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A liquid chromatographic method for the estimation of Class III caramel added to foods

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An ion-pair high-performance liquid chromatographic (HPLC) method has been developed and validated for the estimation of Class III caramel added to foods. The method is based on measurement of a component of the caramel that has been found in all Class III caramels analysed to date and is considered to be a characteristic marker substance. The abundance of this component is correlated to the amount of ammonia incorporated into the caramel. Since the nitrogen content of Class III caramels from different manufacturers and different formulations varied over a 2.3-fold range, whilst still described as Class III caramels, this variability in the composition of the food additive limits the HPLC method quantitatively to a relative standard deviation of 38%.

A variety of beers, biscuits, gravy powders, savoury spreads, confectionery products and baked foods were tested using the method. There were no false positives and no false negatives. The limit of detection of the method was 0.1 g litre^{-1} for beers and 0.3 g kg^{-1} for solid foods. For biscuit samples supplied by manufacturers with a known caramel content, analysis found 38–92% of the caramel concentration declared by the manufacturer. For beers, the values were 34–78%. Since the analytical recovery from biscuits and beers spiked with caramel and analysed immediately was typically 100 ± 10%, this is taken as evidence that the marker component is only partially stable during the manufacture, packaging and storage of the foods. Indirect support for this postulate was the finding that 3-year-old caramels (ex. 1990) contained on average only 34% of the marker substance compared to fresh (ex. 1993) caramels after allowance was made for the nitrogen content. More controlled stability trials are underway. It is concluded, therefore, that the HPLC method provides a simple, robust and semi-quantitative measure of the presence of Class III caramel in foods. Crown copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Caramel usage

Caramels are widely used to colour and flavour a diverse range of foods. In the UK, for example, caramels are the most widely used food colour additives and comprise approximately 90% by weight of the total colouring agents supplied to the food industry (MAFF, 1993). Caramels are formed by heating low molecular weight carbohydrates, such as dextrose or starch hydrolysate, under a variety of reaction conditions to produce brown polymers. The exact reaction conditions and chemical reactants used are selected to give the caramel its desired characteristics. Reactants include alkali, sulphite, ammonia, or ammonium sulphite, to give Class I, II, III and IV caramels, respectively. Each caramel class is suitable for particular applications. Class III caramels (i.e. those prepared using ammonia) are used to colour principally beers and baked goods and account for almost 70% of the total caramel consumed in UK foods (MAFF, 1993).

Legislative background

Chemical analysis of and for caramel serves the purposes of defining the mixture of caramel components and estimating the quantity added to foods. Regarding

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caramel composition, the colour intensity of the product is the single most important parameter commercially. Further knowledge of the physical and chemical characteristics is required to ensure compatibility with the foodstuff to be coloured and also to ensure that the caramel, as a food additive, meets any regulations or specifications in force. There are legal limits of 10 mg kg^{-1} for 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI) and 250 mg kg^{-1} for 4-methylimidazole (MeI) in the caramel (EU, 1994; EC, 1995). Acceptable methods exist for the detection of THI within a caramel colour (Kröplien, 1986; Lawrence & Charbonneau, 1987; Ding *et al.*, 1991), and also for that of MeI (Wilks *et al.*, 1977).

Regarding caramel levels, caramel is approved for use as a food additive in the European Community *quantum statis*—meaning that there is no maximum level of use specified (EU, 1994). Within the *quantum satis* allowance, there is the principle that colour additives should be used according to good manufacturing practice, at a level no higher than is necessary to achieve the intended purpose, and provided that usage does not mislead the consumer. The one exception to the *quantum satis* allowance for caramel is a proposal for so-called 'traditional foods' (EU, 1995) such as beers brewed according to the Reinheitsgebot for which no additives other than propellant gases may be used.

Also within this regulatory framework is a requirement that member states shall, within 3 years of adoption of the directive, establish systems to monitor the consumption and use of colours and report their findings to the Commission (EU, 1994). Furthermore, that within 5 years the Commission will report to the European Parliament on changes that have taken place in the colours market, usage and consumption. Member states were required to implement the directive by 31 December 1995.

Thus, although the use of caramel colour is unrestricted by the recent EU colours directive, it will be necessary in the near future to have an established method for the estimation of caramel in foods for three reasons:

1. to provide a method of detecting when caramel is added to foods to ensure that it is declared on food labels,
2. to provide a means of measuring levels of added caramel to help establish that added levels are no higher than necessary,
3. to provide a means of establishing that caramel is not added to 'traditional foods' where appropriate, as for some beers.

Review of methods used to date

There has been a large number of studies conducted over the past 20 years for the detection and determination of caramel added to foods. The two most common approaches have been to analyse for the main high molecular weight constituents or for minor low molecular weight components.

As examples of the first approach, Stinson and Willits (1963) used gel filtration, through a cross-linked dextran column, to separate high molecular weight caramel colorant (Class IV) from solutions of sugars and salts used as a model for maple syrup. In similar work, gel permeation chromatography (GPC) produced distinctive patterns for the caramel classes (Hellwig *et al.*, 1981; Frischenschlager *et al.*, 1982), but these GPC traces were not recognisable when foods rather than simply pure caramel solutions were analysed (Frischenschlager *et al.*, 1982). Again using GPC, Lee *et al.* (1987) observed broad peaks indicating the addition of caramel to soy sauce samples, but one sample, known to have caramel added, failed to give the GPC peak. They suggested that possibly a different class of caramel had been used in that one sample. Overall, size exclusion chromatography (in GPC, gel filtration or ultrafiltration modes) appears to be more useful for characterising caramels than for demonstrating its presence and determining the level added to foods.

As examples of the second approach, a number of low molecular weight substances have been proposed as 'marker compounds' to indicate caramel addition. Alfonso *et al.* (1980) used an HPLC method to detect 5-hydroxymethylfurfural (HMF) in caramel and Hewala *et al.* (1993) analysed for HMF in pharmaceutical syrups containing caramel. HMF forms early in the Maillard reaction (Porretta, 1992) and is commonly found in caramels of Classes I and IV. However, HMF is also formed in the natural browning of foods (Tiefenbacher *et al.*, 1983) and therefore it is suitable for the detection of added caramel only in limited cases.

4-Methylimidazole is formed when Class III and Class IV caramels are produced, and has been used to distinguish the class of an unknown caramel (Tiefenbacher *et al.*, 1983). However, since it is known that manufacturers can decrease the concentration of this by-product of caramel when required by legislation, quantitative analysis of MeI would not give a quantitative method for the detection of an unknown caramel colour.

Furans, pyrans, furfurals, pyrroles, pyridines, pyrazines, imidazoles and amides have all been detected as pyrolysis products of high molecular weight melanoidins or caramels (Dross *et al.*, 1987; Hardt & Baltes, 1987; Hussain, 1993; Patey *et al.*, 1985, 1987). In the most recent of these studies, pyrolysis gas chromatography–mass spectrometric analysis of foods containing added Class III caramel was undertaken by Hussain (1993). Principal component analysis, followed by canonical variates analysis (CVA), was used to plot the mass spectral data and allow comparison of the samples. No key masses could be identified as having originated from the Class III caramel and the main ions observed were all typical of carbohydrate. Further work

on the pyrolysis of foods with and without caramel may show its suitability for qualitative detection of caramel, but the instrumentation, statistical expertise and databases on authentic samples are not widely available and this will limit its general applicability.

More recently, two of us (Coffey & Castle, 1994) reported in a technical note that ion-pair HPLC had potential for discriminating the various caramel classes and could provide the basis of a method to estimate Class III caramels added to foods. The development and validation of this method is reported here.

MATERIALS AND METHODS

Materials

Caramel samples representative of current production were obtained in 1993 from caramel manufacturers and food processors. Beers and biscuits with known levels of caramel addition were obtained from manufacturers along with samples of the caramel used for addition. Two batches of biscuits were also prepared in the laboratory and either had no caramel added (for method blanks) or contained caramel at 4.5 g kg^{-1} (on a cooked weight basis).

HPLC grade water was from Fisons (Loughborough, UK), methanol from Rathburn Chemicals (Walkerburn, UK) and pentanesulphonic acid from Sigma (Poole, UK).

Sample preparation for beer

Beer samples in cans or bottles were opened 15 h before analysis to allow degassing to occur. Each sample was then diluted 1 + 9 with water prior to HPLC analysis.

Sample preparation for biscuits

A portion of biscuit (2.0 g) was crumbled into a 50 ml flask and water (50 ml) was then added. The flask was capped and the mixture heated at 60°C for 1 h with shaking at intervals to assist extraction. Toluene (5 ml) was added to the cooled contents with further shaking to effect a clean separation of the solvent phase from the aqueous extract and biscuit solids. A portion (1 ml) of the aqueous extract was passed through a syringe filter (2 μm) ready for HPLC analysis.

HPLC analysis

The HPLC system comprised two Gilson Model 302 pumps with dynamic mixer and a Gilson Model 231 autosampler (Anachem, Luton, UK). Separation employed an isocratic mixture of 5 mM pentanesulphonic acid (ion-pair reagent) in 90:10 water:-methanol, followed by a step gradient up to 100% methanol to wash the column after each analysis. Gradient and flow were controlled by an Anachem Remote Switch Module 502 A. Samples (20 μl) were injected onto the column via a Rheodyne valve (Model 7010) under full-loop fill conditions. The HPLC column was an Alphasil ODS 2 column (HPLC Technology, Macclesfield, UK) of dimensions 25 cm×4.6 mm i.d. with a particle size of 5 μm. A guard column and an in-line filter were used.

Estimation of caramel

A Spectra Physics SP8773XR UV detector was set at 275 nm and the output recorded, integrated and

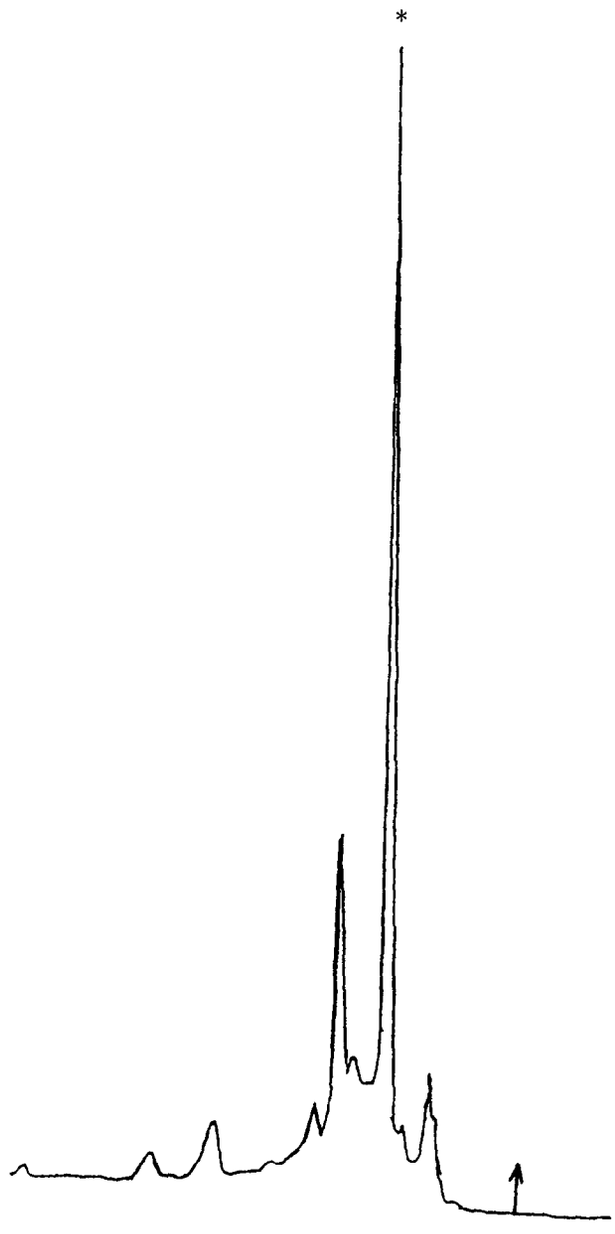

Fig. 1. HPLC trace for a typical Class III caramel (5 μg injected) showing (*) the characteristic marker peak at approx. 3.4 min. *x*-axis, retention time; *y*-axis, = absorbance at 275 nm (2.0 AUFS).

displayed with a Trivector TriLab 2000 (Vinten, Hemel Hempstead, UK) data system. The estimation of caramel content in beers used external standards. For biscuits and other solid foods requiring extraction, the method of standard additions was used. The level of addition was selected to suit the exact sample under analysis, but was typically 0, 3 and 6 g kg^{-1} for biscuits.

RESULTS AND DISCUSSION

Location of a characteristic marker peak in HPLC analysis for caramel Class III

Eleven Class III caramels of 1993 manufacture were provided by three caramel manufacturers and four food producers. These samples were supplemented with a further 13 Class III caramels of 1990 manufacture that were available from earlier work (Coffey & Castle, 1994) and that had been stored in the dark in closed containers at room temperature.

All 24 caramel samples gave a distinctive peak (as yet unidentified) with a retention time of around 3.4 min when analysed by the HPLC method described above. Figure 1 shows a typical chromatogram with the characteristic peak indicated. The more recent (1993) caramel samples showed a 2.3-fold range in nitrogen content (Table 1) and the area of the marker peak reflected this with a 3.5-fold range and with a good correlation with nitrogen content (Fig. 2). The 1990 samples at 3 years old contained on average only 44% of the marker substance (normalised for nitrogen content) compared to the 1993 samples analysed fresh from the manufacturers (compare slopes in Fig. 2). This

Table 1. Class III caramel analysis (1993 production samples)

Sample code	Source	Solids[a] (%)	Nitrogen content[b] (%)	HPLC marker peak area[c]
1	Manufacturer A	69	3.77	19
2	Manufacturer A	70	8.21	54
3	Manufacturer A	66	7.55	34
4	Manufacturer B	67	6.41	44
5	Manufacturer B	67	8.24	52
6	Manufacturer B	67	5.95	34
7	Manufacturer C	60	4.52	16
8	Beer producer 1	60	6.17	20
9	Beer producer 2	70	7.88	43
10	Biscuit maker 1	68	8.70	56
11	Biscuit maker 2	66	8.62	45

[a]Caramel dried to constant weight at 105°C then over phosphorus pentoxide.
[b]Nitrogen content of the dried caramel by CHN combustion analysis.
[c]Arbitrary absorbance/time area units for a constant mass of caramel injected with a 275 nm monitoring wavelength. Values given are the mean of at least three determinations. Repeatability was typically ±2% or better.

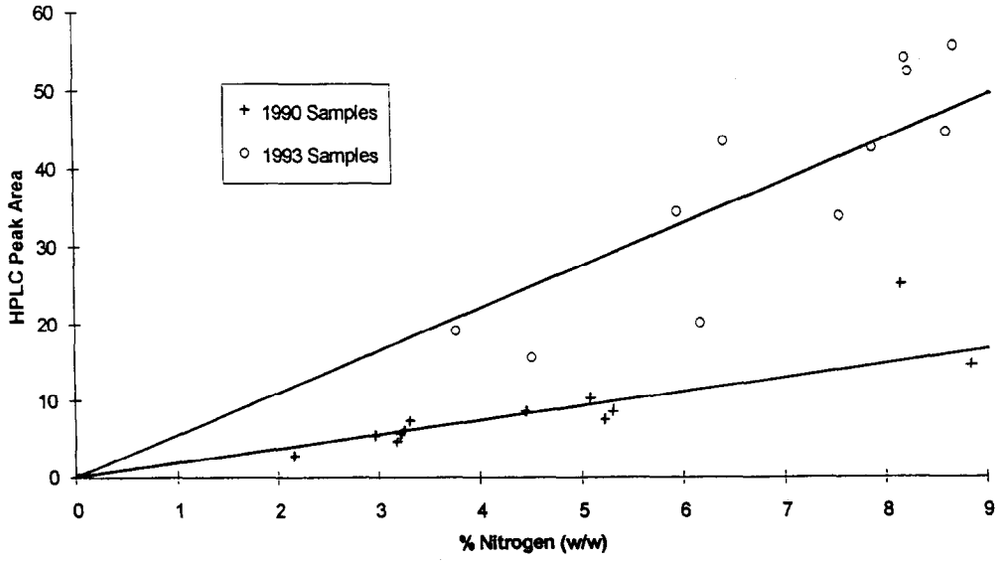

Fig. 2. Correlation of HPLC marker peak intensity versus nitrogen content of Class III caramels. x-axis, nitrogen content (%, w/w) of caramel; y-axis, marker peak area in nominal units per unit mass of caramel injected. ○, 1993 samples; +, 1990 samples.

could be taken as evidence for some instability of the marker substance in the caramel colour, although it should be noted that 3 years is considerably longer than the recommended shelf-life of caramel colours, which is typically given as 6 months. Well-controlled stability tests for the marker substance are underway.

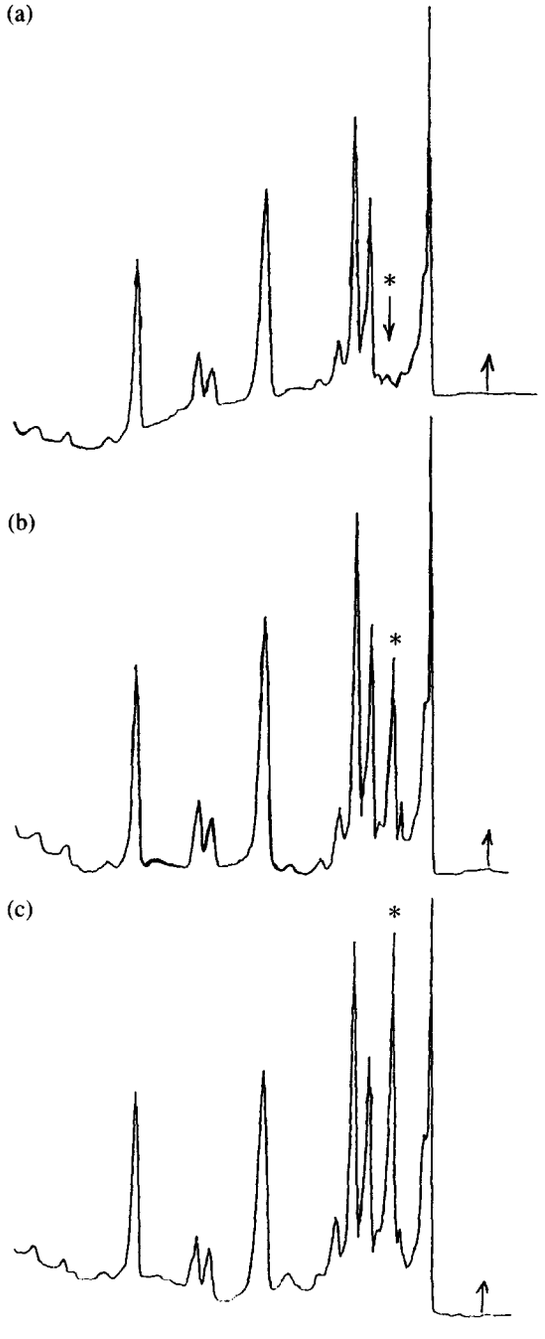

Fig. 3. HPLC traces for the analysis of a lager beer. (a) Sample showing the absence of caramel at expected retention time (*). (b) Sample spiked with caramel at 1.4 g litre^{-1}. (c) Samples spiked with caramel at 2.8 g litre^{-1}.

Analysis of beers

A variety of beers was obtained from brewers with their caramel content declared along with samples of the caramel used. The beers ranged from light beers (e.g. lagers) through medium beers (e.g. bitters) to dark beers (e.g. stouts). The analytical method for beers was very straightforward, involving simple dilution of the sample with water and direct HPLC injection. The beer samples were spiked with caramel standards and then analysed by HPLC for the caramel marker peak at 3.4 min (Fig. 3). The intensity of the marker peak was then used to calculate the recovery of caramel in the analysis. Table 2 gives these recovery data as found/added percentages. Recovery was excellent at between 90–114% for the seven beers tested.

Analytical results for the beers as received are given in Table 3. Quantification of caramel was via the 3.4 min marker peak compared with external standards. For four of the six beer samples in Table 3 made with added caramel, a sample of the caramel used in producing the beer was also available and calibration used that relevant caramel. For two samples, however, a brown ale and a stout beer, no caramel was available from the brewer. For these samples, the calculations were based on a caramel containing 5.5% nitrogen, as a typical example of caramel added for dark beers.

Beers produced without added caramel showed no significant interference at the 3.4 min retention time. Normal beer components eluting at this time gave an effective limit of detection for added caramel of 0.1 g litre^{-1}. This was considered acceptable, since caramel added below this level would serve little useful purpose as a colour additive.

Beers to which the brewer had added caramel all showed the characteristic Class III caramel marker peak at 3.4 min, indicating that its presence was a reliable indicator for the presence of added caramel. When the intensity of this HPLC peak was used to calculate the quantity of caramel found, however, the results were consistently lower than the brewer's stated level of addition. Since recovery from spiked beers was excellent (see above), it is assumed that the marker component is not fully stable during packaging and storage of the beers. As will be discussed later, the identity of the

Table 2. Analytical recovery (%) of caramel from beer samples

Spiked level (g litre^{-1})	Beer sample						
	A	B	C	D	E	F	G
0.6	108	96	86	91	95	99	90
0.9	101	97	91	94	95	103	114

Table 3. Analysis of beer samples for caramel

Sample code and beer type	Caramel declared (g litre^{-1})	Caramel found [a] (g litre^{-1})	Found/ declared (%)
Lager A	Nil	ND	NA
Lager B	Nil	ND	NA
Lager C	Nil	ND	NA
Lager D	Nil	ND	NA
Lager E	Nil	ND	NA
Bitter A	Nil	ND	NA
Bitter B	0.74	0.50	68
Bitter C	1.48	1.08	73
Brown ale A	6.25	2.14	34
Brown ale B	3.1	2.3[a]	74[a]
Stout A	Nil	ND	NA
Stout B	7.38	3.49	47
Stout C	5.4	4.2[a]	78[a]

Nil, none added; ND, not detected (< 0.1 g litre^{-1}); NA, not applicable.
[a]The actual caramel used by the brewer was not stated. Thus, calculations were made on the basis of a caramel standard with 5.5% nitrogen content, which is typical for dark beers.

Table 4. Analysis of biscuit samples for caramel

Biscuit code and type	Caramel declared (g kg^{-1})	Caramel found (g kg^{-1})	Found/ declared (%)
Lab-baked standards	4.60	4.25	92
Lab-baked controls	Nil	ND	NA
Chocolate chip A	Nil	ND	NA
Chocolate chip B	Unknown	4.41	NA
Chocolate-coated	5.97	2.62	44
Bourbon A	5.94	2.28	38
Bourbon B	12.99	11.62	90
Bourbon C	Nil	ND	NA
Ginger A	1.45	1.31	91
Ginger B	Nil	ND	NA
Rich Tea	Nil	ND	NA
Digestive	Nil	ND	NA
Wheat crackers	0.71	0.62	87
Plain	Nil	ND	NA

Nil, none added; ND, not detected; NA, not applicable.

marker component and its stability is the topic of further studies.

Although the added/found values in Table 3 are rather low, they should be viewed in the context of an analysis where the exact brand of caramel added to a food would be unknown to all except the food producer. As discussed above, the intensity of the marker peak showed a 2.3-fold range. Expressed another way, there was a relative standard deviation of ±38% from the population mean for the collection of Class III caramels of current production. This variability was due to the variable composition of Class III caramels with a wide range of nitrogen incorporation seen (Table 1). Thus, where the exact caramel added is not known, a 'typical' caramel must be used for calibration and this would introduce an unavoidable uncertainty.

Analysis of biscuits

As for the beers, a variety of biscuit samples was supplied by producers along with information on the level of caramel added. The biscuits were of a variety of types. Analysis of these baked goods involved a hot water extraction of the caramel followed by de-fatting of the extract using toluene. The aqueous extract thus obtained could be analysed directly by HPLC with quantification by standard additions to the dry biscuit before extraction. Analysis of biscuit samples where caramel had not been used in manufacture revealed the absence of any interferences at the retention time of the caramel marker peak (Table 4). The limit of detection for caramel in these baked goods was around 0.3 g kg^{-1}. Figure 4 shows typical chromatograms for a blank biscuit with and without standard addition of caramel.

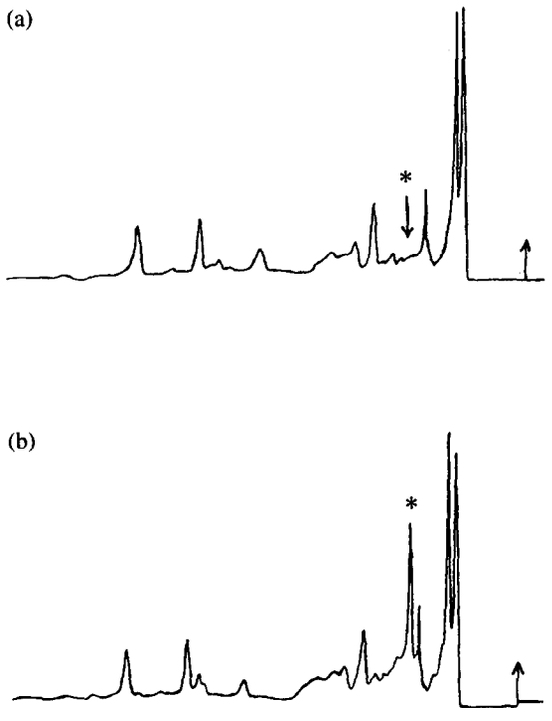

Fig. 4. HPLC traces for the analysis of a Rich Tea biscuit sample. (a) Sample showing the absence of caramel at expected retention time (*). (b) Sample spiked with caramel at 1.5 g kg^{-1}.

The extraction procedure employed had been optimised using the ginger biscuits as a test case. The repeatability of the method is illustrated in Table 5, where five replicate extractions of the lab-baked biscuits were performed. The reproducibility was excellent with a relative standard deviation of 4.6%.

Table 5. Repeatability of the method for biscuit analysis

	Sub-sample					
	1	2	3	4	5	Mean ± SD
Caramel (g kg)$^{-1}$	4.17	4.60	4.72	4.58	4.47	4.5 ± 0.2
Found/added (%)	90.7	100.0	102.6	99.6	97.2	98.0 ± 4.5

Figures 5 and 6 show the HPLC traces for biscuits made with added caramel colour. The biscuit sample for Fig. 5 was a wheat snap cracker made with 0.71 g kg^{-1} caramel. The marker peak is clearly visible (Fig. 5(a)) and was fortified by the addition of caramel standard before extraction and analysis (Fig. 5(b)). The sample for Fig. 6 was a ginger nut biscuit containing 1.45 g kg^{-1} caramel. The chromatogram was more complex around the characteristic retention time, but nevertheless the marker peak was seen clearly (Fig. 6(a)) and again increased in intensity on standard addition (Fig. 6(b)).

Of the six biscuit types made with added caramel, the level of caramel found in four was in reasonably close agreement with the recipe information and found/added values were 87–92% (Table 4). Two samples gave lower values, however, at 38% and 44% (Table 4). These two samples were chocolate-coated biscuits and a Bourbon style biscuit containing a chocolate cream filling. The level of caramel addition by the biscuit manufacturer was declared on a biscuit basis excluding the chocolate or chocolate creams, but this calculation, as with the calculation from recipe formulation to cooked product, must be considered to be subject to some uncertainty.

Analysis of miscellaneous foods

Class III caramels are used to colour many baked products, and foods with high salt content. Thus, in addition to the beers and biscuits, some gravies and savoury spreads, a cake mix, a meringue and confectionery products, including liquorice, were analysed. The extraction method was the same as that used for biscuits and the results are given in Table 6. There was complete

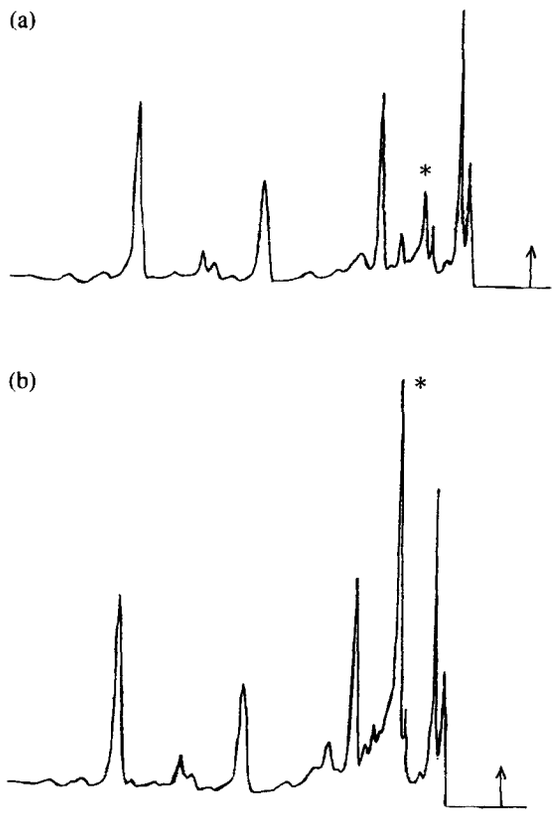

Fig. 5. HPLC traces for the analysis of a wheat snap cracker biscuit sample. (a) Sample showing presence of caramel (*) at 0.62 g kg^{-1}. (b) Sample spiked with caramel at 3 g kg^{-1}.

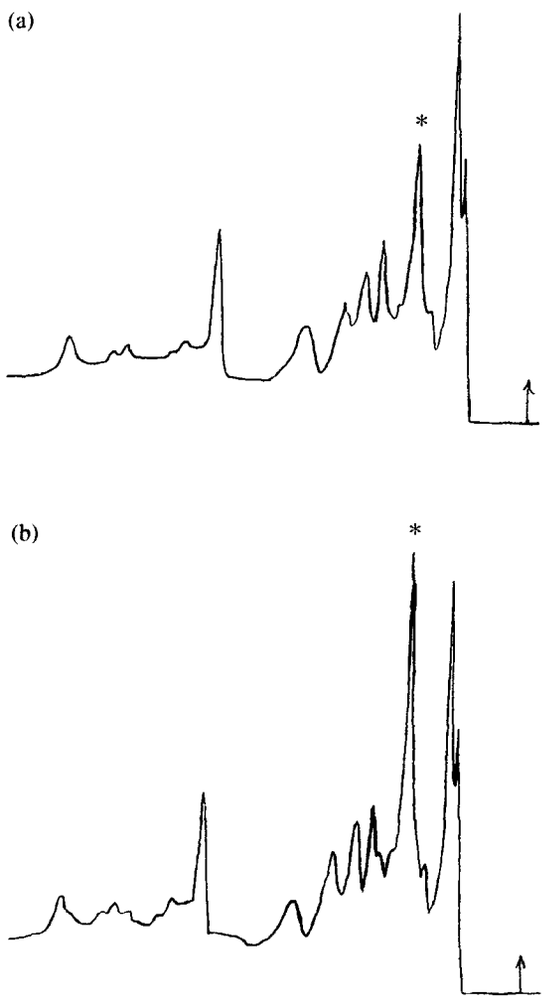

Fig. 6. HPLC traces for the analysis of a ginger nut biscuit sample. (a) Sample showing presence of caramel (*) at 1.31 g kg^{-1}. (b) Sample spiked with caramel at 1.5 g kg^{-1}.

Table 6. HPLC results for caramel content of miscellaneous foods

Sample	Caramel declared on product label?	Class III caramel detected?	Caramel found[a] (g/kg)
Gravy block A	✓	✓	118
Gravy block B	✓	✓	32
Gravy block C	✓	✓	205
Gravy granules A	✓	✓	570
Gravy granules B	✓	✓	38
Yeast extract A	X	X	ND
Yeast extract B	✓	✓	8.2
Beef extract	✓	✓	7.0
Chocolate cake mix	✓	✓	19
Meringue mix	X	X	ND
Confectionery A	X	X	ND
Confectionery B	✓	✓	8.0
Confectionery C	X	X	ND

[a]Based on using a mid-range 1993 caramel as analytical standard (see Fig. 2).
ND, not detected.

agreement between the use of caramel (information from the manufacturer or from the food label) and the detection of caramel by the HPLC method. This indicates that the method is reliable with no false positives or false negatives.

CONCLUSIONS

This ion-pair HPLC method has been shown to be a reliable test for the presence of Class III caramel in foods. The quantitative aspect of the test are limited by two factors:

1. the large variability of caramel composition between manufacturers and even within a single manufacturer's product range intended to cover different food additive applications,
2. evidence for some instability of the caramel marker component.

The variability of caramel composition is well recognised and will thwart all attempts using any *single* chemical index to measure caramel levels more reliably. Further studies are underway in these laboratories to identify the substance(s) that comprise the marker peak used in this analysis and this may assist in understanding the factor of instability. Further chemical analysis is also underway to identify other caramel components that could indicate the level of ammonia incorporation and thus be used to remove some of the uncertainty inherent in the analysis of foods, for which a sample of the actual caramel added is not available.

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