL injection.	FID at 300 ºC	8

(b)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
HPLC	Urine	Extracted with diethyl ether and converted into fluorescent derivatives with 9-anthryldiazomethane	TSK-Gel, octadecyl-silane RP-type	Acetonitrile–water (85:15, v/v), 1.5 mL/min, 20 μL injection	Fluorescence at em 412 nm, ex 365 nm	3
Ion-exclusion chromatography	Ice lollies		TSK-Gel, SCX (H^+ form) at 40 ºC	2 mM H_3PO_4 – 1.5 % acetonitrile at 1 mL/min	210 nm	2

Table 14.2 Summary of statistical parameters for adipic acid in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
GC	Starch	Validated by international collaborative study	Applying ISO 5725 (see Table 4) 1 Total adipate r=60 ppm R=120 ppm 2 Free adipate r=10.1 ppm R=55.4 ppm	8
GC	Crackling candy, soft drinks	Validated by 6 laboratories	Recovery 91.4–99.6 %. See Table 14.3 for collaborative trial details. Orange drink spiked with 125 ppm adipic acid and analysed by 6 laboratories	1
HPLC	Urine	Method performance established	Calibration graph linear 0–342 µmol/L Coefficient of variation (CV) in two specimens were 2.0 and 3.7 % at 34.2 and 137.0 µmol/L. Recovery 92–106 %	3
GC	Food simulants	Method parameters established	Detection limit 10 mg/kg in olive oil, replicates within ±10 % RSD and recoveries 91–101 %	5
GC-MS	Packaging materials	Method parameters established	Recovery 95–131 %, detection limit 0.01 µg/kg	7

Table 14.3 Performance characteristics for adipic acid in orange drink samples[1]

Sample	Orange drink					
Analyte	Adipic acid					
No. of laboratories	6					
Units	ppm					
Spike value	125					
Results	116.3	124.5	114.3	116.7	118.5	116.0
Mean value	117.7					
SD	3.586					
CV	3.05 %					

Table 14.4 Performance characteristics for adipic acid in acetylated adipyl cross-linked starches[8]

Total adipate					
Sample	557/664	455/006	556/161	041/551	545/949
Analyte	adipic acid	adipic acid	adipic acid	adipic acid	adipic acid
No. of laboratories	9	9	9	9	9
Units	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Mean value	430	386	659	90	405
S_r	22	21	36	5	7
S_R	50	32	62	11	40
Free adipic acid					
Sample	557/664	455/006	556/161	041/551	545/949
Analyte	adipic acid	adipic acid	adipic acid	adipic acid	adipic acid
No. of laboratories	9	9	9	9	9
Units	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Mean value	32.6	109.6	17.8	14.6	99.9
S_r	3.4	6.0	2.0	2.3	2.5
S_R	11.4	29.2	6.3	6.0	31.2

Key
Mean The observed mean. The mean obtained from the collaborative trial data.
S_r The standard deviation of the repeatability.
S_R The standard deviation of the reproducibility.

15

E405, E477: Propylene glycol (propan-1,2-diol)

15.1 Introduction

The major food groups containing propylene glycol are dairy-based desserts, fruit-based desserts, low-fat emulsions, cereal-based desserts, emulsified sauces and soft drink concentrates, with a maximum permitted level of 10 000 mg/kg for E405 (propan-1,2-diol alginate) being allowed in emulsified liqueur and 30 000 mg/kg for E477 (propan-1,2-diol esters of fatty acids) being allowed in whipped dessert toppings other than cream, expressed as propylene glycol. The ADI for propylene glycol is 25 mg/kg body weight/day. (Note: propylene glycol is permitted as a carrier for food additives but does not have an E number.)

Propylene glycol (PG) is used as a solvent of various food additives and is frequently added to noodles and some kinds of foods made from wheat flour to increase water coating ability.[1]

15.2 Methods of analysis

There are several methods published for the determination of propylene glycol in foodstuffs. These are summarised in Table 15.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 15.2. The methods are GC–MS–MS,[2] enzymatic digestion[1,3] and HPLC[4] methods and are applicable to anchovies, Japanese commercial foods (i.e. noodles, wantan, gyoza, smoked food, syrup and pudding), fish products, ice-cream and soft drinks.

The gas chromatographic–tandem mass spectrometric (GC–MS–MS) method[2]

was developed for confirming the identity of propylene glycol added to bait fish for preservation. The identity of propylene glycol in anchovy extracts was successfully confirmed using this method.

One enzymatic method[1] is based on the oxidation of PG by propylene glycol dehydrogenase (PGDH) from *Microcyclus eburneus*, which is accompanied by the reduction of NAD^+ to NADH. The use of the enzymatic reaction resulted in almost stoichiometric oxidation of PG and the method was found to be relatively free from interferences and was successfully used to establish the PG content of some commercial Japanese foods. The other enzymatic method[3] uses glycerol dehydrogenase and glycerol kinase. It is a simple and rapid enzymatic analysis for the determination of PG in commercial foods. This method can be applied to foods that contain glycerol because glycerol could be eliminated prior to enzymatic assay of PG through its conversion to glycerol-3-phosphate by incubation with glycerol kinase. Glycerol dehydrogenase showed no activity towards this product. Food ingredients and/or food additives tested (glucose, ethanol, sorbitol, propylene glycol monostearate and glycerol monostearate) did not interfere with this method.

The HPLC method[4] is sensitive for the determination of propylene glycol esters of fatty acids (PGEs) in foods. PGEs in various foods could be selectively detected without interference. Peaks of PGE having fatty acids of C16:0 and C18:0 could be well separated.

15.3 Recommendations

Although various methods have been developed for the analysis of propylene glycol in foods none of these have been validated by collaborative trial. They therefore need to be further developed and validated by collaborative trial.

15.4 References

1 'Enzymatic determination of propylene glycol in commercial foods', Hamano T, Mitsuhashi Y, Tanaka K, Matsuki Y, Nukina M, Oji Y, Okamoto S. *Agric. Biol. Chem.* (1984), **48**(10), 2517–2521.

2 'Confirmation of identities of propylene and ethylene glycols in anchovies by tandem mass spectrometry', Matusik J E, Eilers P P, Waldron E M, Conrad S M, Sphon J A. *Journal of AOAC International* (1993) **76**(6), 1344–1347.

3 'Enzymatic analysis of propylene glycol in foods by the use of glycerol dehydrogenase', Mitsuhashi Y, Hanano T, Tanaka K, Matsuki Y. *Journal of the Food Hygienic Society of Japan* (1985) **26**(3), 290–294. [Japanese]

4 'Determination of propylene glycol esters of fatty acids in oily foods by HPLC', Murakami C, Maruyama T, Niiya I. *Journal of the Food Hygienic Society of Japan.* (1997) **38**(2), 105–109. [Japanese]

Table 15.1 Summary of methods for propylene glycol in foods

Method	Matrix	Sample preparation	Method conditions	Detection	Reference
Enzymatic analysis	Commercial Japanese foods	Homogenised with deionised water and ultrafiltration before enzyme extraction	Filtrate (0.1 mL) + 1 mL 0.5 M $NaCO_3$ buffer (pH 9.5) + 0.1 mL 200 mM NAD^+ (pH 9.5). Reaction started with 0.1 mL of enzyme solution and incubated for 10 min at 37 ºC	Optical density was measured at 340 nm	1
GC–tandem mass spectrometry	Anchovies	Sample (5 g) homogenised with MeOH (5 mL), centrifuged. Supernatant filtered through 0.45 µm filter. Filtrate (2 mL) evaporated under N_2 to 0.2 mL	GC Conditions: column: Nukol fused silica capillary (0.32 mm i.d., 0.25 µm) temp prog 50 ºC for 1 min then 5 ºC/min to 150 ºC for 1 min. Injector temp 220 ºC. MS/MS: 70 eV, 1300 V, 5 keV, 0.35 mA, temp 100 ºC. Isobutane for PICI. Product ions generated with argon set to 1.8 mtorr energy set to –28 eV	MS/MS m/z 77, scanned from 20 to 81 daltons	2
Enzymatic analysis	Smoked dried squid, fish jelly, soft drink, noodle and other flour products, ice-cream	Sample homogenised with water and filtered. Aliquot of filtrate subjected to enzymatic assay of PG using commercial enzymes (glycerol dehydrogenase and glycerol kinase)			3
HPLC	Margarine, shortening, cake powder	PGEs extracted from foods and purified by silica gel column chromatography. PGEs derivatised with 3,5-dinitrobenzoyl chloride. Reaction products dissolved in tetrahydrofuran–acetonitrile (1:1) for HPLC	Inertsil 5C8 column. Acetonitrile–water (90:10) mobile phase	UV at 230 nm	4

Table 15.2 Summary of statistical parameters for propylene glycol in foods

Method	Matrix	Extent of validation	Statistical parameters		Reference
Enzymatic analysis	Commercial Japanese foods	Method parameters established	Recoveries	85–95 % for foods at 2 mg/g of PG 95–99 % for foods at 10 mg/g of PG	1
			Detection limit	2 µg	
HPLC	Margarine, shortening, cake powder	Method parameters established	Recoveries	>93 % for foods spiked with PGE at a level of 0.5 % of 1.0 %	4
			Detection limit	10 µg/g for total PGE	
Enzymatic analysis	Smoked dried squid, fish jelly, soft drink, noodle and other flour products, ice-cream	Method parameters established	Recoveries	>92 % for PG at 0.6–20 mg/g in several foods	3

16

E416: Karaya gum

16.1 Introduction

The major food groups contributing to dietary intake of karaya gum are desserts, emulsified sauces and snacks with the maximum permitted level of 10 000 mg/kg being allowed in emulsified sauces, egg-based liqueurs and nut coatings. The ADI for karaya gum is 12.5 mg/kg body weight/day.

Gum karaya is a dried exudate from deep incisions in the heartwood of large foliate trees of the *Sterculia* family, native to India and Pakistan. Harvesting of the gum is undertaken quickly after the incisions have been made. The colour is very variable, ranging from colourless to a deep pink-brown and the paler the gum, the better the quality. Chemically, it is a glycanorhamnogalactouran, with alternating backbone units of α-D-galactouronic acid linked at C_4 to α-L-rhamnose at the C_2 position. Substitution occurs on the hydroxyl groups by D-galactose and D-glucuronic acid. The gum is employed in the food and textile industries as a stabiliser and adhesive.[1] Among the naturally occurring gums, gum karaya (*Sterculia* spp.) is one of the most profusely used gums at present and ranks only second to gum arabic (*Acacia* spp.) in commercial importance as a food additive.[2]

16.2 Methods of analysis

The only references that could be found for karaya gum were for the analysis of the gum, and as in general[1–3] tabulated in Table 16.1, not for the analysis of karaya gum in foodstuffs.

16.3 Recommendations

There are no recent methods published for karaya gum and these need to be developed.

16.4 References

1 'FT-Raman spectroscopy of gums of technological significance', Edwards H G M, Falk M J, Sibley M G, AlvarezBenedi J, Rull F. *Spectrochimica Acta, Part A: Molecular and Biomolecular Spectroscopy* (1998) **54A**(7), 903–920.
2 'Physico-chemical analysis of gum kondagogu (*Cochlospermum gossypium*): a potential food additve', Janaki B, Sashidhar R B. *Food Chemistry* (1998) **61**(1–2), 231–236.
3 'Determination of pectin in the presence of food polysaccharides', Koseki M, Kitabatake N, Doi E, Yasuno T, Ogino S, Ito A, Endo F. *J. Food Sci*. (1986) **51**(5), 1329–1332.

Table 16.1 Summary of methods for karaya gum

Method	Matrix	Summary	Reference
FT–Raman spectroscopy	Gums	Characteristic bands can be used to identify the gum. Karaya gum; has a prominent feature at 1722 cm^{-1} characteristic of (C=O). Karaya gum contains up to 40 % uronic acids. Spectra for five coloured gum karaya are available and wave numbers and assignments for these gums are given. In an unidentified gum the presence of karaya was confirmed by medium intensity feature at 1720 cm^{-1}	1
Various		Chemical analysis of karaya gum i.e. proximates, cationic profile, physico-chemical properties, per cent sugar composition, compared with gum kondogogu	2
Colorimetric assay		A selective and specific method to assay pectin in mixtures of polysaccharides using pectinase was developed. The mixture was extracted with 99.5 %(v/v) ethanol to remove gum arabic and any other ethanol soluble saccharides and polysaccharides; pectin was then hydrolysed with pectinase. The hydrolysed pectin was recovered by solution in 80 % (v/v) ethanol and assayed by the *m*-hydroxy-biphenyl method. The assay was not affected by karaya gum	3

17

E432–6: Polysorbates

17.1 Introduction

The major food groups contributing to dietary intake of polysorbates are confectionery, ices, desserts, fine bakery wares, milk analogues, emulsified sauces, chewing gum, fat emulsions for baking and dietary supplements with the maximum permitted level of 10 000 mg/kg being allowed in fat emulsions for baking. The acceptable daily intake (ADI) for polysorbates is 10 mg/kg body weight.

Polysorbates are non-ionic emulsifying agents formed by copolymerising sorbitan anhydride and 20 moles of ethylene oxide. A fatty acid is esterified to one terminal hydroxyl group of the polyoxyethylene-oxide side chain. The type of the attached fatty acid molecule is reflected by the numbers in the names of polysorbates:[1]

E432	polyoxyethylene sorbitan monolaurate	(polysorbate 20)
E433	polyoxyethylene sorbitan monooleate	(polysorbate 80)
E434	polyoxyethylene sorbitan monopalmitate	(polysorbate 40)
E435	polyoxyethylene sorbitan monostearate	(polysorbate 60)
E436	polyoxyethylene sorbitan tristearate	(polysorbate 65)

17.2 Methods of analysis

There are several methods published for the determination of polysorbates in foodstuffs. The early methods developed for polysorbates were gravimetric[2,3] and later colorimetry with confirmation by TLC and gas chromatography.[4–6] More recently HPLC[7] methods have been developed. A summary of these methods is given in Table 17.1, together with the matrices for which the methods are applicable. If statistical parameters for them were available these have been

summarised in Table 17.2. There are no recent methods of analysis for polysorbates in foods but some are available for polysorbates in biological samples and in pharmaceutical preparations.

17.3 Recommendations

Gravimetric and colorimetric methods are available for the analysis of polysorbates in foods and an HPLC method has been developed for powdered soup. These methods need to be further developed for other foodstuffs and for all polysorbates. The majority of methods available for foodstuffs are for polysorbates 60 and 80.

17.4 References

1 'Quantitative determination of polysorbate 20 in nasal pharmaceutical preparations by high performance liquid chromatography', Oszi Z, Petho G. *Journal of Pharmaceutical and Biomedical Analysis* (1998) **18**(4–5), 715–720.
2 'AOAC Official Method 974.11 Polysorbate 60 in shortening, oils, dressing. Gravimetric Method. First Action 1974', *AOAC Official Methods of Analysis* (2000) 17 ed. 47.04.01. Chapter 47, p 37.
3 'The determination of polysorbate 60 in foods', Smullin C F, Wetterau F P. Olsanski V L. *J. Am Oil Chem Soc*. (1971) **48**, 18–20.
4 'Determination of polysorbates in foods by colorimetry with confirmation by infrared spectrometry, thin-layer chromatography and gas chromatography', Kato H, Nagai Y, Yamamoto K, Sakabe Y. *JAOAC*. (1989) **72**(1), 27–29.
5 'Detection and determination of polysorbate in powdered soup of instant noodles by colorimetry', Tonogai Y, Nakamura Y, Tsuji S, Ito Y. *Journal of the Food Hygienic Society of Japan* (1987) **28**(6), 427–435. [Japanese]
6 'Determination of polysorbate 60 in salad dressings by colorimetry and thin-layer chromatographic techniques', Daniels D H, Warner C R, Selim S. *JAOAC* (1982) **65**(1), 162–165.
7 'Determination method of polysorbates in powdered soup by HPLC', Takeda Y, Abe Y, Ishiwata H, Yamada T. *Journal of the Food Hygienic Society of Japan* (2001) **42**(2), 91–95. [Japanese]

Table 17.1 Summary of methods for polysorbates in foods

(a)

Method	Matrix	Principle of method	Reference
Gravimetric	Shortening, oils, dressings	Polysorbate 60 extracted with $CHCl_3$:ethanol (93+7). Extract saponified with alcohol KOH and acidified; fatty acids extracted with hexane. Aqueous polyol solution desalted by mixed-bed ion exchange and barium phosphomolybdate used to precipitate polyoxyethylated polyols as insoluble heteropoly acid complex. Precipitate dried to constant weight and polysorbate 60 content calculated using gravimetric factor obtained by analysing known amounts of polysorbate 60	2
Gravimetric	Foods	Extraction of polysorbate 60 from the sample with suitable solvent. Polysorbate polyol moiety recovered, desalted by ion exchange and measured gravimetrically as the barium phosphomolybdate complex. The polysorbate 60 content calculated using a gravimetric factor obtained by analysing known amounts of polysorbate 60	3
Colorimetric with confirmation by IR, TLC and GC	Processed foods	Polysorbates (PS) were extracted from sample with a mixture of dichloromethane and ethanol by using an Extrelut column. The extract is further purified by using a silica gel column. The PS extract is complexed with cobalt–thiocyanate reagent and was determined spectrophotometrically at 620 nm. PS identity was confirmed by IR spectrophotometry of PS extract and GC of fatty acids and TLC of polyoxyethylene-sorbitan residues after saponification	4
Colorimetric and TLC	Powdered soup of instant noodles	Purification of the extract from a sample was carried out by chromatography on a silica gel column and elution with dichloromethane–methanol (2:1) mixture after washing with ethyl acetate. Suitable TLC conditions of polysorbate were as follows: adsorbent, silica gel; developing solvent, dichloromethane–methanol–acetone–water (55:20:15:4) mixture; colour development, Dragendorff reagent. Dragendorff reagent was examined for colorimetry of polysorbate as well as cobalt–thiocyanate reagent	5
Colorimetric and TLC	Salad dressing	Sample partitioned between dichloromethane and water. The dichloromethane extract treated by silica gel chromatography. The isolated polysorbate 60 was complexed with ammonium cobaltothiocyanate and determined spectrophotometrically at 620 nm. Additional evidence is obtained from TLC analysis using the modified Dragendorff reagent for visualisation of spots	6

(b)

Method	Matrix	Sample preparation	Method conditions	Detection	Reference
HPLC	Powdered soup	Extracted with acetonitrile after rinsing with *n*-hexane to remove fats and oils. Extract cleaned up using Bond Elut silica gel cartridge (500 mg). Cartridge washed with ethyl acetate. Polysorbates eluted with acetonitrile–methanol (1:2). Eluate treated with cobalt–thiocyanate solution to form a blue complex with polysorbate	The complex was subjected to HPLC with a GPC column, using a mobile phase of acetonitrile–water (95:5)	620 nm	7

Table 17.2 Summary of statistical parameters for polysorbates in foods

Method	Matrix	Extent of validation	Statistical parameters					Reference
Gravimetric	Foods	Performance of method established and recovery checked with real samples	Gravimetric factor for PSB 60 determined 2.74 (2.71–2.78; SD, 0.026, n=10). Recovery of PSB 60 in foods established:					3
			Food	n	% PSB 60	Recovery		
			Baked bread	10	0.27, 0.45	89 % (87–90 %)		
			Raised doughnuts	10	0.282	91 % (88–93 %)		
			Baked cake	10	0.375	92 % (89–96 %)		
			Chocolate cake mix	10	0.292	99 % 96–101 %)		
			Dried tomato paste	5	0.43, 0.094	100 % (96–106 %)		
			Dried lemon juice	7	0.1, 0.5, 1.0	94 % (82–113 %)		
			Dressing for salad	4	0.27	106 % (104–108 %)		
			Shortening	7	0.6, 1.0, 2.0	101 % (95–108 %)		
			Potato flakes	10	0.09, 0.36	96 % (78–122 %)		
Colorimetric with confirmation by IR, TLC and GC	Processed foods	Performance of method established and recovery for screening test determined by spiking PS-free processed foods	Recovery of PS 80 from processed foods: Detection limit of TLC corresponds to 50 mg PS 80/kg.					4
			Foodstuffs	n	Added PS 80 mg	Recovery	CV	
			Chinese noodle soup	4	5	88.1 %	4.0 %	
			Chocolate	10	5	88.2 %	6.0 %	
			Mayonnaise	3	5	77.0 %	11.3 %	
			Butter	1	5	89.6 %		
			Margarine	2	5	94.0 %		
			Peanut butter	3	5	73.1 %	9.7 %	
			Pickled dill cucumber	1	5	94.6 %		
			Salad dressings	6	5	67.9 %	8.3 %	

Colorimetry and TLC	Salad dressings	Performance of method established with spiked PS-free samples and validated with commercial food products	Recoveries of spiked PS 60-free commercial salad dressing: Level / Av recovery / CV / n 0.1 % / 82 % / 3.7 % / 9 0.3 % / 85 % / 2.4 % / 9 1.0 % / 86 % / 1.3 % / 9 Polysorbate 60 in stable emulsion dressings: (Results in duplicate, corrected for recovery [84.4 %]) Dressing / PS 60, mg/g Creamy Italian-brand A / 2.6 Creamy Italian-brand B / 1.7 Russian / 0.9 Thousand Island / 1.0 Low calorie French / 2.3 Creamy cucumber / 1.7	6
Colorimetry and TLC	Powdered soup of instant noodles	Recovery determined on spiked sample and validated on commercial samples	Sample spiked at 200 ppm, recoveries ranged 94.5–97 % 100–345 ppm of polysorbate was found in imported samples. Range of calibration at 500 nm was 10–50 µg/mL of polysorbate	5
HPLC	Powdered soup	Recovery determined on spiked sample and validated on commercial samples	Recoveries of polysorbate 80 added to powdered soups were more than 75 %. Detection limit was 0.04 mg/g. No polysorbates were detected on 16 commercial samples analysed	7
Gravimetric	Shortening, oil, dressings	AOAC Official Method but no statistical data is available	Method applicable in range 0.1–1.0 % polysorbate 60	2

18

E442: Ammonium phosphatides

18.1 Introduction

The major food groups contributing to dietary intake of ammonium phosphatides are chocolate and cocoa products, imitation chocolate and cocoa mixes. A maximum permitted level of 10 000 mg/kg is allowed in cocoa and chocolate products and cocoa-based confectionery products. The ADI for ammonium phosphatides is 30 mg/kg body weight.

Ammonium phosphatides, or YN as they are called, are synthetic lecithins used in chocolate production in order to obtain similar properties to phospholipids, but without the typical soya bean odour and flavour.[1] The emulsifer YN (ammonium salts of phosphatidic acids) was developed many years ago as a substitute for natural lecithin, for use as an emulsifier in neutral-flavour chocolate. It reduces viscosity and yield value. YN can be used in high dosages, without a negative effect on the viscosity of the chocolate mass. There are four essential steps involved in the manufacture of ammonium phosphatides:

1 Glycerol and partially hydrogenated rape-seed oil are heated together under vacuum to produce a controlled mixture of mono-, di-, and triglycerides.
2 The remaining glycerol is removed by distillation.
3 The reaction product is treated with carefully selected phosphorous pentoxide to produce the phosphatidic acids.
4 Filtration concludes the process.

Ammonium phosphatides have been approved as additives to cocoa and chocolate products under EEC No 442. There is only a tentative specification available (FAO/WHO). The characteristic recommendation is:

Phosphorus content	3.0–3.5 %
Other insolubles	max 2.5 %
pH value	6.0–8.0

18.2 Methods of analysis

No references to the analysis of ammonium phosphatides in foodstuffs could be found. The only references traced for ammonium phosphatides were for its uses and production as an emulsifier. However, methods of analysis for total phosphorus,[2,3] phosphatidycholine[4] and lecithin[1] are available. A summary of these methods is given in Table 18.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 18.2.

A spectrophotometric method for the analysis of total phosphorus was applied to a number of food samples (potato flour, sausage, cold ham, infant formula powder, cheese and skimmed milk powder) in a collaborative study and found to be suitable.[2] A summary of the statistical parameters for this method is given in Table 18.3.

18.3 Recommendations

There are no specific methods available for ammonium phosphatides in foodstuffs. Since they are permitted at high levels in chocolate and cocoa products such methods need to be developed.

18.4 References

1 'Emulsifiers – lecithin and lecithin derivatives in chocolate', Bonekamp-Nasner A. *Confectionery Production* (1992) **58**, 66–68.

2 'Determination of total phosphorus in foods by colorimetric measurement of phosphorus as molybdenum blue after dry-ashing: NMKL Interlaboratory Study', Pulliainen T K, Wallin H C. *Journal of AOAC International* (1994) **77**(6), 1557–1561.

3 'Quantitative method for the survey of starch phosphate derivatives and starch phospholipids by ^{31}P nuclear magnetic resonance spectroscopy', Kasemsuwan T, Jane J-L. *Cereal Chemistry* (1996) **73**(6), 702–707.

4 'Analytical determination of phosphatidycholine: comparison of HPLC and enzymatic method', Boix-Montañés A, Permanyer-Fabregas J J. *Food Chemistry* (1997) **60**(4), 675–679.

Table 18.1 Summary of methods for phosphorus in foods

Method	Matrix	Sample preparation	Extraction	Detection	Reference
Spectro-photometric	Foods and food ingredients	The sample was dry-ashed in the presence of zinc oxide to remove organic material	The acid-soluble inorganic residue is used for a colour reaction based on the formation of a blue complex [$(MoO_2.4MoO_3)_2.H_3PO_4$] between phosphate and sodium molybdate in the presence of ascorbic acid as a reducing agent	The intensity of the blue colour is measured spectrophotometrically at 823 nm	2
^{31}P NMR spectroscopy	Starches	The sample was suspended in acetate buffer and digested with α-amylase. The hydrolysate was frozen (–85 ºC) and dried in a freeze drier	Freeze-dried material was resuspended in 90 % deuterated DMSO and heated in a boiling water bath for 10 min. The solution was mixed with 1.0 mL deuterium oxide and 0.5 mL nicotinamide adenine dinucleotide (NAD) The solution was adjusted to pH 8.0	^{31}P NMR spectra differentiates phospholipid, starch phosphate monoesters and inorganic phosphate content	3
HPLC cf enzymatic analysis (EA)	Cocoa powders and lecithin	Lecithin samples dissolved in chloroform (HPLC) or *t*-butanol–water (1:9) (EA) Cocoa samples blended with benzene–ethanol (1:1) + aq KCl. Shaken. Centrifuged. Aqueous layer re-extracted 3 × with solvent. Benzene extracts were rotary evaporated and dissolved in *t*-butanol–water (1:9) (EA) or trichloroethane (HPLC)	Enzyme Method: Hydrolysed by various enzymes to form (NAD) which is directly proportional to the original phosphatidycholine HPLC Method: Nucleosil 50–5 & 100–5 column, *n*-hexane–isopropanol–acetic acid buffer pH 4.2 (47:47:6) mobile phase, 25 µL injection	Enzyme method: Spectrophotometric by a shift absorbance at 340 nm HPLC method: UV at 206 nm	4

Table 18.2 Summary of statistical parameters for phosphorus in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
Spectro-photometric	Foods and food ingredients	Nordic collaborative study	RSD_r 1.1–5.4 % for 0.96–0.29 gP/100 g RSD_R 3.6–7.7 % for 0.96–0.23 gP/100 g (see Table 18.3)	2
^{31}P NMR spectroscopy	Starches	Method performance established and used to analyse sample (n=7)	Various starches were analysed in duplicate and results for total phosphorus were in agreement with those from a colorimetric chemical method. Method mean 0.031 g/100 g. Standard error 0.0006	3
HPLC cf enzymatic analysis (EA)	Cocoa powders and lecithin	Performance established for both methods and used to analyse a range of samples	Enzymatic Method: intra-assay precision of the whole analysis CV % ranged from 2.3–7.2 % for phosphatidylcholine. HPLC Method: Precision 3.7 %. Calibration linear. Detection limit 0.399 µg/mL, quantitation limit 0.599 µg/mL for phosphatidylcholine. The results given by the 2 methods are highly correlated, although there are systematic differences between them. This is attributed to analytical interference due to the nature of the samples	4

Key
RSD_r The relative standard deviation of the repeatability
RSD_R The relative standard deviation of the reproducibility

Table 18.3 Performance characteristics for total phosphorus in collaborative trial samples[2]

Sample	Potato flour	Sausage	Cold ham
Analyte	Total phosphorus	Total phosphorus	Total phosphorus
No. of laboratories	10	12	11
Units	g/100 g	g/100 g	g/100 g
Mean value	0.0761	0.166	0.233
S_r	0.0016	0.0079	0.0091
RSD_r	2.0 %	4.8 %	3.9 %
r	0.0044	0.022	0.026
S_R	0.0035	0.0115	0.018
RSD_R	4.7 %	6.9 %	7.7 %
R	0.0099	0.032	0.050
Ho_R	0.79	1.3	1.6
Sample	Infant formula	Cheese	Skimmed milk
Analyte	Total phosphorus	Total phosphorus	Total phosphorus
No. of laboratories	11	10	12
Units	g/100 g	g/100 g	g/100 g
Mean value	0.285	0.641	0.960
S_r	0.015	0.0096	0.010
RSD_r	5.4 %	1.5 %	1.1 %
r	0.043	0.027	0.029
S_R	0.017	0.027	0.034
RSD_R	6.1 %	4.1 %	3.6 %
R	0.049	0.074	0.095
Ho_R	1.3	0.97	0.88

Key

Mean 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).

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 the reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

19

E444: Sucrose acetate isobutyrate

19.1 Introduction

The major food groups contributing to dietary intake of sucrose acetate isobutyrate are cloudy soft drinks with the maximum permitted level of 300 mg/kg being allowed in non-alcoholic flavoured cloudy drinks. The ADI for sucrose acetate isobutyrate is 10 mg/kg body weight/day.

Sucrose acetate isobutyrate (SAIB), a mixture of esters of sucrose, with a composition approximating the name sucrose diacetate hexaisobutyrate, has been used for over 30 years in many countries as a 'weighting' or 'density-adjusting' agent in non-alcoholic carbonated and non-carbonated beverages.[1]

19.2 Methods of analysis

The only references that could be found for sucrose acetate isobutyrate were for the chemical and physical properties and for toxicological information with uses as an additive and recommended acceptable daily intakes of this compound. No references could be found for its analysis in foodstuffs since 1980; however, a dated GC method with diethyl ether extraction of soft drinks was published in 1972[2] and 1973[3], and now recently a GC method with solid phase extraction of food additive premixes has been published in 2001.[4] A summary of the method is given in the Appendix and Table 19.1, together with the matrices for which the method is applicable. Statistical parameters for this method are summarised in Table 19.2.

19.3 Recommendations

The recent method published for sucrose acetate isobutyrate[4] needs to be validated by collaborative trial.

19.4 References

1 'Sucrose acetate isobutyrate (SAIB): Historical aspects of its use in beverages and a review of toxicity studies prior to 1988', Reynolds R C, Chappel C I. *Food and Chemical Toxicology* (1998), **36**(2), 81–93.
2 'Gas–liquid chromatographic estimation of sucrose diacetate hexaisobutyrate in soft drinks', Conacher H B S, Chadha R K. *Journal of the AOAC* (1972) **55**(3), 511–513.
3 'Determination of sucrose diacetate hexaisobutyrate in soft drinks by gas–liquid chromatographic analysis of isobutyric and acetic acid components as decyl esters', Conacher H B S, Chadha R K, Iyengar J R. *Journal of the AOAC* (1973) **56**(5), 1264–1266.
4 'Determination of sucrose esters of fatty acids in food additive premixes by gas chromatography and confirmation of identity by gas chromatography/mass spectrometry', Uematsu Y, Hirata K, Suzuki K, Iida K, Kan T, Saito K. *Journal of AOAC International* (2001) **84**(2), 498–506.

19.5 Appendix: method procedure summary

Analysis of food additive premixes[4]

Sample preparation

Method 1 – An SPE RP-select B column was washed with 10 mL methanol and 10 mL water successively before sample solution was transferred to the column. A 1.0 g portion of sample was dissolved in 5–10 mL of water–methanol (1:1). A 1 mL aliquot of the sample solution (<2 mg total SAIB) was transferred to the column and the column was washed with 10 mL water–methanol (1:1). SAIB was eluted with 10 mL methanol–THF (1:1). The fraction was evaporated to dryness under reduced pressure and acetylated with 0.5 mL pyridine and 0.5 mL acetic anhydride for 30 min at 35 ºC. After evaporation of the reagents under a nitrogen stream, the residue was dissolved in 2 mL ethyl acetate. A 1 μL aliquot of the ethyl acetate solution was injected into the gas chromatograph.

Method 2 – The solid sample was ground to a powder. A 1.0 g portion of sample (powder, liquid or cream) was extracted twice with 50 mL THF. The THF phases were filtered and concentrated to 20 mL under reduced pressure to prepare a sample solution for column chromatography. A silica gel column was washed with 10 mL diethyl ether–ethyl acetate (3:7) before the sample solution was transferred to the column. An aliquot of the sample solution (<2 mg SAIB) was evaporated to dryness with a stream of nitrogen. The residue was transferred to the column with a small amount of diethyl ether–ethyl acetate (3:7). SAIB was eluted with 10 mL diethyl ether–ethyl acetate (3:7). The SAIB fraction was evaporated to dryness under reduced pressure, and the residue was dissolved in 2 mL ethyl acetate. A 1 μL aliquot of the ethyl acetate solution was injected into the gas chromatograph.

Method 1 was applied to samples that were soluble in water–methanol (1:1) and contained diglycerides (DG). Method 2 was applied to samples that were not soluble in water–methanol (1:1) or to samples that contained DG.

GC and GC/MS analyses

GC conditions

Column	BPX-5 {SGE International} (0.53 mm × 15 m, liquid phase thickness, 1 µm)
Oven temperature	150 ºC for 2 min; 150–320 ºC at 10 ºC/min; 320 to 350 ºC at 5 ºC/min; hold at 350 ºC for 20 min
Head pressure	70 kPa
Injection	Splitless
Injection temperature	340 ºC
Detection	Flame ionisation detector
Detector temperature	360 ºC

GC/MS conditions

Column	BPX-5 {SGE International} (0.25 mm × 15 m, liquid phase thickness, 0.25 µm)
Oven temperature	150 ºC for 2 min; 150–350 ºC at 20 ºC/min; hold at 350 ºC for 8 min
Injection	Splitless
Injection temperature	340 ºC
Ion source temperature	210 ºC
Interface temperature	300 ºC
Scanned masses	40 to 700 amu

The uncoated capillary tube (0.2 mm × 0.5 m, deactivated) was connected at the end of the separation column in series as a transfer line to the ion source.

Determination of SAIB

The level of SAIB in the sample was calculated by using the calibration curve obtained with standard SAIB.

Table 19.1 Summary of methods for sucrose acetate isobutyrate in foods

Method	Matrix	Sample preparation/extraction	Method conditions	Detection	Reference
GC	Food additive premixes	SAIB fractions were prepared by column chromatography with either C8 or silica gel solid-phase extraction column	Splitless injection. BPX-5 column. FID at 360 ºC.Oven temperature programmed 150–350 ºC	GC with GC/MS for confirmation	4

Table 19.2 Summary of statistical parameters for sucrose acetate isobutyrate in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
GC	Food additive premixes	Requires further validation	Detection limit 0.01 % Recoveries in: annatto extract spiked (10 mg SAIB in 1 g) 99±4.8 % (n=4) vitamin-enriched rice spiked (10 mg SAIB in 1 g) 93±5.9 % (n=4)	4

20

E472e: Mono/diacetyl tartaric acid esters of mono/diglycerides of fatty acids

20.1 Introduction

The major food groups contributing to dietary intake of mono/diacetyl tartaric acid esters of mono/diglycerides of fatty acids (emulsifiers) are desserts, pizza, meat spreads, cakes, ice-cream, bread and poultry with no maximum level of use set. It is used in accordance with good manufacturing practice to achieve the desired technological effect. The acceptable daily intake (ADI) for mono/diacetyl tartaric acid esters of mono/diglycerides of fatty acids is 50 mg/kg body weight.

20.2 Methods of analysis

Very few methods are published for the determination of mono/diacetyl tartaric acid esters of mono/diglycerides of fatty acids in foodstuffs. The most recent publications found were those dating from the 1980s and early 1990s. An HPLC method for diacetyl tartaric acid esters in flour,[1] a GC method for diacetyl tartaric acid ester of diglyceride in coffee cream powders[2] and an HPLC method for the analysis of the emulsifiers, sodium stearoyl lactylate (E481), calcium stearoyl lactylate (E482) and mono/diacetyl tartaric acid esters of mono/diglycerides of fatty acids (E472e).[3] These were for the substances themselves and not for their analysis in foods. A summary of these methods is given in Table 20.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 20.2.

20.3 Recommendations

There are no recent publications for methods of analysis for mono/diacetyl tartaric acid esters of mono/diglycerides of fatty acids. This is therefore an area that requires method development ensuring that the methods are applicable to desserts, pizza, meat spreads, cakes, ice-cream, bread and poultry.

20.4 References

1 'Méthode de detection par chromatographie liquide haute pression de l'acide tartrique constitutive des émulsifiants E 472e et utilisés comme additives dans les farines', Wirsta P, Corbel M. *Industries des Céréales* (1994) **86**, 46–51. [French]

2 'Gas–liquid chromatographic detection and determination of diacetyl tartaric acid esters of diglyceride in dairy and nondairy coffee cream powders', Inoue T, Iwaida M, Ito Y, Tonogai Y. *J. Assoc. Off. Anal. Chem*. (1981) **61**(2), 276–279.

3 'Analytical and structural study of some foods emulsifiers by high-performance liquid chromatography and off-line mass spectrometry', Sudraud G, Coustard J M, Retho C, Caude M, Rosset R, Hagemann R, Guadin D, Virelizier H. *Journal of Chromatogaphy* (1981) **204**, 397–406.

Table 20.1 Summary of methods for mono/diacetyl tartaric acid esters of mono/diglycerides of fatty acids in foods

Method	Matrix	Sample preparation	Method conditions	Detection	Reference
IP-HPLC	Flour	Extraction with ethyl acetate, saponification and acidification of the extract followed by direct injection of a dilute aliquot	Nucleosil C18 column at 30 ºC with acetonitrile:(TBA–H_2PO_4) 40 mM, pH 6.5 (15:85) mobile phase at 1 mL/min and 10 µL injection	UV at 200 nm	1
GC	Coffee cream powder	Extracted under acidic conditions with ethyl acetate, saponified, acidified. Free fatty acids removed with ether and reaction mixture was adsorbed on anion exchange column. Tartaric acid eluted with 2 N HCl–acetone(1:1). Aliquot of TMS derivative of the eluate was injected	Glass column 150 cm × 3 mm packed with 1.5 % silicone SE-30 on 80–100 mesh Chromosorb G at 165 ºC, 5 µL injection at 250 ºC, nitrogen flow 20 mL/min	FID at 250 ºC	2
HPLC–MS	Emulsifiers	Complex mixtures separated by semi-preparative adsorption liquid chromatography using 4 separate isocratic runs using mobile phases of decreasing elution strength	The different products of the more polar compounds were identified by off-line mass spectrometry of the trimethylsilyl derivatives and the medium polar and apolar compounds were identified by their mass spectra as determined by GC–MS	Mass spectrometry	3

Table 20.2 Summary of statistical parameters for mono/diacetyl tartaric acid esters of mono/diglycerides of fatty acids in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
GC	Coffee cream powder	Method used for coffee cream powder	Recoveries of diacetyl tartaric acid esters of diglyceride (DTDG) added at 50, 200, 2000 ppm levels to a coffee cream powder were 85.6–99.5 %.	2

21

E476: Polyglycerol esters of polycondensed fatty acids of castor oil

21.1 Introduction

The major food groups contributing to dietary intake of polyglycerol esters of polycondensed fatty acids of castor oil are yellow spreads, low fat emulsions, imitation chocolate, cocoa and its products with the maximum permitted level of 5 000 mg/kg being allowed in cocoa-based confectionery, including chocolate. The ADI for polyglycerol esters of polycondensed fatty acids of castor oil is 7.5 mg/kg body weight/day.

Polyglycerol polyricinoleate (PGPR) is prepared by the esterification of condensed castor oil fatty acids with polyglycerol. This substance is a strong water-in-oil emulsion that is used as a greasing agent for baking tins and as an emulsifier with lecithin in chocolate couverture and block chocolate.[1]

21.2 Methods of analysis

References could only be found for polyglycerol polyricinoleate for the manufacture, chemistry, uses of this compound and its analysis in emulsifiers[2–4] (tabulated in Table 21.1) and not for its analysis in foodstuffs.

21.3 Recommendations

There are no recent methods published for polyglycerol esters of polycondensed fatty acids of castor oil, so these methods need to be developed.

21.4 References

1 'Overview of the preparation, use and biological studies on polyglycerol polyricinoleate (PGPR)', Wilson R, Van Schie B J, Howes D. *Food Chemical Toxicology* (1998) **36**, 711–718.
2 'Analyses of polyglycerol esters of fatty acids using high performance liquid chromatography', Garti N, Aserin A. *J. Liq. Chromatogr.* (1981) **4**(7), 1173–1194.
3 'Analysis of polyglycerols by high performance liquid chromatography', Kumar T N, Sastry Y S R, Lakshminarayana G. *J. Chromatography* (1984) **298**(2), 360–365.
4 'Non-aqueous reversed-phase high-performance liquid chromatography of synthetic triacylglycerols and diacylglycerols', Lin J-T, Woodruff C L, McKeon T A. *Journal of Chromatography A* (1997) **782**, 41–48.

Table 21.1 Summary of methods for polyglycerol polyricinoleate in foods

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
HPLC	Emulsifiers i.e. polyglycerol esters of fatty acids	Samples dissolved in isopropanol (up to 15 % w/w)	LiChrosorb Diol	Gradient elution with *n*-hexane and isopropanol at 1 mL/min, 10 µL injection	UV at 220 nm	2
HPLC	Mixtures of polyglycerols	Samples dissolved in acetonitrile–water (30:70)	Carbohydrate analysis	Acetonitrile–water (83:17) at 1.5 mL/min	Refractometric	3
HPLC	Synthetic mixtures of triacylglycerols and diacylglycerols	Diacylglycerols and unsaturated triacylglycerol standards dissolved in isopropanol and saturated triacylglycerol standards dissolved in dichloromethane	Ultrasphere C18	Linear gradient elution from 100 % methanol to 100 % isopropanol	UV at 205 mn	4

22

E481–2: Stearoyl lactylates

22.1 Introduction

The major food groups contributing to dietary intake of stearoyl lactylates are fruit-based desserts, dairy-based drinks, infant formulae and weaning foods with the maximum permitted level of 10 000 mg/kg being allowed in fat emulsions. The acceptable daily intake (ADI) for stearoyl lactylates is 20 mg/kg body weight.[1]

Sodium stearoyl-2-lactylate, a dough conditioner and emulsifier, is the reaction product of two naturally occurring food components, stearic acid and lactic acid, neutralised to the sodium salt.[1] The commercial product is a mixture of sodium salts of a homologous series of stearoyl lactylic acids.

22.2 Methods of analysis

Very few methods are published for the determination of stearoyl lactylates in foodstuffs. No recent publications could be found; the latest were publications dated in the 1980s. A GC method for determining sodium stearoyl-2-lactylate (SSL) in baked wheaten products[2] and an HPLC method for the analysis of the emulsifiers, sodium stearoyl lactylate (E481) and calcium stearoyl lactylate (E482).[3] These were for the substances themselves and not for their analysis in foods. A summary of these methods is given in Table 22.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 22.2.

22.3 Recommendations

There are no publications for methods of analysis for stearoyl lactylates. This is therefore an area that requires method development and it should be ensured that the methods are applicable to desserts, dairy-based drinks, infant formulae and weaning foods.

22.4 References

1 'Estimation of the distribution of the maximum theoretical intake for ten additives in France', Verger P, Chambolle M, Babayou P, Le Breton S, Volatier J L. *Food Additives and Contaminants* (1998) **15**(7), 759–766.

2 'The determination of sodium stearoyl-2-lactylate in baked wheaten products', Kokot M L, March E R. *8th biennial congress, SAFOST conference 1985 Pretoria (South African Assoc for Food Science and Tech, Pretoria CSIR)* (1985) 206–214.

3 'Analytical and structural study of some food emulsifiers by high-performance liquid chromatography and off-line mass spectrometry', Sudraud G, Coustard J M, Retho C, M Caude, R Rosset, R Hagemann, D Guadin, H Virelizier. *Journal of Chromatography*. (1981) **204**, 397–406.

Table 22.1 Summary of methods for stearoyl lactylates in foods

Method	Matrix	Sample preparation	Method conditions	Detection	Reference
GC	Bread	Extraction using bacterial α-amylase, triphosphate buffer and chloroform–methanol. Extract was methylated with boron trifluoride–methanol complex and taken up in hexane	SE30 capillary column 50 m × 0.3 mm. Isothermal at 280 ºC with He carrier gas. 3 µL injection with injector and detector temperatures being 290 ºC	FID	2
HPLC and MS	Emulsifiers	Added to 1 M HCl and extracted with diethyl ether. Organic phase washed with water then with saturated NaCl solution	Adsorption chromatography provided good resolution according to lactoyl group number. Reverse-phase chromatography for C-number fatty acids	Identification by off-line high resolution mass spectrometry	3

Table 22.2 Summary of statistical parameters for stearoyl lactylates in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
GC	Bread	Method used on bread containing varying levels of SSL and fat	Correlation coefficient = 0.91	2

23

E483: Stearyl tartrate

23.1 Introduction

The major food groups contributing to dietary intake of stearyl tartrate are fruit-based desserts, cakes, cookies and pies, and other fine bakeryware with the maximum permitted level of 5 000 mg/kg being allowed in desserts. The acceptable daily intake (ADI) for stearyl tartrate is 20 mg/kg body weight.

23.2 Methods of analysis

There are no methods published for the determination of stearyl tartrates in foodstuffs.

23.3 Recommendations

Analytical methods need to be developed for the determination of stearyl tartrate in foodstuffs.

24

E491–2, E493–4, E495: Sorbitan esters

24.1 Introduction

The major food groups contributing to dietary intake of sorbitan esters are fruit-based desserts, dairy-based desserts, cereal-based desserts, other fine bakery ware, cakes, cookies and pies, emulsified sauces with the maximum permitted level of 10 000 mg/kg being allowed in fat emulsions and fine bakery wares. The acceptable daily intake (ADI) for sorbitan esters (1) E491–2 is 2.5 mg/kg body weight and for sorbitan esters (2) E493 is 5 mg/kg body weight.

24.2 Methods of analysis

There are several methods published for the determination of sorbitan esters in foodstuffs. These are mainly HPLC[1] or GC[2–3] and more recently supercritical fluid chromatography (SFC)[4] methods have been developed and a summary of these is given in Table 24.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 24.2. Methods are available for the determination of sorbitan tristearate in vegetable oils and fats[1] and for Span-20 (sorbitan monolaurate) in human plasma.[2]

24.3 Recommendations

There are only limited publications for sorbitan esters in foodstuffs, therefore method development is required for the specific foodstuffs in which these additives are permitted.

24.4 References

1 'Determination of sorbitan tristearate in vegetable oils and fats', Thyssen K, Andersen K S. *Journal of the American Oil Chemists Society* (1998) **75**(12), 1855–1860.
2 'GLC-determination of Span-20 (sorbitan monolaurate) in human plasma', Giovanetto S H. *Analytical Letters Part B-Clinical and Biomedical Analysis* (1983) **16**(12), 867–876.
3 'A determination of emulsifiers in various foods', Yomota C, Toyoda M, Ito Y. *Journal of the Food Hygienic Society of Japan* (1986) **27**, 37–43. [Japanese]
4 'Separation of T-MAZ ethoxylated sorbitan fatty acid esters by supercritical fluid chromatography', Ye M Y, Hill K D, Walkup R G. *Journal of Chromatography A* (1994) **662**, 323–327.

Table 24.1 Summary of methods for sorbitan esters in foods

Method	Matrix	Sample preparation	Extraction	Detection	Reference
HPLC	Vegetable oils and fats	Sample melted in an electric oven at 60±5 ºC	SPE using a silica cartridge and hydrolysis of sorbitan tristearate (STS) [E492] to sorbitol. Separated on HPLC column: Shodex SUGAR SC1011 at 50 ºC with water–acetonitrile (985:15) mobile phase at 0.8 mL/min and 50 µL injection	RI at 40 ºC	1
GC	Human plasma	To samples (1.0 mL) containing Span 20 (sorbitan monolaurate) [E493] ethanol (100 µL), internal standard (pentadecylic acid) (100 µL) and diethyl ether (6.5 mL) were added	Extracted into diethyl ether and saponified with methanolic KOH. Fatty acids (lauric and myristic acids) were acidified, extracted with diethyl ether, methylated using ethereal dizomethane. GC Column: 6′ × 0.125″ glass column packed with 3 % OV-17 on 80/100 mesh Chromosorb W. Isothermal at 205 ºC, 1 µL injection	FID at 250 ºC	2
GC	Ice-cream, margarine, soy milk, pudding and cake powder	Monofatty acids of sorbitan were extracted from foods with tetrahydrofuran	Extract purified by silica gel column chromatography. Polar substances were washed out with ether–chloroform (1:99), then isosorbide monoesters were eluted with methanol–chloroform (5:95) and sorbitan monoesters were eluted with methanol. These separated emulsifiers were converted to their trimethyl silyl ether derivatives and determined by GC		3
SFC	T-MAZ ethoxylated sorbitan fatty acid esters		SFC separation utilises a density programming technique and a 50 µm i.d. SB-biphenyl-30 capillary column with 0.25 µm film thickness	FID at 390 ºC	4

Table 24.2 Summary of statistical parameters for sorbitan esters in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
HPLC	Vegetable oils and fats	Method parameters established	Spiked recovery of STS = 97 %, SD=5.7, n=7 Recovery of STS from commercial products = 109 %, SD=11.2, n=3 Linearity over range 8–50 g STS/kg fat, r = 0.9961 S_r = 0.19 %, r = 0.53 %, S_R = 0.18 %, R = 0.51 % LOD = 0.4 %, LOQ = 1.4 % of STS	1
GC	Human plasma	Basic method parameters were established	Correlation coefficient r = 0.999 for lauric acid and 0.998 for myristic acid from 3 standard curves. Coefficient of variation about an individual point was <10 %	2
GC	Ice-cream, margarine, soy milk, pudding and cake powder	Basic method parameters were established	Recoveries from foods spiked at 0.1 % were more than 90 % Detection limit was 50 ppm	3

25

E520–3, E541, E554–9, E573: Aluminium

25.1 Introduction

The major food groups contributing to dietary intake of aluminium are such items as various processed cheeses and dried powdered foodstuffs. The maximum permitted levels of 10 g/kg for E554–9 are allowed in sliced cheese, dried powdered foods, salt substitutes, 200 mg/kg for E520–3 in glacé fruit and 32 mg/kg in dried and liquid egg. The acceptable daily intake (ADI) for aluminium (from all sources) is 1 mg/kg body weight.

The permitted food additives containing aluminium are E520–3 aluminium sulphates, E541 sodium aluminium phosphate and E554–9 aluminium silicates. It should be noted that the permitted levels for E520–3 and E541 are expressed as aluminium and levels for E551 and E554–9 are expressed as the relevant aluminium salts, which contain between 2 % Al (E556) and 21 % Al (E559).

25.2 Methods of analysis

Aluminium is the most abundant metal in the earth's crust. It has a high affinity for oxygen and therefore it is only found in combination with other elements such as silicon, oxygen and phosphorus, i.e. as aluminium oxides, silicates and phosphates and their combinations. Although aluminium is abundant in our environment, foods, animal and plant tissues contain only trace amounts of the element because of the insolubility of its compounds. Levels in foods generally range from around 0.1 mg/kg to 100 mg/kg.[1]

There are several methods published for the determination of aluminium in foodstuffs. These methods require a digestion stage to decompose the sample and measurement by atomic absorption spectrometry,[1–10] (graphite furnace (GFAAS),

flame (FAAS), electrothermal (ETAAS)), inductively coupled plasma (ICP), atomic emission spectrometry (AES)[1,11] or spectrophotometry.[9,10] A summary of these methods is given in Table 25.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 25.2.

An IUPAC check sample survey of analytical performance for aluminium determination of foods matrices was carried out.[1] Twenty-four laboratories participated in the survey using their own method for the determination of aluminium. The performance characteristics for milk powder are given in Table 25.3 with a summary of key steps of procedures used for the survey given in Table 25.4.

25.3 Recommendations

Various methods have been developed for aluminium in foods. What method is used depends on the matrix, the detection limit required and the analytical instrument available for measurement. These methods need to be adapted for use with all relevant foodstuffs where aluminium is permitted.

25.4 References

1 'Aluminium determination in food matrices IUPAC check sample survey of analytical performance', Vaessen H A M G, van de Kamp C G, Szteke B. *Z Lebensmittel-untersuchung Forschung* (1992) **194**(5), 456–460.
2 'Direct analysis of coffee and tea for aluminium determination by electrothermal atomic absorption spectrometry', Magalhes C E C, Lima E C, Krug F J, Arruda M A Z. *Mikrochimica Acta* (1999) **132**, 95–100.
3 'Aluminum levels in Canadian infant formulae and estimation of aluminium intakes from formulae by infants 0–3 months old', Dabeka R W, McKenzie A D. *Food Additives and Contaminants* (1990) **7**(2), 275–282.
4 'Aluminum in wine – its measurement and identification of major sources', McKinnon A J, Cattrall R W, Scollary G R. *American J Enology & Viticulture*. (1992) **43**(2), 166–170.
5 'Determination of aluminium in the edible parts of fish by GFAAS after pre-treatment with microwave activated oxygen plasma', Ranau R, Oehlenschlager J, Steinhart H. *Fresenius J Anal Chem* (1999) **364**, 599–604.
6 'Determination of trace levels of aluminium in biological materials (fish) by graphite-furnace AAS', Ezoe Y, Takatsu A, Kuroiwa T, Eyama S, Uchiomi T. *Bunseki Kagaku* (1999) **48**(11), 1013–1018. [Japanese]
7 'Improved determination of aluminium in port wine by electrothermal atomic absorption spectrometry using potassium dichromate chemical modification and end-capped graphite tubes', Almeida A A, Cardoso M I, Lima J L F C. *Journal of Analytical Atomic Spectrometry* (1997) **12**(8), 837–840.
8 'Determination of aluminium and chromium in slurried baby food samples by electrothermal atomic absorption spectrometry', Vinas P, Pardo-Martinez M, Hernandez-Cordoba M. *Journal of AOAC International* (2001) **84**(4), 1187–1193.
9 'Occurrence of aluminium in some foodstuffs', Nabrzyski M, Gajewska R, Czuprynska-

Rzepko A, Sandak-Bosak K. *Roczniki Panstwowego Zakladu Higieny* (1994) **45**(3), 181–190. [Polish]

10 'Aluminium determination in foods by using spectrophotometric oxine and flame AAS methods', Nabrzyski M, Gajewska R. *Nahrung-Food* (1998) **42**(2), 109–111.

11 'Microwave Digestion with HNO_3–H_2O_2–HF for the determination of total aluminum in seafood and meat by inductively coupled plasma atomic emission spectrometry', Sun D, Waters J K, Mawhinney T P. *J. Agric. Food Chem* (1997) **45**, 2115–2119.

Table 25.1 Summary of methods for aluminium in foods

Method	Matrix	Sample preparation	Method conditions	Reference
ICP and AAS	Duplicate diets and milk powders	Various methods used including dry ashing, wet open and wet pressurised	Various, including ETAAS, ICP–AES, FAAS, ZETAAS and DCP–AES	1
ETAAS	Coffee and tea	For slurry analyses the samples were ground, sieved at 105 µm and then suspended in 0.2 % HNO_3 and 10 % Triton X-100 medium. For liquid phase Al, the samples were prepared the same way and only the liquid was introduced into the graphite furnace	The slurry was diluted, sonicated and transferred to autosamples cup and analysed	2
GFAAS	Infant formula and evaporated milk	Added 2 mL conc HNO_3 to sample (0.3 g) + water (1.7 mL) for powdered sample, or 2 g sample for ready to use, into tube. Capped tube and left in water-bath at 55 ºC overnight. Cap removed and solution diluted to 10 mL with water	Al standards containing 0, 10, 20, 40, 60 and 80 ng/mL were prepared in 20 % nitric acid without digestion. Al determined by GFAAS in the presence of a Pd modifier	3
GFAAS	Wine	Samples filtered through a 0.45 µm membrane filter to remove any sediment and diluted with water either 20-fold or 50-fold	Al determined at 309.3 nm using a Zeeman Z7000 GFAAS with normal graphite cuvettes. 20 µL injected, temperature programmed with atomisation at 2700 ºC (6 s)	4
GFAAS	Fish	Frozen samples were freeze dried, finely ground, homogenised and stored in polyethylene bags at room temperature. 1 g was placed into petri-dishes and put in a plasma asher chamber for mineralisation in a closed low-temperature microwave oxygen plasma processor system. After mineralisation the remaining ash was dissolved with 0.2 % HNO_3	Al determined at 309.6 nm using a PE 4100ZL AAS with Zeeman-background correction and THGA using pyrolytically coated graphite tubes. 20 µL injected, temperature programmed with atomisation at 2300 ºC (5 s)	5

Table 25.1 cont'd

Method	Matrix	Sample preparation	Method conditions	Reference
GFAAS	Fish	Samples were digested with HNO_3. Ashing temperature was fixed at 1500 ºC. Prior to the Al analysis, samples were diluted with water to ensure that interfering inorganic components were lower than the tolerable concentrations. The concentration of the samples should be maintained at 0.1 % HNO_3	Al determined at 394.4 nm with Zeeman-background correction using pyrolytically coated graphite tubes. 20 µL injected, temperature programmed with atomisation at 2600 ºC (3 s)	6
ETAAS	Port wine	Samples diluted 1+9 with 1 g/L potassium dichromate and 0.2 % Triton X-100 directly in the cups of the autosampler	Al determined at 309.3 nm using a Perkin Elmer 4100 ZL GFAAS with end-capped traverse heated graphite tubes. 10 µL injected, temperature programmed with atomisation at 2200 ºC (4 s)	7
ETAAS	Baby foods	Samples without previous treatment were introduced into the atomiser as suspensions, prepared in a medium containing 10 % ethanol, 5 % H_2O_2 and 5 % HNO_3. Slurries were homogenised with a potter and introduced into the furnace	Al determined at 309.3 nm using a Perkin Elmer 1100B GFAAS with pyrolytically coated graphite tubes. 20 µL injected, temperature programmed with atomisation at 2650 ºC (3 s)	8

Spectroscopic and FAAS	Foods	Sample digested with H_2SO_4 + HNO_3 + $HClO_4$. Solutions of destroyed samples kept in polyethylene bottles	Spectroscopic method with 8-hydroxyquinoline extraction and AAS method with nitrous oxide–acetylene flame	9
Spectrophoto-metric oxine (SO) and FAAS	Foods	Sample digested with H_2SO_4 + HNO_3 + $HClO_4$. Solutions of destroyed samples kept in polyethylene bottles	Spectroscopic method with 8-hydroxyquinoline extraction absorbance measured at 385 nm and AAS method with nitrous oxide–acetylene flame	10
ICP-AES	Seafood and meat	Microwave digestion with HNO_3, H_2O_2 and HF. Lyophilised samples were digested in closed vessels with HNO_3 and HF. An additional digestion then proceeded in open vessels with H_2O_2. H_3BO_3 was employed to eliminate HF	Measurements performed on ARL 3410+ sequential ICP spectrometer with Minitorch. Samples introduced by a Meinhard concentric nebuliser. Al was calculated from a linear regression equation on the basis of an average intensity of 4 separate determinations	11

Table 25.2 Summary of statistical parameters for aluminium in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
ICP and AAS	Duplicate diets and milk powders	Various methods used (see Table 25.4) to validate samples	See Table 25.3 for details of milk powder samples Duplicate diet samples: A. (n-20) Mean 11.04 (6.32–18.68) mg/kg SD=4 mg/kg CV=36.2 % B. (n-20) Mean 25.28 (17.6–37.81) mg/kg SD=4 mg/kg CV=16.2 % Detection limit ranged from 0.02–10 mg/kg	1
GFAAS	Infant formula and evaporated milk	Precision of method established with reference samples and validated with real samples	Data for 12 batches for the reference sample: Mean = 0.316 µg/g, S_r = 0.012 µg/g, RSD_r = 3.7 %, S_R = 0.034 µg/g, RSD_R = 10.8 %, detection limit 0.0126 µg, sample spike recovery 88–127 % (av 106 %) Method applied to 282 commercial samples	3
ETAAS	Coffee and tea	Precision of method established with standards and validated with real samples	Calibration performed by aqueous standards in the linear range between 50 and 250 µg/L. Characteristic mass of Al = 45 pg/L and detection limit = 2 µg/L. Using 0.1 % coffee slurry sample (n=15) RSD_r = 8.2 % and RSD_R = 9.8 % (n=5)	2
GFAAS	Wine	Precision of method established with aqueous spiked samples and validated with real samples	Linear calibration 0–100 µg/L. 3 wine samples gave RSD 2–4 % (n=5). Recovery for wine spiked with aqueous Al 96–106 %. Method applied to 267 commercial wines	4
ICP-AES	Seafood and meat	Precision of method established and validated with reference materials and applied to 12 samples	Recoveries for spike 95.2–97.6 %. Analyses of NIST RM 1566a and 1577b demonstrated reliability of the method. Method applied to 12 representative seafoods and meats	11

ETAAS	Baby foods	Precision of method established CRMs and validated with different types of baby food samples	Detection limit 50 pg; characteristic mass 18 pg. Reliability of the procedure checked statistically comparing results with those obtained with a previous microwave mineralisation stage and by analysis of several CRMs. Calibration graph linear between 0 and 90 ng/mL. Repeatability RSD = 5.1 % (n=10) Al content of baby foods ranged from 0.4–3.0 µg/g	8
ETAAS	Port wine	Precision of method established and applied to samples (n=10)	Method proved to be accurate. Reproducibility for 10 samples in 3 runs 2.7 % (0.81–4.73 %). Repeatability <1.0 %. Detection limit 1.3 µg/L	7
GFAAS	Fish	Precision of method established and applied to fish tissue samples	The linearity of the calibration line as well as of different standard addition lines were very good within 0–60 µg Al/L. Detection limit in dried ocean perch fillet was 0.03 µg Al/g on a dry weight basis	5
GFAAS	Fish	Precision of method established and applied to fish tissue samples	The Al concentrations in biological reference materials (NRCC DORM-2 and DOLT-2) agreed well with the reported reference values. Detection limit in samples of fish was 1 ng Al/g on a dry weight basis	6
Spectro-scopic and FAAS	Foods	Method applied to real samples	Recovery of spikes: For 8-hydroxyquinoline extraction method 88.5–106.5 % For AAS method 75–100 % Methods applied to commercially available foods	9
Spectro-scopic and FAAS	Foods	Method applied to real samples	For SO method Recovery 86.4–109.3 % (mean 98.6 %±5.2 %) Detection limit 0.5 µg/mL For AAS method Recovery 83.3–100 % (mean 94.2 % ±8.1 %) Detection limit 6 µg/mL. Methods applied to commercially available foods	10

Table 25.3 Performance characteristics for aluminium in milk powder[1]

Sample	Milk powder
Analyte	Aluminium
No. of laboratories	16
Units	mg/kg
Mean value	15.87
S_r	0.83
RSD_r	5.2 %
r	2.33
S_R	2.90
RSD_R	18.3 %
R	8.21

Key

Mean	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).
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).

Table 25.4 Summary of key steps of procedures used in IUPAC sample survey[1]

Lab	Test portion (g)	Type	Decomposition Aid	Temp (°C)	Measurement	Detection limit (mg/kg)
1	1	Wet-open	HNO_3	100	ETAAS	0.1
2	0.5	Dry-ashing	No	450	ICP-AES	2.8
3	0.2	Wet-pressurised	HNO_3	140	ETAAS	0.02
4	1	Wet-open	$HNO_3/HClO_4$	200	FAAS	10
5	5	Wet-open	HNO_3/H_2SO_4	?	FAAS	5
6	0.5	Dry-ashing	No	550	ICP-AES	0.08
7	0.15–0.20	Wet-open	HNO_3	110	ZETAAS	1.7
8	0.2	Wet-open	$HNO_3/HClO_3/HClO_4$	150	ZETAAS	5
9	0.3	Wet-pressurised	HNO_3	270	ETAAS	0.1
10	0.3	Wet-pressurised	HNO_3	150	ICP-AES	1
11	1	Dry-ashing	HNO_3	500	FAAS	0.1
12	0.20–0.25	Wet-pressurised	HNO_3/HCl	150	ETAAS	0.5
13	1	Wet-open	HNO_3/HF/$HClO_4$	105	ICP-AES	1.5
14	2.5	Dry-ashing	H_2SO_4	500	FAAS	0.5
15	1	Dry-ashing	$Mg(NO_3)_2$	550	ETAAS	5
16	2.5	Wet-open	$HNO_3/HClO_4$	200	ICP-AES	1
17	0.3	Wet-pressurised	HNO_3	150	DCP-AES	0.3
18	0.15–0.60	Dry-ashing	No	550	ZETAAS	0.1–0.5
19	2–4	Wet-open	$HNO_3/HClO_4$	200	ETAAS	0.7
20	0.25	Wet-open	HNO_3	110	ETAAS	0.02
21	0.2	Wet-pressurised	HNO_3	150	ZETAAS	0.1
22	0.8	Wet-pressurised	HNO_3	?	ETAAS	?
23	1	Wet-pressurised	HNO_3/HCl/HF	150	ICP-AES	0.25
24	0.25–0.50	Wet-open	HNO_3	55	ETAAS	0.05

Measurement methods are abbreviated as:

ETAAS	Electrochemical atomic absorption spectrometry
ZETAAS	Zeeman backround corrected ETAAS
AES	Atomic emission spectrometry
DCP	Direct current plasma
ICP	Inductively coupled plasma
FAAS	Flame atomic absorption spectrometry

26

E954: Saccharin

26.1 Introduction

The major food groups contributing to dietary intake of saccharin include a wide variety of foods with maximum permitted levels of 100–200 mg/kg (mustard 240 mg/kg; weight control diets 500 mg/kg; vitamin preparations, chewing gum 1200 mg/kg; 'Essoblaten' 800 mg/kg) and soft drinks 80 mg/kg; 'Gaseoza' 100 mg/kg; various beers and cider 80 mg/kg). The acceptable daily intake (ADI) for saccharin (expressed as saccharin acid) is 5 mg/kg body weight.

26.2 Methods of analysis

There are various methods published for the determination of saccharin in foodstuffs. Methods available for saccharin in foodstuffs include spectrometric,[1–3] differential pulse polarography,[4] sublimation,[5] potentiometric,[6–10] micellar electrokinetic capillary chromatography (MECC)[11] and HPLC.[12–19] A summary of these methods is given in Table 26.1, together with the matrices for which the methods are applicable. If statistical parameters for these methods were available these have been summarised in Table 26.2. Three of these methods are AOAC Official Methods of Analysis,[4,5,16] and three methods have been accepted as European Standards.[1,12,13]

The three AOAC Official Methods for the determination of saccharin in foods are the following:

1 Saccharin in food, differential pulse polarographic method.[4]
2 Saccharin in food, sublimation method.[5]
3 Benzoate, caffeine and saccharin in soda beverages, liquid chromatographic method.[16]

They were developed in the 1980s and there are no performance characteristics available for them. However, performance characteristics are available for all the three European Standards. They are the following:

1 BS EN 1376:1997. The spectrometric method for the determination of sodium saccharin and saccharin content in solid table-top sweetener preparations prepared from cyclamates/saccharin or saccharin has been collaboratively tested on sweetener tablets.[1] A sample test solution is prepared by dissolving table-top sweetener in sodium hydroxide solution with photometric determination of the sodium saccharin content at the absorption maximum of about 265 nm. A summary of the procedure for this method is given in the Appendix and the performance characteristics are given in Table 26.3.
2 BS EN 1379:1997. The high performance liquid chromatography method for the determination of sodium cyclamate and saccharin in liquid table-top sweetener preparations has been collaboratively tested on liquid sweetener preparation.[12] The method involves the determination of sodium cyclamate, saccharin and sorbic acid in an appropriate dilution of a liquid table-top sweetener preparation in water by HPLC and subsequent photometric detection in the ultraviolet (UV) range with identification on the basis of the retention times, and quantitative determination by the external standard method using peak areas or peak heights. A summary of the procedure for this method is given in the Appendix and a summary of the statistical parameters in Table 26.4.
3 BS EN 12856:1999. The high performance liquid chromatography method for the determination of acesulphame-K, aspartame and saccharin in foodstuffs has been collaboratively tested on foods.[13] The sample is extracted or diluted with water. If necessary, the sample solution with the intense sweeteners is purified on a solid phase extraction column or with Carrez reagents. The intense sweeteners in the sample test solution are separated on an HPLC-reversed phase chromatography column and determined spectrometrically at a wavelength of 220 nm. A summary of the procedure for this method is given in the Appendix with a summary of the statistical parameters in Table 26.5.

Another suitable liquid chromatography method for the determination of benzoate, caffeine and saccharin beverages based on the Official AOAC Method 979.08[16] has been collaboratively tested on beverages, sweets and desserts.[17,18] The sample is decarbonated and filtered for beverages and extracted with ethanol for sweets prior to LC. The sample test solution is separated on an HPLC-reversed phase chromatography column with UV detection at 254 nm. A summary of the procedure for this method is given in the Appendix and a summary of the statistical parameters in Tables 26.6 and 26.7.

26.3 Recommendations

There are many methods available for the analysis of saccharin in foods and the decision as to which one should be used depends on the expected level of saccharin

and the matrix to be analysed. Further method development may be required to ensure the methods are applicable for all matrices.

26.4 References

1 'Foodstuffs – determination of saccharin in table top sweetener preparations – spectrometric method', BS EN 1376:1997.
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5 'AOAC Official Method 947.10. Saccharin in Food, Sublimation Method', *AOAC Official Method of Analysis* (2000) 47.6.15 p 50.
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15 'Foodstuffs. Determination of acesulfame-K, aspartame and saccharin. High performance liquid chromatographic method', Czech Republic Standard CSN EN 12856. Cesky Normalizacni Inst, Biskupsky Dvur 5, CZ-1133 47 Prague 1, Czech Republic. [Czech].
16 'AOAC Official Method 979.08. Benzoate, caffeine and saccharin in soda beverages, liquid chromatographic method', *AOAC Official Method of Analysis* (2000) 29.1.14 p 2.
17 'Liquid chromatographic determination of saccharin in beverages and sweets: NMKL collaborative study', Sjoeberg A M K, Alanko T A. *Journal of AOAC* (1987) **70**(1), 58–60.
18 'Liquid chromatographic determination of saccharin in beverages and desserts: complementary collaborative study', Sjoeberg A M K. *Journal of AOAC* (1988) **71**(6), 1210–1212.
19 'Simultaneous determination of five sweeteners in foods by HPLC', Kobayashi C, Nakazato M, Ushiyama H, Kawai Y, Tateishi Y, Yasuda K. *Shokuhin Eiseigakyu Zasshi* (1999) **40**(2), 166–171. [Japanese]

26.5 Appendix: method procedure summaries

Foodstuffs – determination of saccharin in table-top sweetener preparations – spectrometric method[1]

Scope
A spectrometric method for the determination of sodium saccharin and saccharin content in solid table-top sweetener preparations prepared from cyclamate/saccharin or saccharin

Principle
Preparation of the sample test solution by dissolving table-top sweetener preparation in sodium hydroxide solution. Photometric determination of the sodium saccharin content at the absorption maximum of about 265 nm.

Procedure

Determination of average tablet mass:
Determine the mass of at least 20 sweetener tablets to the nearest 0.1 mg and calculate the average mass (m_2) of one tablet.
(Note: For improved accuracy the use of 100 tablets is recommended.)

Determination of loss in mass on drying of standard substance:
Weigh about 1.0 g to the nearest 0.1 mg of the reserved finely ground sodium saccharin standard substance used for the preparation of the stock solution, dry this portion to constant mass at 105 °C±2 °C and determine the loss in mass on drying in per cent (LD) by weighing.

Preparation of the sample test solution:
Dissolve an amount (m_0) of finely ground table-top sweetener preparation equivalent to about 35 mg of sodium saccharin weighed to the nearest 0.1 mg in the sodium hydroxide solution in a 50 mL volumetric flask and dilute to the mark. Pipette 20.0 mL of this solution into a 100 mL volumetric flask and dilute to the mark with sodium hydroxide solution.

Determination:
Measure the absorption spectrum of the standard sodium saccharin solution containing about 100 mg of anhydrous sodium saccharin in 1000 mL between wavelengths of 230 nm and 300 nm in quartz cuvettes with sodium hydroxide solution as reference and determine the wavelength of the absorption maximum (about 265 nm).

Prepare the calibration graph by measuring the absorptions of the standard sodium saccharin solutions at the absorption maximum determined. Measure the absorption spectrum of the sample test solution as described and determine the absorption in the absorption maximum. If the shape of the curve obtained for the sample test solution differs from that of the standard solution, it is probable that an interfering substance is present. In this case, the method is not applicable.

Verify the applicability by determining the absorptions 15 nm above and below the wavelengths of the absorption maximum (about 265 nm). Absorption ratios between these values and the maximum absorption shall not differ from those obtained when using the sodium saccharin standard solutions.
(For further details see BS EN 1376:1997[1]).

Foodstuffs – determination of cyclamate and saccharin in liquid table-top sweetener preparations – method by high performance liquid chromatography[12]

Scope
An HPLC method for the determination of sodium cyclamate and saccharin in liquid table-top sweetener preparations. It also allows the determination of sorbic acid in liquid table-top sweetener preparations.

Principle
Determination of sodium cyclamate, saccharin and sorbic acid in an appropriate dilution of a liquid table-top sweetener preparation in water by HPLC and subsequent photometric detection in the ultraviolet (UV) range. Identification on the basis of the retention times, and quantitative determination by the external standard method using peak areas or peak heights.

Procedure

Determination of loss in mass on drying of standard substances

Determination of loss in mass on drying of sodium cyclamate standard substance
Weigh, to the nearest 0.1 mg, about 1.0 g of the reserved finely ground sodium cyclamate standard substance. Dry this portion to constant mass at (105±2) °C and determine the loss in mass on drying (LD) in per cent by weighing. The loss in mass on drying shall not exceed 1 %.

Determination of loss in mass on drying of sodium saccharin standard substance
Weigh, to the nearest 0.1 mg, about 1.0 g of the reserved finely ground sodium saccharin standard substance. Dry this portion to constant mass at (105±2) °C and determine the loss in mass on drying (LD) in per cent by weighing. The loss in mass on drying shall not exceed 15 %.

Preparation of the sample test solution
Dilute 10 mL of the liquid table-top sweetener preparation to 100 mL (V_1) with the mobile phase and filter through a membrane filter. Dilute 10 mL of this solution to 100 mL.

Identification by HPLC
Identify the sweeteners to be determined and the sorbic acid either by comparing the retention times in the sample with those of the standard substances or by

comparing the absorption properties of the sample with those of the standard substance after either recording the absorption curve or taking measurements at different wavelengths in the relevant range for both sample and standard.

Laboratories equipped with fixed-wavelength detectors should carry out separate runs for determination of cyclamate and saccharin at the wavelengths concerned. This method allows the determination of sorbic acid at the same conditions as chosen for saccharin (of wavelength 265 nm). Whenever the determination of sorbic acid with improved sensitivity is required, an additional wavelength switch to 260 nm is recommended.

Note 1. As sorbic acid is a late-eluting compound, the risk of interference in the next run has to be taken into account.

Note 2. If the separating column and mobile phase (as stated in Table 26.1) are used, it has been found satisfactory to adopt the following experimental conditions.

Flow	1.7 mL/min
UV detection	200 nm (sodium cyclamate)
	265 nm (saccharin)
	260 nm (sorbic acid)
Volume injected	20 μL

Determination by HPLC

To carry out the determination by the external standard method, integrate the peak areas or determine the peak heights and compare the results with the corresponding values for the standard substance with the nearest peak area/height, or use a calibration curve. In the case of a calibration curve additional solutions with concentrations within the linear range may be prepared for the calibration graph.

Inject equal volumes of the sample and standard test solutions. Check the linearity of the calibration function.

(For further details see BS EN 1379:1997.)[12]

Foodstuffs – determination of acesulphame-K, aspartame and saccharin – high performance liquid chromatographic method[13]

Scope

An HPLC method for the determination of acesulphame-K, aspartame and saccharin. It also allows the determination of caffeine, sorbic acid and benzoic acid in foodstuffs.

Principle

The sample is extracted or diluted with water. If necessary, the sample solution with the intense sweeteners is purified on a solid-phase extraction column or with Carrez reagents. The intense sweeteners in the sample test solution are separated on an HPLC-reversed phase chromatography column and determined spectrometrically at a wavelength of 220 nm.

Procedure

Preparation of the sample test solution

Clear liquid products (e.g. lemonades, cola, beverages)
Dilute 20 mL of the sample in a 100 mL volumetric flask with water. Filter the solution through a membrane filter of pore size 0.45 μm before injection.

Cloudy liquid products (e.g. juices, flavoured milk drinks)
Dilute 20 mL of the homogenised sample in a 100 mL volumetric flask with 50 mL water, add 2 mL of Carrez solution No. 1, mix and add 2 mL of Carrez solution No. 2. Shake vigorously and allow the solution to stand at room temperature for 10 min. Dilute to the mark with water. Filter through a fluted filter paper, discarding the first 10 mL of the filtrate. Pass the filtrate through a membrane filter of pore size 0.45 μm before injection.

To make allowance for the volume of any precipitate, if the fat-free insoluble matter in the sample volume (here 20 mL) exceeds approximately 3 g, it is advisable to centrifuge the clarified sample mixture for 10 min at least 1400 g before filtering it quantitatively into the 100 mL volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 100 mL volumetric flask and then dilute the solution to the mark with water. This procedure may also be followed when the amount of insoluble matter is less than 3 g.

Jams, preserves, marmalades and related products (except fruit curds)
Weigh, to the nearest 1 mg, about 20 g of homogenised sample into a 100 mL volumetric flask. Add about 60 mL of water and place the flask in an ultrasonic bath at 40 °C for 20 min. The temperature should not exceed 40 °C since aspartame can be degraded.

Cool the solution to room temperature. Add 2 mL of Carrez solution No. 1, mix and then add 2 mL of Carrez solution No. 2. Shake vigorously and allow the solution to stand at room temperature for 10 min. Dilute to the mark with water. Filter the solution through a fluted filter paper, discarding the first 10 mL of the filtrate. Pass the filtrate through a membrane filter of pore size 0.45 μm before injection.

To make allowances for the volume of any precipitate, if the fat-free insoluble matter in the initial sample mass exceeds approximately 3 g, it is advisable to centrifuge the clarified sample mixture for 10 min at least 1400 g before filtering it quantitatively into the 100 mL volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 100 mL volumetric flask and then dilute the solution to the mark with water. This procedure may also be followed when the amount of insoluble matter is less than 3 g.

Semi-solid and solid products (e.g. curd cheese desserts, yogurt products, delicatessen salads, except custard powder)
Weigh, to the nearest 1 mg, about 10 g to 20 g of the thoroughly homogenised sample into a 100 mL volumetric flask. Add about 50 mL of water and place the

volumetric flask in the ultrasonic bath at 40 °C for 20 min. The temperature should not exceed 40 °C since aspartame can be degraded.

Cool the solution to room temperature. Add 2 mL of Carrez solution No. 1, mix, add 2 mL of Carrez solution No. 2. Shake vigorously and allow the solution to stand at room temperature for 10 min. Dilute to the mark with water. Filter the solution through a fluted filter paper, discarding the first 10 mL of the filtrate. In the case of very complex matrices, additional purification using the solid phase extraction column may be necessary to protect the separating column, since colourings, flavourings and fat cannot be separated by Carrez clarification. In this case, add 2 mL of the clarified filtrate to the cartridge, previously activated with 3 mL of methanol and 20 mL of water, and elute with about 20 mL of mobile phase. Pass the filtrate through a membrane filter of pore size 0.45 µm before injection.

To make allowance for the volume of any precipitate, if the fat-free insoluble matter in the initial sample mass exceeds approximately 3 g, it is advisable to centrifuge the clarified sample mixture for 10 min at least 1400 g before filtering it quantitatively into the 100 mL volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 100 mL volumetric flask and then dilute the solution to the mark with water. This procedure may also be followed when the amount of insoluble matter is less than 3 g.

Custard powder

Weigh, to the nearest 1 mg, about 10 g of the sample into a 500 mL volumetric flask. Add about 400 mL of water and proceed as described above, i.e. add 6 mL of Carrez solution No. 1, mix, add 6 mL of Carrez solution No. 2 for clarification.

To make allowance for the volume of any precipitate, if the fat-free insoluble matter in the initial sample mass exceeds approximately 3 g, it is advisable to centrifuge the clarified sample mixture for 10 min at least 1400 g before filtering it quantitatively into the 500 mL volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 500 mL volumetric flask and then dilute the solution to the mark with water. This procedure may also be followed when the amount of insoluble matter is less than 3 g.

Identification

Identify the intense sweeteners in the sample solution by comparing the retention times of the analyte concerned in the sample solution with that of the standard substance, or by simultaneous injection of the standard solution and the sample test solution, or by adding the standard solution to the sample test solution and recording an absorption curve in the relevant wavelength range.

Inject equal volumes of the sample test and standard solutions. Intervals between successive injections of the standard solutions should not be less than 15 min. To minimise the risk that substances eluted from earlier injections will be confused with components from subsequent samples, successive injections of the sample test solutions should be made at sufficiently long intervals.

In case of possible interferences washing of the columns is recommended. A

suitable mobile phase for washing would have the following composition: 50 parts per volume of mobile phase + 50 parts per volume of acetonitrile.

Suitable chromatographic conditions for identification are:

Chromatographic columns	
Type	reversed phase (RP)
Stationary phase and column	spherical particles of 3 µm, for column lengths of 100 mm, up to 10 µm for lengths of 300 mm
Internal diameter	4.0 mm
Guard column	recommended (optional)
Examples	Lichrospher® 100 RP 18, Superspher® RP Select B Nucleosil 100-5 C18 AB, µBondapak C18 Partisil ODS 3
Flow rate	0.8 mL/min up to 1 mL/min
Injection volume	10 µL up to 20 µL
Detection	
Photometrical (UV) at a wavelength of	217 nm for aspartame 227 nm for acesulphame-K 265 nm for saccharin 220 nm for all intense sweeteners, if detector does not allow a wavelength switch in one run

Mobile phase

In general the following proportions have given satisfactory results:

- phosphate buffer solution I + acetonitrile [90:10, volume parts, (V/V)]
- phosphate buffer solution II + acetonitrile (80:20, V/V)
- phosphate buffer solution II + acetonitrile (85:15, V/V)
- phosphate buffer solution II + acetonitrile (90:10, V/V)
- phosphate buffer solution II + acetonitrile (95:5, V/V)
- phosphate buffer solution II + acetonitrile (98:2, V/V)
- phosphate buffer solution II + phosphoric acid pH = 2.8

Determination

For the determination by the external standard method, integrate the peak areas or determine the peak heights and compare the results with the corresponding values for the standard substance with the nearest peak area/height, or use a calibration graph. To prepare a calibration graph, inject a suitable amount of standard solutions of appropriate mass concentrations. Plot the peak heights or peak areas of the standard solutions against the corresponding mass concentrations in milligrams per litre. Check the linearity of the calibration graph. Alternatively, the calibration may also be evaluated mathematically by the regression. Check the linearity of the regression graph.

(For further details see BS EN 12856:1999.)[13]

Liquid chromatographic method for saccharin in beverages and sweets[17, 18]

Principle
Saccharin is determined in soft drinks and juices by reverse phase LC with UV detection at 254 nm; sweets must first be extracted with ethanol.

Apparatus

a) *Liquid chromatograph* – isocratic instrument with possibility of using 2 mobile phases, step-gradient, or gradient system. Operating conditions: column temperature ambient; flow rate 2.0 mL/min; wavelength 254, 280, 207 or 214 nm; injection volume 10–30 µL: sensitivity 0.005–0.02 AUFS; chart speed 1 cm/min
b) *Reverse phase LC column* – particle size 10 µm, 30 cm × 3.9 mm i.d., e.g. µBondapak C18
c) *Guard column* – particle size 37–50 µm C18
d) *Membrane filters* – For aqueous solutions, pore size 0.45 µm
e) *Centrifuge* – minimum 2000 g.

Reagents

a) *Mobile phase* – prepare 1 % (v/v) acetic acid solution. Mobile phase I: Mix 950 mL 1 % acetic acid solution and 50 mL methanol; de-gas. Mobile phase II: mix 300 mL 1 % acetic acid solution and 700 mL methanol; de-gas.
b) *Ethanol* – 99 %
c) *Sodium saccharin standard solution* – 100 mg/L. Transfer exactly 25 mg sodium saccharin dihydrate, $C_7H_4NNaO_3S{\cdot}2H_2O$, to 250 mL volumetric flask and dilute to volume with water.

Sample preparation

a) *Soft drinks and juices* – Decarbonate, filter if particulate matter is present, and inject
b) *Sweets* – Weigh 10.00 g samples containing ≥0.5 mg saccharin and add 100.0 g water. Dissolve sample with mixing. Weigh 10.00 g homogenate into centrifuge tube and add 50 mL ethanol. Mix and let stand overnight. Centrifuge and decant aqueous ethanol phase. Wash precipitate twice with 30 mL ethanol and combine washings with aqueous ethanol phase. Evaporate to dryness and dissolve and dilute residue to 5.00 mL with water. Filter and inject directly.

Determination
Inject 10 µL standard solution to determine peak height of saccharin. Repeat injections until results agree 2 %. Inject sample solution containing *c.* 1 µg saccharin twice: chromatograph samples from sample preparation (a) with mobile phase I and samples from sample preparation (b) with mobile phases I and II.

Measure peak heights of standard solutions and samples.

sodium saccharin in soft drinks $mg/L = C' \times (H/H') \times (V'/V)$ [26.1]

sodium saccharin in sweets $mg/kg = C' \times (H/H') \times (V'/V) \times 5.5$ [26.2]

where:

C'	= concentration of standard, mg/L
H and H'	= average peak height of sample and standard, respectively
V and V'	= volumes injected (μL) of sample and standard, respectively
5.5	= 5/10.00 × 110.0/10.00

Table 26.1 Summary of methods for saccharin in foods

(a)

Method	Matrix	Sample preparation	Method conditions	Reference
Spectrophotometric	Sweetener preparations	Ground tablets dissolved in sodium hydroxide solution	Photometric determination at 265 nm	1,2
Derivative UV spectrophotometric	Artificial sweeteners	Dissolve powdered tablet in distilled water, acidify with HCl and extract 5 times with chloroform–EtOH (9+1). Evaporate extracts, dissolve residue in sodium hydroxide solution	D^2 and D^4 calibrated in concentration range 28–98 µg/mL and wavelength range 240–320 nm. ZP and PP at 285.5 nm and 290 nm. Scan speed 120 nm/min, ordinate axis ±0.025 absorbance nm^{-2} (or nm^{-4}), respectively. Differential wavelength 4 nm for D^2 and 12 nm for D^4. Both with 11 points of noise attenuation	3
Differential pulse polarographic	Foods	2 g suspension + 1 mL HCl (1+1). Add 5 g diatomaceous earth. Place on chromatography column and elute with 40 mL water saturated with $CHCl_3$. Collect eluate and evaporate. Add 0.1 M NaOH to dissolve residue	Add 15 mL electrolyte to polarographic cell and bubble N_2 through solution 5 min. Sweep N_2 over solution, and polarograph. Pipette 2 mL test solution into polarographic cell containing electrolyte, bubble N_2 through solution 1 min and polarograph exactly as before. Repeat with standards and plot calibration curve detected by differential pulse polarography	4
Sublimation	Foods	Acidify test portion with HCl and extract with CCl_4. Discard CCl_4. Extract aqueous phase with ether and evaporate	Transfer to sublimator with alcohol and evaporate to dryness. Sublime residue. Wash saccharin from condenser bulb of sublimator with warm alcohol into weighed beaker. Repeat sublimation until no further residue appears on condensing bulb. Evaporate on water bath, heat residue for 2 h at 100 ºC, cool and reweigh beaker	5

Table 26.1 cont'd

Method	Matrix	Sample preparation	Method conditions	Reference
Potentiometric	Dietary products	Sample dissolved in 0.2 mol/L MacIlvaine buffer solution (pH 2.5). 1 mL transferred to measuring cell and saccharin determined by successive addition method	Coated graphite saccharin ion-selective electrode was developed, 5:30:65 % m/m (toluidine blue O cation) (saccharinate anion)/dibutylphthalate/PVC. The electrode potential was measured in 10 mL of a 0.2 mol/L MacIlvaine buffer solution (pH 2.5) with stirring at 25 ºC	6
Ion-selective electrode	Aqueous solutions		Brillant green–hydrogen phthalate ion-pair in nitrobenzene with PVC support was used to prepare a liquid membrane electrode which responded to saccharin (1×10^{-4}–0.1 mol/L) ions with sub-Nernstian slope	7
Flow injection potentiometric	Dietary products	Sample dissolved in 0.2 mol/L MacIlvaine buffer solution (pH 2.5). 500 µL inserted in the flow system and transported by carrier stream to the tubular ion-selective electrode	A tubular ion-selective electrode coated with an ion pair formed between saccharinate ion and toluidine blue O cation incorporated on a PVC matrix was constructed and adapted in a flow-injection (FI) system. Optimum conditions: analytical path 120 cm, injection sample volume 500 µL, pH of 2.5, flow rate 2.3 mL/min and tubular electrode length of 2.5 cm. Analytical frequency 40 determinations/h	8

Flow injection potentiometric	Dietary products	Sample dissolved in distilled water	Saccharin is precipitated as mercurous saccharinate and the excess of the mercurous cation is potentially measured by using a silver wire coated with mercury film as the working electrode. A filter unit is used to avoid contact between the precipitate and the electrode surface. With relocation in the manifold, the accumulated precipitate is removed on-line	9
Potentiometric	Dietary products	Sample dissolved in 0.2 M $NaNO_3$, pH 3	Saccharin is potentiometrically titrated with mercurous nitrate solution using a silver wire coated with a metallic mercury film as the working electrode and the end point was found using a Gran's plot	10
Micellar electrokinetic chromatography (MECC)	Cola beverages and jams	Butyl paraben was used as an internal marker	Additives were separated using a 20 mM berate buffer with 35 mM sodium cholate, 15 mM sodium dodecyl sulphate and 10 % methanol added at pH 9.3	11

Table 26.1 cont'd
(b)

Method	Matrix	Sample preparation/extraction	Column	Mobile phase	Detection	Reference
HPLC	Liquid sweetener preparations	Dilute sample with mobile phase and filter through a membrane filter. Dilute 10 mL of this solution to 100 mL	C18 reversed phase (4.6 × 250 mm, 10 µm)	Potassium dihydrogen orthophosphate solution–MeOH (70:30) adjusted to pH 4.5. Flow 1.7 mL/min, injection volume 20 µL	UV at 265 nm	12
HPLC	Foodstuffs	Aqueous extraction of sample with water. Clean-up on solid phase extraction or with clarification reagents, and filtered through a 0.45 µm membrane filter before injection	C18 reversed phase (4.0 × 100–300 mm, 3 µm)	Phosphate buffer + acetonitrile. Flow 0.9 mL/min, injection volume 20 µL	UV at 220 nm	13,14,15
LC	Soda beverages	Decarbonate solution, filter through 0.45 µm filter or centrifuge if necessary	µBondapak C18, 300 × 3.9 mm	20 % CH_3COOH (v/v) buffered to pH 3.0 with saturated sodium acetate solution. Flow 2 mL/min, injection volume 10 µL	UV at 254 nm	16

LC	Beverages and sweets	Beverages: decarbonate, filter. Sweets: extract with EtOH, evaporate to dryness, dissolve in water, filter	µBondapak C18, 300 × 3.9 mm, 10 µm	Acetic acid (1 %)–MeOH (95 +5) I; MeOH–acetic acid (1 %) (70 +30) II	UV at 254 nm	17
LC	Beverages and desserts	Beverages: decarbonate, dilute with water (1+1), filter. Sweets: extract with EtOH, evaporate to dryness, dissolve in water, filter	µBondapak C18, 300 × 3.9 mm, 10 µm	Acetic acid (1 %)–MeOH (95 +5) I; MeOH–acetic acid (1 %) (70 +30) II	UV at 254 nm	18
HPLC	Foods	Chopped or homogenised samples packed into cellulose tubing with HCl solution containing NaCl and dialysed. Tetra–*n*-butylammonium bromide and pH 5.0 phosphate buffer were added to the dialyzate. Solution added to Sep-Pak Vac C18 cartridge, washed with water and MeOH–H_2O (1:9) and eluted from cartridge with MeOH–H_2O (45:55)	Intersil ODS-2	MeOH–water (1:3) containing 0.01 mol/L tetra-*n*-propylammonium hydroxide adjusted to pH 3.5 with phosphoric acid	UV at 210 nm	19

Table 26.2 Summary of statistical parameters for saccharin in foods

Method	Matrix	Extent of validation	Statistical parameters	Reference
Spectrophotometric	Sweetener preparations	Full collaborative trial	see Table 26.3	1
HPLC	Foods	Full collaborative trial	see Table 26.4	12
HPLC	Foods	Full collaborative trial	see Table 26.5	13,14
LC	Beverages and sweets	Full collaborative trial	see Table 26.6	17
LC	Beverages and desserts	Full collaborative trial	see Table 26.7	18
Potentiometric	Dietary products	Precision of method established with standards (n=9)	Linear from 8.1×10^{-5} to 1.4×10^{-2} mol/L LOD = 6.3×10^{-5} mol/L Recovery of 97.8–102.7 % of saccharin from 5 dietary products (n=6). Results were obtained for saccharin for nine commercial products using the potentiometric standard addition method and these compared well to a UV spectrophotometric method and label values	6
Flow injection potentiometric	Dietary products	Precision of method established with standards (n=9)	Linear from 1×10^{-4} to 2×10^{-2} mol/L, with a slope of –53.2±0.4 mV/decade LOD = 8×10^{-5} mol/L Recovery of 96.7–103.4 % of saccharin from 5 dietary products (n=6) Results were obtained for saccharin for nine commercial products using the FI potentiometric method and these compared well to a spectrophotometric method and label values	8

Potentiometric	Dietary products	Precision of method established with standards (n=6)	Calibration curve linear LOD for sodium saccharin was 0.5 mg/mL, the best pH range was from 2.0 to 3.5 Recovery of 95.2–103.2 % of saccharin from 5 dietary products (n=6) Results obtained for saccharin for six commercial products using the potentiometric method compared well to a spectrophotometric method	10
Flow injection potentiometric	Dietary products	Precision of method established with standards (n=4)	Linear from 2×10^{-3} to 1×10^{-2} M For 4 mM saccharin solution RSD was 2.78 % (n=8) Sampling frequency is 60/h and only 0.76 mg Hg_2^{2+} is consumed in each determination Results were obtained for saccharin for four commercial products using the FI potentiometric method and these were comparable to those obtained by UV-spectrophotometry The correlation coefficient between methods is 0.9930	9
Derivative UV spectrophotometric	Artificial sweeteners	Precision of method established and applied to real samples	Method: D^2 correlation coefficient 0.9999, RSD 0.7082 % D^4 correlation coefficient 0.9999, RSD 0.5182 % Saccharin tablets: RSD 1.41 % for D^2 and 0.83 % for D^4, recoveries ranged from 95.51 % to 99.72 % with D^2 and 98.77 to 104.48 % with D^4	3
HPLC	Foods	Precision of method established	LOD = 10 µg/g in samples Recovery checked by spiked 7 different food matrices with 200 µg/g saccharin and analysing each 3 times Recovery ranged from 80 % for biscuit sample to 102 % for jam sample	19
Ion-selective electrode	Aqueous solutions	Precision of method established	Linear concentration range from 1×10^{-4} to 0.1 mol/L LOD = 3.2×10^{-6} mol/L over pH range 3.0–9.0. RSD <2 %	7

Table 26.3 Performance characteristics for saccharin in sweetener tablets[1]

Sample	Saccharin–cyclamate tablets
Analyte	Saccharin
No. of laboratories	7
Units	mg/100 g
Mean value	5.80
S_r	0.51
RSD_r	2.59 %
r	0.42
S_R	0.30
RSD_R	5.23 %
R	0.85

Identified in interlaboratory test conducted by the Max von Pettenkofer Institute, Germany.

Key

Mean 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).

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).

Horrat The HORRAT value for reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

Table 26.4 Performance characteristics for saccharin in liquid sweetener[12]

Sample	Liquid table-top sweetener
Analyte	Saccharin
No. of laboratories	7
Units	g/100 mL
Mean value	1.06
S_r	0.01
RSD_r	1.01 %
r	0.33
S_R	0.03
RSD_R	2.70 %
R	0.08

Identified in interlaboratory test conducted by the Max von Pettenkofer Institute, Germany.

Key

Mean 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).

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).

Horrat The HORRAT value for reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

Table 26.5 Performance characteristics for sodium saccharin in marzipan, yogurt, orange juice, cream, cola and jam[13,14,15]

Sample	Marzipan *	Fruit yogurt *	Orange juice beverage*	Cola **	Jam **
Year of test	1992	1992	1992	1993	1993
No. of laboratories	6	8	12	8	8
Units	mg/kg	mg/kg	mg/L	mg/L	mg/L
Mean value	228.0	116.0	50.8	75	60
S_r	10.0	2.7	1.2	1.4	1.8
RSD_r	4.4 %	2.4 %	2.4 %	1.9 %	3.0 %
r	28.2	7.7	3.4	4	5
S_R	13.5	16.1	8.1	12.1	16.8
RSD_R	5.9 %	14.0 %	16.2 %	16.2 %	28.0 %
R	37.9	45.5	23.0	34	47
Horrat value	4.1	1.8	2.0	1.7	2.8

Sample	Orange juice beverage ***	Cola ***	Cream ***	Yogurt ***	Orange juice ***
Year of test	1995	1995	1995	1995	1995
No. of laboratories	10	11	10	10	8
Units	mg/L	mg/L	mg/kg	mg/kg	mg/kg
Mean value	82	64.9	68.4	71.4	16.1
S_r	2.0	2.0	5.5	8.9	2.3
RSD_r	2 %	3 %	8 %	12 %	14 %
r	6	5	15	25	6
S_R	6.7	10.6	11.3	15.8	6.9
RSD_R	8 %	16 %	17 %	22 %	43 %
R	19	30	32	44	19
Horrat value	1.0	1.9	1.9	2.6	4.1

* Max von Pettenkofer Institute, Germany
** French Institute for Beverages, Brewing and Malting, France
*** MAFF, UK[14]

Key

Mean 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).
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).
Horrat The HORRAT value for reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

Table 26.6 Performance characteristics for sodium saccharin in juice, soft drink and sweets[17]

Sample	Juice		
No. of laboratories	7	8	7
Units	mg/L	mg/L	mg/L
Spike value	40.0	80.0	85.0
Mean value	39.0	77.9	82.3
S_r	1.3	1.3	1.0
RSD_r	3.2 %	1.7 %	1.2 %
S_R	1.9	7.2	5.9
RSD_R	5.8 %	9.4 %	7.3 %
Recovery	99.8 %	97.4 %	96.8 %
Sample	Soft drink		
No. of laboratories	8	8	8
Units	mg/L	mg/L	mg/L
Spike value	50.0	100.0	67.0
Mean value	48.9	94.2	62.9
S_r	1.2	2.4	1.1
RSD_r	2.4 %	2.6 %	1.8 %
S_R	10.5	13.9	11.2
RSD_R	21.7 %	15.1 %	17.9 %
Recovery	97.8 %	94.2 %	93.9 %
Sample	Sweets		
No. of laboratories	4	4	4
Units	mg/kg	mg/kg	mg/kg
Declared value	336	672	112
Mean value	267.1	485.3	102.7
S_r	5.2	23.5	3.5
RSD_r	1.9 %	4.8 %	3.4 %
S_R	12.2	34.4	40.4
RSD_R	5.0 %	8.6 %	39.5 %
Recovery	79 %	72 %	92 %

Key

Mean	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).
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).
Horrat	The HORRAT value for reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

Table 26.7 Performance characteristics for sodium saccharin in juice, soft drink and dessert[18]

Sample		Juice	
No. of laboratories	5	5	5
Units	mg/L	mg/L	mg/L
Mean value	25.9	59.6	89.9
S_r	0.71	0.45	0.71
RSD_r	2.72 %	0.75 %	0.79 %
S_R	1.82	3.81	6.52
RSD_R	6.98 %	6.39 %	7.26 %
Recovery	99.6 %	96.1 %	99.9 %
Sample		Soft drink	
No. of laboratories	7	6	6
Units	mg/L	mg/L	mg/L
Mean value	35.4	58.5	73.3
S_r	2.07	2.92	0.91
RSD_r	5.84 %	4.98 %	1.24 %
S_R	7.31	7.96	6.73
RSD_R	20.63 %	13.62 %	9.19 %
Recovery	107.4 %	104.5 %	100.5 %
Sample		Dessert	
No. of laboratories	7	6	7
Units	mg/kg	mg/kg	mg/kg
Mean value	53.0	114.8	152.6
S_r	3.97	3.47	24.75
RSD_r	7.49 %	3.02 %	16.22 %
S_R	7.13	17.68	24.75
RSD_R	13.43 %	15.40 %	16.22 %
Recovery	94.8 %	104.3 %	103.8 %

Key

Mean 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).

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).

Horrat The HORRAT value for reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

Index

acesulphame-K 235–8
adipic acid and its salts 174–82
  free adipic acid 178
  in orange soft drinks 175, 176, 182
  in packaging materials 174
  in starch 177, 182
  total adipate 177
almond paste 52, 70
aluminium 220–9
  in milk powder 228
ammonium phosphatides 196–200
  in chocolate 196
  in cocoa 196
anchovies 183
anionic resin adsorption 131
annatto extracts 30–4
  in butter 30
  in flavoured milk 30
AOAC Official Methods of Analysis
  and benzoic acid 54–5, 57–8, 58–9
  and BHA 153, 155–6
  and gallates 142, 144–5
  and L-tartaric acid and its salts 166
  and nitrites 98–9
  and saccharin 230–1
  and sorbic acid and its salts 35–6, 37–9
apples
  apple juice 52, 70, 128, 131, 141
  dried apples 93
apricots 94
aspartame 235–8
atomic absorption spectrometry 220
azorubine 15–23
  in beverages 18
  in bitter samples 23
  in flour-based products 15–16, 17–18
  in skimmed milk 16

baby food 127, 200
beer 93, 129, 131, 141
beetroot 53
benzoic acid 54–72
  in almond paste 70
  in apple juice 70
  in cheese 57
  in cola drinks 72
  in fish homogenate 70
  in orange juice 58–9, 71
  in orange squash 72
  in pie filling 72
BHA 153–65
  in butter oil 153, 155–6, 165
  in fats 153, 155–6
  in lard 165
  in oils 153, 155–6, 165
bitter samples 23
butter 30
  fats 142, 144–5, 152
  oil 153, 155–6, 165

cadmium reduction, and nitrites 99, 106–11
calcium stearoyl lactylate 212–14
capillary electrophoresis (CE)
  and adipic acid and its salts 174
  and azpribome 15
  and caramel class III 27
  and L-tartaric acid and its salts 166
  and nitrites 98
  and sulphites 73

and sunset yellow 1
capillary isotachophoresis (cITP), and fumaric acid and its salts 128
caramel class III 27–9
carmoisine 15–23
caseinates 106, 108, 109
caseins 106, 108, 109
cheese
and annatto extracts 30
and benzoic acid 57
and nitrites 99, 106–7, 108, 109–10, 111–12, 115
and phosphorus 200
Red Leicester 30
and sorbic acid and its salts 38
whey cheese 107, 108, 109–10
chlorophylls and chlorophyllins 24–6
chocolate 196
coal-tar dyes 1, 15
cocoa 196
cola drinks 53, 72, 250
cold ham 200
colorimetric methods
and annatto extracts 31
and gallates 142
and karaya gum 189
and nitrites 98, 99
and polysorbates 190
and sulphites 73
copper complexes of chlorophylls and chlorophyllins 24–6
corned beef 126
cream 250
custard powder 237

deproteination 108–10
desserts 236–7
differential pulse polarography (DPP)
and saccharin 230
and sulphites 73, 74, 81–2
differential pulse voltammetry (DPV), and nitrites 98
dried fruit 81
apples 93
apricots 94
sultanas 93
dried milk 106, 108–9, 112, 113
dried potato products 81, 94, 95, 96
dried whey 106, 108, 109

E110 (sunset yellow) 1–14
E122 (azorubine) 15–23
E141 (copper complexes of chlorophylls and chlorophyllins) 24–6
E150c (caramel class III) 27–9
E160b (annatto extracts) 30–4
E200–3 (sorbic acid and its salts) 35–53
E210–13 (benzoic acid) 54–72
E220–8 (sulphites) 73–97
E249–50 (nitrites) 98–127
E297 (fumaric acid and its salts) 128–41
E310–2 (gallates) 142–52
E320 (BHA) 153–65
E334–7 (L-tartaric acid and its salts) 166–73
E354 (L-tartaric acid and its salts) 166–73
E355–7 (adipic acid and its salts) 174–82
E359 (adipic acid and its salts) 174–82
E405 (propylene glycol) 183–6
E416 (karaya gum) 187–9
E432–6 (polysorbates) 190–5
E442 (ammonium phosphatides) 196–200
E444 (sucrose acetate isobutyrate) 201–4
E472e (mono/diacetyl tartaric acid esters) 205–8
E476 (polyglycerol esters) 209–11
E477 (propylene glycol) 183–6
E481 (sodium stearoyl lactylate) 212–14
E482 (calcium stearoyl lactylate) 212–14
E483 (stearyl tartrate) 215
E491–2 (sorbitan esters) 216–19
E493–4 (sorbitan esters) 216–19
E495 (sorbitan esters) 216–19
E520–3 (aluminium) 220–9
E541 (aluminium) 220–9
E554–9 (aluminium) 220–9
E573 (aluminium) 220–9
E954 (saccharin) 230–52
electrometric methods, and sulphites 73
electrothermal atomic absorption spectrometry (ETAAS) 221
enzymatic methods
and propylene glycol 184
and sulphites 73, 74, 79–81
European Standards
and L-tartaric acid and its salts 166
and nitrites 99
and saccharin 231

fats 142, 144–5, 153, 155–6
butter fats 142, 144–5, 152
fish 183
anchovies 183
seafood 91, 94, 95, 96
shrimp 94, 96
fish homogenate 52, 70
flame atomic absorption spectrometry (FAAS) 221

flavoured milk 30
flour-based products 15–16, 17–18, 200
flow injection analysis (FIA)
  and nitrites 98, 99–100, 111–15, 115–17
  segmented flow analysis 99, 111–15
  and sulphites 73, 75, 84–6
food additive premixes 202–3
free adipic acid 178
fruit, dried 81, 93, 94
fruit juices 80, 92, 167–8, 236, 251, 252
  apple 52, 70, 128, 131, 141
  grape 74, 166, 167–8, 173
  lemon 93, 95
  orange 58–9, 71, 94, 250
FT–Raman spectroscopy 189
FTIR (vapour phase Fourier transform infrared spectrometry) 74
fumaric acid and its salts 128–41
  in apple juice 128, 131, 141
  in beer 129, 131, 141

gallates 142–52
  in butter fats 142, 144–5, 152
  in fats 142, 144–5
  in lard 152
  in oils 142, 144–5, 152
gas chromatography (GC)
  and adipic acid and its salts 174, 176–8
  and benzoic acid 54, 57–8
  and BHA 153
  and fumaric acid and its salts 128
  and mono/diacetyl tartaric acid esters 205
  and polysorbates 190
  and sorbic acid and its salts 35, 37–9
  and sorbitan esters 216
  and stearoyl lactylates 212
  and sucrose acetate isobutyrate 201
gas chromatographic-tandem mass spectrometric, and propylene glycol 183–4
grape juice 74, 166, 167–8, 173
graphite furnace atomic absorption spectrometry (GFAAS) 220
gravimetric methods
  and polysorbates 190
  and sulphites 74
Greiss diazotisation procedure 98

ham 200
hominy 92
HPLC (high pressure liquid chromatography)
  and adipic acid and its salts 174
  and ammonium phosphatides 198, 199
  and annatto extracts 30
  and azorubine 15
  and benzoic acid 54, 55, 59–61
  and BHA 153–65
  and caramel class III 27
  and copper complexes of chlorophylls/chlorophyllins 24
  and fumaric acid and its salts 128
  and gallates 142
  and L-tartaric acid and its salts 166, 167–8
  and mono/diacetyl tartaric acid esters 205
  and polyglycerol esters 211
  and polysorbates 190
  and propylene glycol 183, 184
  and saccharin 230–1, 234–8
  and sorbic acid and its salts 35, 36, 39–41
  and sorbitan esters 216
  and stearoyl lactylates 212
  and sulphites 73
  and sunset yellow 1, 2, 4
HPTLC (high performance thin layer chromatography)
  and benzoic acid 54
  and sorbic acid and its salts 35

ice-cream 183
inductively coupled plasma atomic emission spectrometry 221
infant formula 127, 200
International Standards 99
ion exclusion chromatography (IEC), and sulphites 73, 74–5, 83
ion-exchange chromatography (IC), and nitrites 98, 99, 104–6

jam 81, 236, 250
Japanese foods 183

karaya gum 187–9

L-tartaric acid and its salts 166–73
  in fruit juices 167–8
  in grape juice 166, 167–8, 173
  in vegetable juices 167–8
lager beer *see* beer
lanthanide-sensitised luminescence 54
lard 152, 165
lemon juice 93, 95
liquid chromatographic methods

and benzoic acid 55, 58–9
and BHA 153, 155–6
and gallates 142, 144–5
and saccharin 239–40
see also HPLC; HPTLC

marmalade 236
marzipan 250
meat and meat products 99, 101–4, 104–6, 126, 127
cold ham 200
corned beef 126
cured meat 99
sausages 102, 126, 127, 200
MECC (micellar electrokinetic chromatography)
and benzoic acid 54
and BHA 153
and gallates 142
and saccharin 230
and sorbic acid and its salts 35, 127
milk and milk products 99–100, 106–11, 112, 113
cream 250
dried milk 106, 108–9, 112, 113
flavoured milk drinks 30, 236
powdered milk 228
skimmed milk 16, 200
modified Jones reduction method, and nitrites 99
Monier–Williams method, and sulphites 73, 74, 77–9
mono/diacetyl tartaric acid esters 205–8

nitrates 101–4, 110, 114
nitrites 98–127
in baby food 127
in cheese 99, 106–7, 108, 109, 111–12, 115
in cured meat 99
deproteination 108–10
in dried milk 106, 108–9, 112, 113
in dried whey 106, 108, 109
in meat and meat products 99, 101–4, 104–6, 126, 127
in milk and milk products 99–100, 106–11, 112, 113
in paté 127
reduction to nitrates 110
in salami 127
in whey cheese 107, 108, 109–10
NMKL-AOAC methods *see* AOAC Official Methods of Analysis

oils 153, 155–6, 165
butter oil 153, 155–6, 165
orange juice 58–9, 71, 94, 250
orange soft drinks 53, 72, 175, 176, 182

P NMR spectroscopy 198, 199
packaging materials 174
paper chromatography
and azorubine 15
and sunset yellow 1
paté 127
peas 94
phosphorus 196–200
in cheese 200
in cold ham 200
in infant formula 200
in potato flour 200
in sausage 200
in skimmed milk 200
photoacoustic spectrometry (PAS), and annatto extracts 30
pie filling 53, 72
pineapple 96
polarographic methods
and fumaric acid and its salts 128
and sulphites 73
polyglycerol esters 209–11
polysorbates 190–5
potato products 81, 94, 95, 96
potato flakes 93, 94
potato flour 200
potentiometric methods
and benzoic acid 54
and saccharin 230
powdered milk 228
propylene glycol 183–6
in anchovies 183
in fish products 183
in ice-cream 183
in Japanese foods 183
in soft drinks 183

Red Leicester cheese 30
reduction to nitrates 110

saccharin 230–52
in cola drinks 250
in cream 250
in custard powder 237
in desserts 236–7
in fruit juices 236, 251, 252
in jam 236, 250
in marzipan 250
in milk drinks 236

in orange juice 250
in soft drinks 236, 239–40, 251, 252
in sweeteners 233–5, 248–9
in sweets 239–40, 251
in yogurt products 236–7, 250
salad cream 53
salami 127
sausages 102, 126, 127, 200
seafood 91, 94, 95, 96
segmented flow analysis, and nitrites 99, 111–15
sequential injection analysis (SIA), and sulphites 73
shrimp 94, 96
skimmed milk 16, 200
sodium copper chlorophyllin 24
sodium hydroxymethylsulphonate (HMS) 78
sodium stearoyl lactylate 212–14
soft drinks 2, 4, 183, 236, 239–40, 251, 252
cola drinks 53, 72, 250
orange drinks 53, 72, 175, 176, 182
sorbic acid and its salts 35–53
in almond paste 52
in apple juice 52
in beetroot 53
in cheese 38
in cola drinks 53
in fish homogenate 52
in orange squash 53
in pie filling 53
in salad cream 53
sorbitan esters 216–19
spectrometric methods
atomic absorption spectrometry 220
and nitrites 99, 101–4, 106–11
and saccharin 230, 233–4
spectrophotometric methods
and ammonium phosphatides 198, 199
and azpribome 15
and benzoic acid 54
and BHA 153
and gallates 142
and phosphorus 197
and sorbic acid and its salts 35
and sunset yellow 1
spectroscopic determination 98
spices 81
starch 95, 177, 182
stearoyl lactylates 212–14
stearyl tartrate 215
sublimation methods, and saccharin 230
sucrose acetate isobutyrate 201–4
sulphites 73–97
in beer 93
in dried apples 93
in dried apricots 94
in dried fruit 81
in dried potato products 81, 94, 95, 96
in fruit juices 80, 92
in grape juice 74
in hominy 92
in jam 81
in lemon juice 93, 95
in orange juice 94
in peas 94
in pineapple 96
in potato flakes 93, 94
in seafood 91, 95
in shrimp 94, 96
in spices 81
in starch 95
in sultanas 93
in wine 75, 80, 84, 85–6, 93, 95, 96, 97
sulphur dioxide 73, 74
sultanas 93
sunset yellow 1–14
in beverages 5
in soft drinks 2, 4
supercritical fluid chromatography (SFC) 216
sweeteners 233–5, 248–9
sweets 239–40, 251

tartaric acid
L-tartaric acid and its salts 166–73
mono/diacetyl tartaric acid esters 205–8
titration methods
and L-tartaric acid and its salts 166
and sulphites 74, 79
titrimetric methods, and sulphites 73
TLC methods
and annatto extracts 30
and azpribome 15
and BHA 153
and gallates 142
and polysorbates 190
and sunset yellow 1
total adipate 177

vapour phase Fourier transform infrared spectrometry (FTIR) 74
vegetable juices 167–8
voltammetric methods
and BHA 153
and gallates 142

and nitrites 98
and sunset yellow 1

whey 106, 107, 108, 109–10

wine 75, 80, 84, 85–6, 93, 95, 96, 97

xylenol method, and nitrites 99

yogurt products 236–7, 250