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Methods for the determination of EU-permitted added natural colours in foods: a review

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Abstract

Coupled to increasing consumer demand, food manufacturers have moved towards increased usage of approved natural colours. There is a legal requirement for governments to monitor the consumption of all food additives in the EU to ensure the ADIs are not exceeded, especially by young children. Validated analytical methods are needed to fulfil this requirement. The aim of this paper is to review the available literature on methods of extraction for approved natural colours in food and drink. Available analytical methods for the determination of EU-permitted natural food colour additives in foods and beverages have been assessed for their fitness-for-purpose in terms of their the key extraction and analysis procedures, selectivity and sensitivity, especially with regard to maximum permitted levels, and their applicability for use in surveillance and in an enforcement role. The advantages and disadvantages of available analytical methods for each of nine designated chemical classes (groups) of natural colours in different food and beverage matrices are given. Other important factors such as technical requirements, cost, transferability and applicability are given due consideration. Gaps in the knowledge and levels of validation are identified and recommendations made on further research to develop suitable methods. The nine designated natural colour classes covered are: 1.Curcumin (E100), 2. Riboflavins (E101i-ii), 3. Cochineal (E120), 4. Chlorophylls – including chlorophyllins and copper analogues (E140-141), 5. Caramel Classes I – IV (E150a-d), 6. Carotenoids (E160a-f, E161b, E161g), 7. Beetroot red (E162), 8. Anthocyanins (E163) and 9. Other colours - Vegetable carbon (E153), Calcium carbonate (E170), Titanium dioxide (E171) and Iron oxides and hydroxides (E172).

Keywords: additives, analysis, colours, determination, extraction, food, ingredients, methods, natural, pigments, regulation, validation,

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Introduction

There is a legal requirement to monitor the consumption of all food additives in the EU (and elsewhere) to ensure that acceptable daily intakes (ADIs) are not exceeded, especially by young children. Validated analytical methods are needed to fulfil this requirement. Following the UK Southampton study on the effect of certain azo dyes on children's behaviour, the UK Food Standards Agency recently called for a voluntary withdrawal of certain artificial colours. Coupled to increasing consumer demand, food manufacturers have moved towards increased usage of approved natural colours, especially in children's food.

There are relatively very few standardised or validated methods for the determination of added natural colours in food hence the available literature on methods of extraction and analysis for approved natural colours in food and drink has been reviewed in order to inform and direct future research in this area with special consideration given to validated analytical techniques and their applicability for use in surveillance and in an enforcement role. There is no legal definition of a natural colour hence several disparities in categorization arise. For the purposes of this review natural colours are defined as those which occur in nature but can also be synthesized as nature-identical forms. These may include therefore, mineral, plant and animal products with or without some form of chemical modification. Moreover, the terms 'dye' and 'pigment' have various definitions and are therefore considerd synonymous within the context of this review to avoid confusion. The available information on methods of extraction and analysis of approved natural colours in food and drink has therefore been divided into nine main sections (groups) for the review, which related to the main chemical classes:

1. Curcumin (E100)
2. Riboflavins (E101i-ii)
3. Cochineal – including carminic acid (E120)
4. Chlorophylls – including chlorophyllins and copper analogues (E140-141)
5. Caramels – Classes I – IV (E150a-d)
6. Carotenoids – (E160a-f, E161b, E161g)

7. Beetroot red, betanin (E162)
8. Anthocyanins (E163)
9. Others: Vegetable carbon (E153), Calcium carbonate (E170), Titanium dioxide (E171) and Iron oxides and hydroxides (E172)

For reference, the chemical structures of classes 1-4 and 6-7 are given in Figure 1 and the resonance structures for class 7 is given in Figure 2.

The diversity of the chemical classes of natural colours, the complexities of their structures and the wide range of applications in foods and beverages presents an analytical challenge that in turn requires a diverse range of analytical procedures. The development of efficient extraction procedures is necessary, especially since many of the natural colouring components are unstable and clean up techniques are required to separate the colours from other sample components and co-extractives that are often present in an overwhelming excess. In most cases chromatographic separation techniques are requisite, particularly where a mixture of colouring components is present and where the sample extract contains analytes, degradation products and interfering compounds covering a range of polarities. The identification and quantification of the separated colouring components is usually achieved using spectroscopic procedures such as UV/VIS absorbance that require readily-available instrumentation in common usage in analytical laboratories. While other more sophisticated techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy are becoming more popular as research tools, the relatively high capital costs is still an impediment to their routine use in enforcement laboratories – a fact that must be taken into consideration.

The aim of this review is not to provide detailed critical comparison of an exhaustive list of methods for natural food colours, but rather to provide a review of methods available for the detection and measurement of natural colours present as permitted food additives with a view to their potential for use in a regulatory context. Where knowledge gaps are evident consideration has been given to analytical methods developed for matrices other than foodstuffs and where appropriate, specific analytical techniques are described in more detail.

Group 1: Curcumin E100

Curcumin (Figure 1, I) is the principal colour present in the rhizome of the turmeric plant (*Curcuma longa*). Curcumin is obtained by solvent extraction of turmeric rhizomes to produce turmeric oleoresin, which contain in the region of 37 to 55% curcumin. The permitted solvents are ethyl acetate, acetone, carbon dioxide, dichloromethane, n-butanol, methanol, ethanol and hexane.The purified colouring material (generally referred to as curcumin) is produced by crystallisation from the oleoresin and has a purity level of around 95%, which is the standard commercially-available quality.

Curcumin colour is essentially comprised the major diferoylmethane pigment curcumin (CUR) along with its demethoxy (DMC) and bisdemethoxy (BDMC) analogues (Scotter, 2009). It is an orange-yellow crystalline product that is soluble in ethanol and in glacial acetic acid, to which it imparts a greenish-yellow hue. A solution of curcumin in ethanol is characterised by a pale green fluorescence. It is insoluble in water and in ether. Curcumin is degraded in alkaline solution and is unstable to light, a factor that usually limits its applications in foods (Henry, 1992). No method of assay is prescribed in the EU purity criteria for curcumin (EU, 2008). However, the JECFA specification for curcumin prescribes a method based on spectrophotometric procedure (JECFA, 2010).

Prior to the development of HPLC, the methods most widely used for the determination of curcumin utilised direct spectrophotometry. Reaction with boric acid to form an intensely coloured complex has also been used a basis for the determination of curcumin (AOAC, 1970) but is reported to be prone to interference from co-extractives, however the strong fluorescence exhibited by curcumin has been exploited for its direct determination (Karasz etal, 1973). A method for the determination of curcumin in yoghurt and mustard using direct and derivative spectrofluorimetry has been reported but does not appear suitable for routine application, since the fluorescence intensity is markedly reduced in aqueous systems which limits its use with reverse-phase HPLC systems employing aqueous-based mobile phases that are usually preferred to effect separation (Navaz-Diaz and Ramon-Peinado, 1992).

In recent years, curcumin has undergone extensive preclinical and some early clinical evaluation as a putative cancer chemopreventive agent, and *in vitro* acts as a powerful antioxidant (Heath et al., 2003; Marczylo, et al., 2009). As a result, a number of sensitive analytical methods have been developed for the determination of curcumin in various biomatrices and in turmeric root or preparations thereof in order to study their pharmacological properties and biological effects (Scotter, 2009). According to Scotter (2009), available methods for the determination of curcumin alone in foods are sparse. Curcumin and annatto are often used in combination to achieve a desrired colour shade but because methods for the determination of annatto in foods usually employ alkaline extraction, these are not suitable for curcumin. Navaz-Diaz and Ramon-Peinado (1992) have reported a spectrofluorimetric procedure for the determination of curcumin in yoghurt and mustard, which does not require the use of alkaline extraction media. Samples were directly extracted using acetonitrile followed by filtration and dilution with measurement at λex=397nm λem=508nm. The limit of quantitation calculated from the raw data ranged between 0.1 to 0.4 mg/kg. Replicate analysis (n=3) of three different fruit yoghurts gave mean curcumin levels in the extract of between 85.70±0.34 to 88.69±1.12 ng/mL total curcumin, and for two different mustard samples of 0.29±0.020 and 0.89±0.025 ng/mL. Allowing for sample weight and dilution, these results equate to total curcumin contents of 2.1-2.2 mg/kg in yoghurt (2g sample) and 0.05-0.15 mg/kg in mustard (0.3g sample). Mean (n=3) recovery of curcumin from spiked samples was 105% for yoghurt spiked at 0.25 mg/kg and 94.2% for mustard spiked at 83 mg/kg.

High performance TLC has been used recently for the determination of curcuminoids in *Curcuma longa* germplasm, using silica gel plates chloroform:methanol mobile phase (Paramasivam et al., 2009). Quantitation was achieved using densitometry in the absorption-reflection detection mode at 425nm. Samples were extracted with hexane to remove volatile oils using a soxhlet apparatus and then with benzene to extract the curcuminoids. The toxicity of the latter solvent nowadays precludes its use in most laboratories for these purposes. The detection limit of the method was determined by the minimum mass of curcuminoid that could be detected in a TLC spot, which was 0.1ug. Spiked rhizome extracts were used to determine recoveries of each curcuminoid which ranged from 96.29-98.71% (n=3). Clearly, this method is not

likely to be employed due to the health and safety implications associated with the regular use of benzene as a solvent. A very similar procedure is reported by Phattanawasin, Sotanaphun and Sriphong, (2009) in which the ground rhizomes were extracted with methanol prior to TLC with detection using imaging analysis. The limit of quantitation for the three curcuminoids was in the range 525-972 ng respectively. Reverse-phase TLC using acetonitrile:THF:oxalic acid solvent with quantitation by scanning densitometry has been used to determine turmeric oleoresin (and gardenia yellow and annatto) in a total of 89 foodstuffs in Japan (Ozeki et al., 2000).

HPLC methods for curcumin are reasonably numerous. As far back as 1983, HPLC separation of the three curcuminoids was reported using an amino (-NH_2) bonded-phase column and ethanol (i.e. non-aqueous) mobile phase but the peaks were broad and tailed significantly (Tønnesen and Karlsen, 1983). Nevertheless, this method was reported to give a 10-fold increase in sensitivity when fluorescence detection was used *i.e.* Ex = 420 nm, Em = 470 nm, compared to UV-visible (UV-VIS) detection. The use of UV (254nm) and electrochemical (Ag-AgCl) detectors were compared for the determination of curcumin in turmeric using a similar extraction procedure to that described by Phattanawasin, Sotanaphun and Sriphong, (2009) and a reverse-phase column (Smith and Witowska, 1984). Similar results were found for both detection systems but solvent compatibility was an issue with electrochemical detection and baseline resolution of the three curcuminoids was not achieved.

Where curcumin and annatto have been used in mixtures to provide a required colour shade, a method that could partially separate the three curcuminoids and in turn separate annatto colour components simultaneously using isocratic and non-linear gradient elution has been reported (Rouseff, 1988). An ODS (C_{18}) column was used with a THF: water mobile phase. A much improved separation using gradient reverse-phase HPLC with methanol, acetonitrile and dilute acetic acid, and UV-VIS detection was reported by Jayaprakasha, Rao and Sakariah, (2002) for the determination of curcuminoids in turmeric.

Hiserodt et al (2003) developed a reverse-phase gradient HPLC method for the characterization of the three main curcuminoids as well as other components in turmeric using mass spectrometric detection. Baseline separation of the main

curcuminoids was achieved using a mobile phase comprising ammonium acetate buffer and acetonitrile. Both thermospray and particle beam interfaces allowed for the detection of the curcuminoids and quantitation was achieved using an external standard (technical grade curcumin, 80%) while assuming equal spectrophotometric responses for the curcuminoids at 423nm. Samples were extracted into methanol using a Soxhlet apparatus but no clean up was used. Apart from response linearity and precision (repeatability) data, no other validation date was reported. The validation of an LC-electrospray MS method for the determination of major curcuminoids in foods has been reported (Inoue et al., 2003). Eleven foodstuffs (3 tablets, 3 teas and 5 candies) were analysed for the three major curcuminoids. Samples with expected high curcumin content were extracted into methanol using ultrasonication. Samples with expected low curcumin content were dispersed in water prior to clean up and concentration on a reverse-phase (C_8) SPE cartridge, followed by washing with water and elution with methanol. Extracts were then dried under N_2 at 40°C and reconstituted in a small volume of methanol. LC-MS detection of the curcuminoid ions was achieved in negative ionization mode by monitoring the m/z channels at 307 (BDMC), 337 (DMC) and 367 (CUR). Linearity of response was > 0.999 for all three curcuminoids and recoveries from water spiked at 1 and 10 ug/g ranged between 85.8-92.9% and 91.6-96.5% respectively. The detection limit was reported as 1.0 ng/mL but no limit of quantitation was reported.

HPLC with fluorescence detection has been used for the quantification of curcuminoids in turmeric products (powder, tablet, dressing, beverage and tea) based on a simple methanol extraction using ultrasonication followed by centrifugation if necessary and filtration (Zhang et al., 2009). Quantitation was achieved using an internal standard (2,5-xylenol) and the HPLC system comprised a C_{18} column maintained at 30°C with an isocratic mobile phase consisting of acetate buffer and acetonitrile. The detection wavelengths were 287nm (λ_{ex}) 303nm (λ_{em}) for the internal standard and 426nm (λ_{ex}) and 539nm (λ_{em}) for curcuminoids respectively. Separation was achieved in under 30 mins with baseline resolution and the calibration showed correlation coefficients of >0.993 for all three curcuminoids. The instrumental detection limits for CUR, DMC and BDMC (s/n ratio = 3) were 1.5, 0.9 and 0.09 ng/mL respectively. The relative standard deviations of intra- and inter-day assays for

curcuminoids spiked into turmeric powder at levels of between 1.25 and 15.00 ug/mg were < 6.1% (n=5) with mean recoveries ranging between 94.1 and 104.7%. Wichitnithad et al (2009) used reversed phase HPLC at 33°C with isocratic elution employing a mobile phase of acetonitrile and dilute acetic acid with UV-VIS detection at 425nm, for the determination of curcuminoids in commercial turmeric extracts. Samples were extracted into acetonitrile with ultrasonication and diluted with 50% acetonitrile prior to HPLC analysis. Good baseline separation was achieved for the three curcuminoids and detailed system suitability data reported. Correlation coefficients from calibration standards were > 0.999 and the limits of quantitation were 2.73 ug/mL (CUR), 2.53 ug/mL (DMC) and 0.23 ug/mL (BDMC). The relative standard deviations of intra-day precision were1.22-1.76, 1.07-1.52 and 0.94-1.31 for CUR, DMC and BDMC respectively, and those for the inter-day accuracy (recovery) were all within 98-102%, indicating good method accuracy.

Curcuminoids and annatto have been simultaneously extracted from fish by grinding with Celite and HCl in the presence of ascorbyl palmitate, followed by a hexane wash to remove oils and extraction into acetonitrile in the presence of antioxidants (Scotter, 2009). RP-HPLC with photodiode array (PDA) detection at 422nm with a 10nm bandwidth was used to monitor peaks and confirm their identities. The curcuminoid peaks were clearly discernable from norbixin isomers and interfering peaks. The necessity for controlling the water content of sample extracts in order to achieve optimal peak separation was highlighted. From a simple spiking experiment, the average recovery of curcumin added to minced fish at 8.4 ug/g was 85% (n=10) with an residual standard deviation (RSD) value of 5.0%.

While HPLC is the most common technique used for the determination of curcuminoids, the separation and individual quantitation of the three curcuminoids is important because their extinction coefficients and stabilities vary (Péret-Almeida et al., 2005; Price and Buescher, 2009; Scotter, 2009). Moreover, when used in combination with annatto, the isomers of the yellow coloured thermal degradation products of annatto exhibit similar chromatographic behaviour to the curcuminoids and thus clearly need to be differentiated. Since the curcuminoids are not readily available as pure reference materials they require preparation from turmeric extracts, which is very time consuming. Literature references for the synthesis of pure

curcumin and its bisdemethoxy and demethoxy analogues are available. However, the latter analogue has an asymmetric molecular structure and may only be synthesised as part of a curcuminoid mixture and therefore requires isolation and purification (Scotter, 2009).

The scope of analytical methods for curcumin in foods and beverages must necessarily encompass the diverse range of foodstuffs defined in 94/36/EC that will necessitate the inclusion of appropriate extraction regimes (EU, 1994). For example, curcumin is permitted for use in smoked fish (maximum limit 100 mg/kg) and is often used in combination with annatto. Savoury snacks may also be coloured with curcumin at maximum levels of between100 and 200 mg/kg. Several food types (jams, jellies and marmalades, sausages, patés and terrines, edible external coatings and dried potato products) are permitted to contain curcumin *quantum satis* and hence while quantitative compliance is not a key issue, labelling compliance may be. Sauces and seasonings, and mustard are permitted to contain curcumin up to levels of 500 and 300 mg/kg respectively (94/36/EC). Moreover, compliance with the colouring materials in food regulations is not always straightforward for the food industry. Smoked fish products are a case in point. These are often coloured with curcumin (and annatto) but the addition of these colours to fish, via the most commonly-practised method of brine dipping, is not easily controlled.

Overview and recommendations

Annex III of 94/36/EC prescribes maximum limits for curcumin of between 20 mg/kg (Americano; bitter soda; jams, jellies and marmalades; sausages, pâtés and terrines) and *quantum satis* (margarine, minarine and other fat spreads; pasurmas; dried potato flakes and granules). Annex V Part 2 allows all foodstuffs specified in the range to contain between 50 and 500 mg/kg except for edible cheese rind and edible casings (*quntun satis*). Most modern published methods are able to achieve limits of quantitation of well below 20 mg/kg using HPLC with HPLC-PDA and fluorescence modes of detection. Extraction conditions are generally very simple and various clean up techniques have been reported. but have not been validated for all of the foodstuffs permitted under 94/36/EC. LC-MS(MS) appears to offer enhanced selectivity and sensitivity but there are few methods available and which have yet to be validated. However, methods developed for the analysis of turmeric extracts and biomatrices

could be suitably adapted. Methods must be capable of detecting and quantifying all three of the main curcuminoids CUR, DMC and BDMC so it is necessary to have access to standards of the main colouring principles of known purity. Methods may also be readily adapted to enable the simultaneous determination of curcumin and annatto.

The extraction and analysis conditions for a selection of available methods for curcumin are summarized in Table 2.

Group 2: Riboflavin E101

Commission Directive 2008/128/EC defines riboflavin (vitamin B_2, Figure 1, III) as a synthetically produced yellow or orange-yellow crystalline solid with a slight odour in colour (EU, 2008). It very slightly soluble in water and practically insoluble in alcohol, chloroform, acetone and ether, but very soluble in dilute alkali solutions. It is susceptible to degradation by light. No method of assay is prescribed in the EU purity criteria for E101 (EU, 2008). However, the JECFA specification for riboflavin prescribes a method based on spectrophotometric procedure which is identical to that prescribed for riboflavin-5′-phosphate (JECFA, 2010). Riboflavin is found at low levels in almost all biological tissues and is particularly abundant in meat at ca. 2 mg/kg and in liver at ca. 30 mg/kg, and found at lower levels in cheese at ca. 5 mg/kg) and milk at ca. 1.5 mg/kg (Coultate, 2009). Free riboflavin reportedly occurs very rarely in nature except in milk (Van Niekerk, 1988) and is usually present as riboflavin-5′-phosphate (FMN) and riboflavin-5′-adenosyldiphosphate (FAD). In addition to being used as a food colouring, riboflavin is also used to fortify certain foods for nutritional purposes.

Historically, the levels of riboflavin in foods were determined routinely using microbiological assay, which though sensitive were difficult to carry out. Thus, before HPLC became available, riboflavin was assayed chemically by treatment of the sample with dilute HCl at high temperature to release the protein-bound riboflavin followed by extract clean up and measurement using fluorimetry (Coultate, 2009). The use of HPLC coupled with fluorimetric detection has enabled specific and sensitive methods to be developed for the determination of free riboflavin, FMN and FAD in foodstuffs. Among the very many methods available, riboflavin is often

determined in the free form following hydrolysis of the phosphorylated analogues and/or those bound to proteins during the extraction step prior to HPLC analysis (Ndaw et al., 2000). The extraction usually consists of an acid hydrolysis (typically 0.1M HCl or H_2SO_4 at 100-120°C) to release the analyte from bound protein and to aid the conversion of polysaccharides such as starch to sugars thereby facilitating sample work up. The enzymatic treatment is used to dephosphorylate FMN and FAD, which is usually achieved using acid phosphorylase though takadiastase, clarase and mylase have all been used with varying levels of success. While the EU (and JECFA) specifications do not contain any reference to riboflavin-protein complexes and their use as food colouring materials is therefore not permitted, in the absence of methods developed specifically for permitted riboflavin analogues as food colours, methods available for riboflavin-protein complexes are referred to.

A collaborative study involving twelve laboratories re-evaluated a proposed official method for the determination of vitamins B_1 and B_2 (riboflavin) in nine different foods: baby food, powdered milk, meal with fruits, yeast, cereal (2), chocolate powder, food complement and tube-feeding solution (Arella et al., 1996). Samples were hydrolysed in 0.1M HCl at 100°C for 30 min and after cooling, adjusted to pH 4.5 whereupon enzymatic hydrolysis was performed using β-amylase and takadiastase at 37°C for 18h. HPLC was accomplished on a reverse-phase (C_{18}) column with an isocratic mobile phase consisting of methanol and 0.05M sodium acetate. Fluorimetric detection was used at λ_{ex} = 422nm and λ_{em} = 522nm. The calibration range of standard vitamin B_2 was 0.2 – 1.0 ug/mL. The results for the food analyses ranged from 2.1 to 871 mg/kg and the reproducibility standard deviations (RSD_R%) were generally between 13 and 21% and did not appear to depend upon vitamin concentration. The recovery rate in all foodstuffs analysed was > 89% except for chocolate powder (75%).

Van den Berg et al., (1996) reported on an intercomparison study on methods for the determination of vitamin B_2 and other vitamins in food involving 16 laboratories, arising from an earlier study where it was concluded that the observed high variability in the results obtained was largely due to differences in extraction and hydrolysis procedures. Samples of lyophilized pig's liver, mixed vegetables and wholemeal flour

were analysed by each laboratory using their own 'in-house' method as well as an 'optimized extraction protocol'. Both normal- and reverse-phase HPLC chemical methods and microbiological assay were used. For their 'in-house' extraction and hydrolysis method, all laboratories used acid hydrolysis with HCl at elevated temperature and a combination of enzymes, apart from one laboratory that used H_2SO_4 for hydrolysis. The 'optimal' extraction procedure used 0.1M HCl at 121°C for 30 min followed by adjustment of pH to 4.0 and incubation with 100mg takadiastase at 37-45°C for 18h. After filtration, an appropriate aliquot of extract was taken for HPLC (or microbiological) analysis and results expressed as total riboflavin. Only 2 of the 16 participating laboratories used microbiological assay, the remainder used reverse-phase HPLC with fluorescence detection. The authors concluded that while good agreement was achieved between laboratories using their own 'in-house' methods and no apparent differences between the 'in-house' and 'optimal' extraction protocols were obtained, care had to be taken in selection of the enzymes used as well as the sample:enzyme ratio.

In a study on extraction procedures for the HPLC determination of riboflavin (and other B vitamins) in foodstuffs, the HCl hydrolysis step (100°C 30min) was considered to be superfluous if the protease activity of the selected takadiastase was sufficient or a selection of specific enzymes (protease, phosphatase and α-amylase) were used (Ndaw et al., 2000). Good agreement was obtained between analytical results and certified reference material (CRM) values. The foods studied were yeast, powdered milk, pork, veal, mackerel, wheat flour, porridge oats, rice, peas, orange juice and carrots, and pig's liver and milk powder CRMs. Variable results were obtained when enzymes from various other sources were used highlighting the influence of extraction protocol on riboflavin recovery. However, the method was optimised for the determination of vitamins B-1 and B-6 as well as riboflavin hence hydrolysis protocols were developed accordingly, and diastase with low protease activity did not allow the complete release of protein bound riboflavin.

Tang, Cronin and Brunton (2006) describe a simplified approach to the determination of riboflavin (and thiamine) in beef, pork and pig's liver using reverse phase HPLC with fluorimetric detection, in which extraction was achieved by digestion in 0.1M

HCl followed by enzymatic hydrolysis with readily-available acid phosphatase of defined activity (0.5U/mg). According to the authors, the clara-diastase used by Ndaw et al., (2000) was not commercially available (during 2003 and since discontinued), highlighting a critical issue with methods that prescribe the use of enzymes whose activity and availability cannot be guaranteed. Good agreement was obtained between the analytical result and certified reference material (CRM) value for pig's liver. In a similar study, Jakobsen (2008) optimized an extraction and hydrolysis procedure using a mixture of α-amylase, proteinase and phosphatase in combination with ultrasonication for the determination of riboflavin and thiamine analogues in 16 different foods representing the food groups dairy, meat, vegetables, cereals and yeast. HPLC with fluorescence detection was used to quantify the riboflavin. For comparison a standardised extraction method using HCl followed by takadiastase (45°C 18h) hydrolysis was used for analysis of the samples. Changes in α-amylase amount showed no difference in riboflavin content of liver, whereas varying the amount of phosphatase showed a significant effect on the riboflavin extracted from broccoli, oat flour and dried yeast. No significant effects were observed in the riboflavin content of when varying the amount of protease or the conditions used for ultrasonication. However, the ultrasonication process enabled the enzymatic treatment to be performed within 1h instead of 18h. The detection limit was reported as 20pg, equivalent to a limit of quantitation of 0.1 mg/kg, which is more than adequate for detection of riboflavin at levels necessary for a colouring effect. Both the precision and accuracy of the method were good as was agreement between results for pig liver and milk powder CRMs. Recovery experiments performed on standard solutions and FMN-spiked samples were reported as acceptable (81% and 77% respectively) although results for FMN were marginally lower but nevertheless were judged as acceptable in the light of the low purity of the FMN standard.

Other analytical techniques that have been developed recently for the determination of riboflavin include a biosensor method based on surface plasmon resonance with on-chip measurement (Caelen, kalman and Wahlström,, 2004) and a biochemical fluorimetric method with front-face light emission for the determination of free riboflavin in milk (Zandomeneghi, Carbonaro and Zandomeneghi, 2007). A high throughput planar chromatography method with confirmatory electrospray LC-MS

has been developed for energy drinks (Aranda and Morlock, 2006) and supercritical fluid extraction followed by capillary electrophoresis with fluorimetric detection for the analysis of chicken liver and milk powder (Zougagh and Rios, 2008).

Overview and recommendations

Under Annex III of 94/36/EC, E101 is permitted for use in specific beverages at a maximum concentration of 100 mg/L and in vegetables in vinegar, brine or oil *quantum satis*. Under Annex V Parts 1 and 2 E101 is permitted in a wide range of foodstuffs *quantum satis*. Riboflavin and its analogues are present naturally in foods at a variety of different levels the highest being liver (ca. 30 mg/kg). The levels necessary to achieve the required additive (colouring) effect of riboflavin are expected to be at least as high. The generally accepted analytical technique for determining the riboflavin content of foods is acid hydrolysis followed by enzymatic hydrolysis with measurement by reverse-phase HPLC and fluorimetric detection. While the HPLC conditions are well established and display adequate sensitivity and selectivity, the hydrolysis steps are reported to be not only time-consuming but also to vary in efficacy depending upon the enzymes used and the conditions employed. Conditions for the extraction and hydrolysis protocols require careful consideration since they will be dependent upon sample type. Despite this, methods are readily available to enforcement laboratories, many of which are likely to be familiar with these techniques through the need to determine levels of riboflavin analogues in foods. Published methods are adequately sensitive for the levels of riboflavin added for colouring purposes, such as that described by van den Berg et al (1996) and could be used as a basis for development of a method sufficient in scope to cover all foods permitted to contain riboflavin.

The extraction and analysis conditions for a selection of available methods for riboflavin are summarized in Table 2.

Group 3: Cochineal, carminic acid, carmines E120

Carmines and carminic acid (Figure 1, II) are obtained from aqueous, aqueous alcoholic or alcoholic extracts from cochineal, which consists of the dried bodies of the female insect *Dactylopius coccus* Costa (2008/128/EC). The colouring principle is carminic acid. Carmines are aluminium lakes of carminic acid in which the

aluminium and carminic cid are thought to be in the molar ratio 1:2. Commission Directive 2008/128/EC prescribes the identification and the minimum content of the colouring material (measured by spectrophotometry). The JECFA specification prescribes separate specifications for cochineal extract (JECFA, 2010a) and carmines (JECFA, 2010b) but share a common assay procedure based on spectrophotometric measurement at 494nm after boiling with dilute hydrochloric acid to produce the free carminic acid. The usual product used by the food industry is an alkaline solution of carmine, which is stable at all pH values above ca. 3.5 and is stable to heat, light and oxygen. The hue of carmines can be affected by different cations. Carmine can be formulated as water-soluble powders, lakes and encapsulated products depending upon the desired food application.

Prior to 1986, there were no reported methods for the determination of cochineal in foods using HPLC. Marshall and Horobin (1974) reported a simple spectrophotometric assay procedure for carmine (and carminic acid) stains based on hydrolysis to carminic acid using dilute HCl which gave a linear concentration relationship at 494nm up to ca. 22 mg/L carminic acid. Andrzejewska (1981) developed a method for the identification and determination of cochineal in sausages based on extraction of the colour from defatted sample, adsorption onto polyamide to remove impurities followed by TLC. Three different adsorbents were used along with different mobile phases. The detection limit using silica gel plates with fluorescent indicator was 6 ug, whereas for silica gel without indicator and cellulose, the limit of detection was 15ug. The reported recovery values were non specific at ca. 90%.

A method for the identification of cochineal in raw sausage has been discussed by Wellnitz (1986), where it was demonstrated that cochineal could be recovered by decomposition of the defatted sample using 10% nitric acid followed by filtration, pH adjustment and concentration. Attempts to use cellulose and silica TLC, and RP-HPLC to identify cochineal were not very successful due to the poor solubility of the dye in the mobile phases used, giving rise to poor retention characteristics and peak shape. Moreover, measurement of the colour using spectrophotometry was unsuccessful. Methods employing TLC or direct measurement of cochineal in foods by spectrophotometry permit only at best a semi-quantitative approach.

Jalón, Peña and Rivas (1989) reported the first successful method for the determination of carminic acid in a foodstuff (yoghurt) using HPLC. Samples were incubated with papain at pH 5 to release the colour from the proteinaceous matrix and after boiling with phosphoric acid, the carminic acid was adsorbed on to a column of polyamide. The colour extract was purified by sequential washing with water/acetone/water and desorbed using dilute NaOH solution, concentrated by rotary evaporation after pH adjustment and analysed by HPLC. The HPLC system comprised a reverse-phase (C_{18}) column, a mobile phase of acetonitrile:aqueous formic acid and photodiode array detection at 280 and 500nm. Small variations in the composition of the mobile phase were reported to affect the retention of the carminic acid considerably. Detector response was linear over the range 0.02-5.0 ug/mL (R^2 = 0.9997). The limit of quantification was reported as 0.1 ug/g carminic acid, equivalent to ca. 20-fold improvement on previously published methods. Yoghurt with a mean carminic acid content of 9.52 ug/g analysed 10 times gave an RSD value of 3.0%. Recoveries of carminic acid added to yoghurt at 5, 10, 15 and 20 ug/g gave recoveries of between 87.2 and 95.3% (mean 90.2%). The authors recommended that sample extracts should not be filtered since carminic acid is adsorbed onto filter membranes.

Yamada et al (1993) used methylation with diazomethane as a means of chemical derivatization to facilitate the extraction, isolation and measurement of cochineal by HPLC. Reverse-phase SPE was used to isolate the carminic acid from the food extract prior to derivatisation but several sample preparation regimes were required, especially for meat products were enzyme digestion followed by partition was used. The method was applied to the analysis of beverages and ice flavours, candies, jellies, steamed rice cake, milk beverage, ice cream, ham and sausage. RP-HPLC was used to detect and quantify the colour using acetonitrile:0.1% phosphoric acid as mobile phase and a dual detection system of UV-VIS at 495nm and fluorescence at E_x = 365nm and E_m = 565nm. A limit of quantitation of 0.1 ug/g was achieved with fluorescence detection and was reported to be 10-fold higher than UV-VIS detection. Jelly and milk samples spiked with cochineal gave recoveries of 91.2% (RSD=2.1%) and 89.8% (RSD=2.7%) respectively. Cochineal was detected in 23 of 65 food products analysed over the concentration range 0.9 to 137.7 ug/g. While this method is clearly sufficiently sensitive and shows scope for development, the derivatisation

procedure involves the used of highly toxic diazomethane and also requires the preparation and characterization of a reference standard.

Lancaster and Lawrence (1996) used gradient RP-HPLC with methanol:6% acetic acid mobile phase and UV-VIS detection for the simultaneous determination of carminic acid and annatto colouring components in fruit beverages, yoghurt and candies. The extraction procedures developed by Jalón, Peña and Rivas (1989) were used with modifications although recoveries of carminic acid spiked into yoghurt were in the range 76-83%. The authors reported that cellulose filter membranes in polypropylene housing were suitable for filtration for water-based extracts. Solutions of citric acid and sugar spiked with known amounts of carminic acid (20-200 ug/g) and extracted using a RP-SPE procedure gave recoveries of 91-99%. The acetonitril:dilute formic acid mobile phase reported by Jalón, Peña and Rivas (1989) was not found to be suitable due to rapid deterioration of the column, however modern HPLC phases are far more stable to low pH mobile phases. A simplified extraction procedure for carminic acid in yoghurt, cheese, cookie filling and alcoholic beverage, in which high protein samples were treated with strong alkali followed by strong acid prior to centrifugation, filtration and RP-HPLC analysis has been reported (Carvalho and Collins, 1997). Samples with high lipid content were defatted with hexane with back extraction of the carminic acid into water, whereas aqueous samples were filtered prior to direct injection. Both diode array and fluorescence detection were used with limits of detection of 1 and 1.5 ug/mL respectively. Diode array detection was preferred because it gave better recoveries for carminic acid spiked into water and milk at 99 and 96% respectively.

The most significant study by far for the development and validation of a quantitative method for the determination of carmine in foods is the collaborative study reported by Merino, Edberg and Tidriks, (1997). Carminic acid was extracted by digesting samples for 2 min in boiling 2M HCl, cleaned up using RP-SPE and analysed using C_{18} RP-HPLC with methanol:phosphate buffer mobile phase and UV detection at 280nm. The method was evaluated using internal quality control and collaboration between 11 laboratories. Samples of fruit jelly, liqueur, juice, yoghurt and ice cream were analysed and the limit of detection was 0.1 mg/L. External calibration was used and the calibration curve was linear up to 20 mg/L carminic acid. The mean recovery

range for spiked samples was 85-94% with RSD_R values all below 12%. The authors concluded that the glucose link of carmine has a remarkable resistance to acid hydrolysis, hence reasonably strong acid could be used to break down sample matrices without significant analyte losses. Moreover, when compared to the enzymatic extraction procedure described by Jalón, Peña and Rivas (1989) for yoghurt samples, similar recovery figures were obtained.

Other methods reported for the determination of cochineal in foods using TLC include samples of raw sausage (Brockmann, 1998), retail foods (Itakura et al., 1999) and processed food by TLC (Hirokado et al., 1999). Other HPLC methods include those described by González, Gallego and Valcárcel, (2003) for the determination of carmine and other colorants in dairy samples (LOD 0.02 ug/mL), Lei et al (2007) for sudan dyes, amaranth and carmine in foods (LOD 0.12-0.15 ug/mL, recovery range 76.8-105.9%), and Yu et al (2008) for carmine in carbohydrate foods and meat products (LOD 0.04mg/L, recovery range 88-99%).

A competitive enzyme immunoassay (EIA) procedure using monoclonal anti-carminic acid antibody obtained from mice immunized with carminic acid-IgG conjugate, was reported to have a limit of quantitation comparable to HPLC methods (0.2 ug/g) (Yoshida et al., 1995). Carminic acid was extracted with water from beverage, jelly, candy, pasta sauce, yoghurt and ice cream, whereas ham and fish paste samples required predigestion with pronase and extraction with dilute sodium hydroxide. Recoveries of carminic acid were >95% for milk beverage and jelly, and >85% for yoghurt and fish paste. The method was applied to the analysis of 26 different food samples, 7 of which contained carminic acid in the range 3.5- to 356 ug/g. The production of the antibody is clearly an important limitation to the applicability of this method for routine enforcement purposes. Moreover, the EIA system also responded to laccaic acid, a structural analogue of carminic acid. Capillary electrophoresis has been used for the determination of carmine in beverages, ice lollies and fruit syrups with an LOQ of 7 mg/L and recovery range 95.6-98.0% (Berzas Nevado et al., 1999), in sweets with an LOD of 2 mg/L (Xu, Tang and Wu, 2007), and in milk beverages with an LOD of 0.05-0.40 ug/mL and recovery rate of 101.6% (Huang, Shih and Chen, 2002). Other less well known methods used for carmine determination in foods

include microemulsion electrokinetic chromatography (Huang et al., 2005) and stripping voltammetry (Alghamdi et al., 2009).

Overview and recommendations

The scope of analytical methods for cochineal/carmine in foods and beverages must necessarily encompass the diverse range of foodstuffs defined in 94/36/EC that will necessitate the inclusion of appropriate extraction regimes, many of which are covered by literature reports (EU, 1994). Extraction conditions are generally very simple involving acid hydrolysis with or without SPE, but enzymatic digestion can be used for difficult matrices, particularly meat products which do not feature heavily in published methods. Annex III of 94/36/EC prescribes maximum limits of between 20 mg/kg and *quantum satis*. Annex V Part 2 allows all foodstuffs specified in the range to contain between 50 and 500 mg/kg except for edible cheese rind and edible casings (*q.s*). Most modern published methods are able to achieve limits of quantitation of well below 1 mg/kg using HPLC with UV-VIS or fluorescence detection.

There are relatively few available HPLC-based methods for the determination of E120 in foods, but they are reasonably well established and have been validated for a number of different sample types. RP-HPLC with UV-VIS detection is generally the preferred choice of analysts and will therefore be amenable to application by enforcement laboratories e.g. the method described by Merino et al (1997).

The extraction and analysis conditions for a selection of available methods for cochineal are summarized given in Table 2.

Group 4: Chlorophylls E140(i) and Chlorophyllins E140(ii), and Copper chlorophylls E141(i) and Copper chlorophyllins E141(ii)

E140(i) chlorophylls are obtained by solvent extraction of natural strains of edible grass material, grass, lucerne and nettle. The principal colouring matters are the phaeophytins and magnesium chlorophylls (EU, 2008). The green colour is due to the pigments chlorophyll a (Chl*a*, blue-green) and chlorophyll *b* (Chl*b*, yellow-green) that occur together in a ratio of about 3:1 (Hendry, 1992). The structure of chlorophyll *a* is shown in Figure 1, V. Removal of magnesium from the chlorophylls gives the corresponding phaeophytins *a* and *b* (PP*a*, PP*b*), both of which are olive brown. Replacing the Mg^{2+} with copper (Cu^{2+}) retains the green colour. E140(ii) chlorophyllins are obtained by the saponification of chlorophylls which removes the methyl and phytol ester groups and may partially cleave the cyclopentenyl ring. The acid groups are neutralized to form the sodium or potassium salts of chlorophyllin a and b. E141(i) copper chlorophylls are obtained by the addition of a salt of copper to extracts of chlorophylls *a* and *b* to give the corresponding copper phaeophytins *a* and *b*. Likewise, E141(ii) copper complexes of chlorophyllins are obtained from the addition of copper salt to chlorophyllins to give the sodium or potassium salts copper chlorophyllin *a* and *b*.

It is important to consider that despite a joint initiative introduced by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry, a substantial body of long-established trivial names for chlorophyll and its analogues remains in popular use by both the food colour industry and scientific researchers (Hendry, 1992; Schoefs, 2005). The term 'chlorophyllin' covers a range of compounds identical to, or structurally related to, the porphoryns. These are historically known as chlorin e6 (Ce6), isochlorin e4 (iCe4), their hydroxy derivatives, purpurins 5 and 7 and their corresponding rhodins. In this review, no strict adherence to nomenclature has been made so chlorophyll and its analogues are named as reported in the literature. However, where possible, chlorophyll and its analogues have been described uniformly.

Commission Directive 2008/128/EC prescribes separate definitions and purity criteria for E140(i), E140(ii), E141(i) and E141(ii). The purity specifications include

definition of the source materials (edible plant material, grass, lucerne and nettle), the solvents permitted for extraction (actone, methylethyl ketone, dichloromethane, carbon dioxide, methanol, ethanol, propan-2-ol and hexane) and the identification and the minimum content of the colouring material (measured by spectrophotometry). Seven separate specifications for similar products are prescribed by JECFA, depending upon the production method (e.g. JECFA, 2010a,b), in which the prescribed assay methods are similarly based on spectrophotometry.

4.1. E140(i) and E140(ii)

Due to their complex chemistry, it is important to understand not only the general properties of chlorophylls but also their molecular and spectroscopic properties in particular, since chlorophylls are known to have fragile stability (Schoefs 2002). Almost any type of food processing, alone or combined with another treatment or storage, causes some deterioration of native chlorophyll pigments (Davídek, Velíšek, and Pokornyŷ, 1990; Hendry, 1992; Belitz, Grosch and Schierberle,, 2004). There are many literature references available on the determination of naturally-occurring chlorophylls, which have largely concentrated on their determination in fresh and processed fruit and vegetables (Schwartz, Woo and von Elbe, 1981; Saag, 1982; Roy, 1987; Suzuki, Saitoh and Adachi, 1987), while relatively few are applied to analysis of foodstuffs (Schoefs, 2002, 2003, 2004, 2005). The main strategies of methods for chlorophylls have focussed on solvent extraction, clean up using liquid-liquid partition and measurement using reverse-phase HPLC. Because chlorophylls are particularly labile pigments, appropriate care is required during extraction and analysis. It is generally recommended that manipulations should be carried out in rapidly in darkness or in dim light to prevent photodestruction or photoisomerization, and at relatively low temperatures (Bertrand and Schoefs, 1997). The extended double bond system of the pigments renders them susceptible to oxidation by air, especially during shaking/homogenization and native enzymes such as chlorophyllase should be inactivated. The concentration of acids should be kept very low during extraction in order to minimise loss of chelated metals (i.e. Mg^{2+}). Ammonium hydroxide is sometimes added to acetone extraction solvent in order to ameliorate this effect. Chlorophylls (and carotenoids) analysis in food products has been reviewed by Schoefs (2002) with reference to the difficulties associated with the analysis of complex foodstuffs including their processing and storage, in which an overview of

invasive and non-invasive methods is given. A practical view of the methods is illustrated in a case-by-case review which covers aspects of stability, isolation and analysis (Schoefs, 2003). A range of examples are given which includes details on solvent extraction and partition techniques, chromatographic separation and identification and measurement of chlorophylls in juices and drinks, oils, pasta, fish flesh, leaf and vegetable pureés, and cheese. These last two articles are referenced among others in a comprehensive review of the properties, analysis and degradation of plant pigments (including carotenoids, anthocyanins and alkaloids (betalains)) by Schoefs (2004, 2005). The spectroscopic, molecular and chemical properties are discussed in detail with particular reference to spectral characteristics, chemical modifications occurring during storage, food treatments, enzymatic and heat degradation, acidification and exposure to oxygen and light. The detection of adulterants and quality control issues are discussed within the context of the methods used for analysis and provides a useful review of spectroscopic methods. Additional information on the structure of chlorophyll (and carotenoid) analogues can also be obtained using NMR (Schoefs, 2004). For example, Valverde and This (2008) used ^{1}H NMR (1D and 2D) for quantitative determination of photosynthetic pigments from green beans and reported that it provides more structural information on chlorophyll allomers and epimers than UV/VIS spectroscopy. Twenty-eight discrete chemical shift assignments were reported to give a measurement precision comparable to other methods but with higher discriminatory power compared to UV/VIS spectroscopy which can only discriminate between Chl*a*, Chl*b* and total carotenoids. While NMR is a powerful research tool, it is not likely to find common use in enforcement laboratories due to high capital costs. Other detection modes such as infra-red and circular dichroism have been discussed by Schoefs (2004) but are essentially outside the scope of this review.

Most methods for chlorophyll extraction employ homogenization of the sample with acetone and/or methanol, especially for samples of high water content such as vegetables and algae (the latter outside the EU), which help to break down the pigment:protein complex. Other solvents such as petroleum ether, hexane and *N,N*-dimethylformamide have also been used. Extracts require filtering or centrifugation to obtain clear solutions but back partition into ether facilitated by the addition of sodium chloride or sodium sulfate has been used. Care needs to be exercised when the

relatively large amounts of solvent necessary for pigment extraction require removal prior to TLC or HPLC analysis using techniques such as vacuum-assisted rotary evaporation and blow-drying under nitrogen, due to the degradative effects of heat (Bertrand and Schoefs, 1997; Schoefs, 1998). Solvent partitioning may be used to isolate and purify chlorophyll analogues from crude extracts and especially carotenoids, however allomerization of Chl*a* and Chl*b* can occur (Bertrand and Schoefs, 1997). The authors recommend that solvent partitioning is not convenient for quantitative study since recovery rates may vary.

Reverse-phase (C_{18}) solid-phase extraction (SPE) has been used to fractionate acetone extracts of plants where dephytylated pigments are eluted with 70% acetone and phytylated pigments with 90% acetone (Johnson-Flanagan and Thiagarajah, 1990). Oshima et al (2004) developed a simple RP-SPE method for the isolation and cleanup of PPa and PPb in health foods (chlorella, spirulina, aloe, kale, Jews mallow and green tea leaves) prior to HPLC analysis. Samples were extracted with 85% acetone, which was acidified with HCl prior to SPE isolation. The pigments were eluted with methanol: 0.025M ammonium acetate (88:12). Counter-current chromatography has been used to isolate Chl*a* and Chl*b* from spinach , with particular attention to minimising the effects of light, heat, oxygen, acids and bases (Jubert and Bailey, 2007). Chlorophyll structures and purities were established by HPLC. However, the use of counter-current chromatography as a routine technique is not widespread.

Since chlorophylls and chlorophyllins are not as widely used for the colouring of food compared to carotenoids and anthocyanins, and because chlorophylls have no metabolic importance for animals consuming them, there is correspondingly less work reported on their HPLC analysis in food systems apart from fresh leafy vegetables and olive oils. Chlorophylls have distinct spectroscopic properties; hence absorption or fluorescence can be employed for their identification and quantitation (Eder, 2000). The pigment may be characterized by its absorbance spectrum in a given solvent and quantified using its molar extinction coefficient. This approach becomes complicated when more than one pigment is present. Usually wavelengths of between 430 and 440, and 645 and 660 respectively are used for detection, especially if carotenoids are also present and detection limits of between 1-80ng chlorophyll are reported to be achievable. The JECFA method of assay for chlorophylls relies on the measurement

of absorbance at six specific wavelengths representing the absorption maxima of Chl*a*, Chl*b*, PP*a* and PP*b* respectively in diethyl ether (JECFA, 2010a). The difference in absorption measurements before and after treatment with oxalic acid are used to calculate the content of each analogue. Mínguez-Mosquera et al (1990) used a rapid spectrophotometric method to quantify pigments in fermented olives. Pigment separation was carried out using TLC and the spots scraped from the plate and eluted with ether or acetone. The *a* and *b* analogues of chlorophyll, chlorophyllin, phaeophytin and phaeophorbide were quantified by spectrophotometry using wavelength maxima and extinction coefficient values from literature. Greater sensitivity and specificity can be achieved using fluorescence detection at excitation wavelengths of 409-468nm and emission wavelengths of 650-670nm (Canjura and Schwartz, 1991). The use of scanning wavelength and photodiode array spectrophotometers and spectrofluorimeters allows chlorophyll and its analogues to be characterized and identified following isolation and purification. The fluorescence of some pigments helps aid their identification after TLC or column chromatography. In solution, quantitative detection is achievable provided the solution is dilute enough (ca. mM concentration) to prevent quenching (Bertrand and Schoefs, 1997). The nature of the solvent must also be taken into account since significant differences in emission spectra will be observed between polar and non-polar solvents. The importance of proper procedure for spectrophotometric determination of chlorophyll has been reported by Kouřil et al (1999), who discussed the limitations of the applicability of spectrophotometric methods for the determination of chlorophyll *a/b* ratio in barley leaves extracted with 80% acetone at ambient temperature. They proved that the accuracy of measurement of the chlorophyll content of samples with a Chl *a/b* ratio was dependent upon the absorbance of the measured samples. Moreover, Chl *a/b* ratio measurements can also be distorted by the chlorophyll fluorescence signal , the extent of which is dependent upon sample-detector geometry and is higher in more concentrated samples when light intensity is low.

The importance of pigment environment during spectroscopic determination has been discussed in detail by Schoefs (2005), where solvent, temperature, ligation to protein etc are shown to strongly influence the position and shape of UV/visible spectra, and how this in turn determines which quantitative approach to use i.e. equations or extinction coefficients. The presence of multiple pigments highlights the major

limitations of spectroscopic methods but the use of other techniques such as spectrofluorimetry can be used in structural diagnosis because of the selective excitation of chlorophyll analogues. Infra red spectroscopy can be used to identify pigments on the basis of their particular structural features. The author concludes however that spectroscopic methods usually permit crude identification of pigments in an extract, but in most cases, the specific composition remains obscure. Therefore, obtaining details on the composition of a mixture of pigments requires additional separation (chromatographic) techniques.

Open column chromatography has been used to achieve chlorophyll and carotene separation using phases such as powdered sucrose, DEAE-Sepharose, cellulose or MgO/Hyflosupercel (Schoefs, 2005). Thin layer chromatography is a relatively cheap and easy technique for the separation of pigments but is generally used for qualitative purposes (e.g. JECFA, 2010a). Low-pressure (column) chromatography has been used mainly to separate fractions containing groups of compounds of similar polarity. Among the solid phases used are sucrose, DEAE-sepharose or cellulose. The limitations of TLC have been discussed by Schoefs (2005) particularly in the selection of phase and the separation of similarly structurally compounds. While these techniques have been largely superceded by HPLC, they are still useful for extract cleanup.

HPLC has clear advantages (resolution, speed, reproducibility and sensitivity) over other analytical techniques for the identification and quantitation of chlorophyll analogues and has become the method of choice for analysis of food. The use of photodiode array and fluorimetric detectors allows chlorophyll and its analogues to be characterized and identified following separation by HPLC. Liquid chromatography with mass spectrometric detection is finding increasing use in the identification and quantitative determination of many chlorophyll analogues. While normal phase HPLC has been used to separate chlorophyll and its analogues (especially from carotenes), reverse-phase HPLC is the most widely used technique for routine analysis largely due to the availability of a wide range of reverse-phases (Lea, 1988; Bertrand and Schoefs, 1997; Eder, 2000; Schoefs, 2004; Schoefs, 2005). Simple solvent systems based on e.g. methanol, water and ethyl acetate may be used in conjunction with modern stationary phases where retention is affected largely by analyte polarity. Retention time is generally found to increase in the order: chlorophyllides *b* <

chlorophyllides *a* < phaeophorbides < chlorophyll *b* < chlorophyll *a* < phaeophytin *b* < phaeophytin *a* (Canjura and Schwartz, 1991). Twelve different chlorophyll derivatives from spinach were analysed in under 30 min using gradient elution. Peak identification was based on UV-VIS spectra using photodiode array detection and was confirmed by using fast atom bombardment mass spectrometry. This work was extended to the identification of two zinc analogues using tandem mass spectrometry (MS/MS) to obtain structural information with respect to the presence or absence of the phytyl chain and a localised β-keto ester group (Van Breemen, Canjura and Schwartz, 1991). HPLC has also been used to evaluate the purity of commercially available chlorophyllin (Chernomorsky et al, 1997; Schoefs, 2001, 2002).

LC-MS(MS) methods for chlorophyll and its analogues have beenn developed over recent years to improve ionization efficiency and sensitivity, Airs and Keely (2000), used post column addition of formic acid to improve the LC-MS sensitivity of chlorophyll extracted from spinach, by an order of magnitude (ca. 1uM). Linear response was achieved using atmospheric pressure chemical ionization (APcI). The differences in response between chlorophylls and phaeophytins were due to the presence or absence of Mg^{2+} and overall response exceeded those that could be achieved by absorbance detection. For comparison, this technique was applied to the analysis of bacterial pigment distributions in non-food matrices using high resolution RP-HPLC with ternary and quaternary gradient systems comprising mixtures of 0.01M ammonium acetate, methanol, acetonitrile and ethyl acetate (Airs et al, 2001). Gauthier-Jaques et al (2001) developed a method to track chlorophyll degradation in plant extracts employing RP-HPLC coupled to UV-VIS PDA and LC-MS/MS detectors. The method was successfully applied to the analysis of rehydrated spinach powder and canned beans. Over thirty chlorophyll related compounds were identified by their UV-VIS spectral characteristics and by key MS fragment ions produced using APcI in positive mode. The mass spectra of the chlorophyll species identified by their HPLC retention characteristics and UV-VIS spectra were characterized further by intense protonated molecular ions. MS/MS analysis showed that a major fragment at [M+H-278] is observed with all chlorophyll derivatives possessing the phytyl chain, and at [M+H-338] corresponding to the elimination of $CH_3COOC_{20}H_{39}$. Other characteristic fragment ions were assigned to specific chlorophyll derivatives and

chemical moieties, including several zinc-containing analogues present in processed beans. Allomerization of chlorophyll during sample preparation was identified as a problem since Chl*a* was almost entirely transformed into methoxylactone Chl*a* within 24 hours. The separation and detection of allomers was reported to be more complicated as a result. This key work highlights the importance of being able to identify the main chlorophyll analogues in a foodstuff as well as the potentially large number of degradation products arising not only from food processing but also as artefacts of the extraction process itself. The reader is referred to the paper by Schoefs (2004) which provides an overview of extraction, chromatographic separation and detection of pigments.

RP-HPLC based methods have been used for the determination of chlorophylls and their analogues in a limited range of foodstuffs using UV-VIS, fluorimetric and/or mass spectrometric detection. For example, in green tea leaves (Gross, 1980), olive oils (Hsieh and Karel, 1983; Mínguez-Mosquera et al, 1992), celery leaves (Daood et al, 1989), green olives (Mínguez-Mosquera et al, 1991), spinach (Canjura, Schwartz and Nunes, 1991; Khalyfa et al, 1992; Gauthier-Jaques et al, 2001), kiwi fruit (Cano, 1991), canola seeds (Johnson-Flanagan and Thiagarajah, 1990), beans (Lopez-Hernandez et al, 1993; Gauthier-Jaques et al, 2001), stored fruit (Almela et al, 2000), green peas (Edelenbos et al, 2001), major teas (Suzuki and Shioi, 2003) and health foods (Oshima et al (2004). Very few of these methods have been reported with analytical validation data.

For further information, the reader is referred to the chromatographic and MS techniques reviewed in detail by Schoefs (2005), which includes less widely used methods such as super- and sub-critical HPLC and capillary HPLC. The application of mass spectrometry to pigment analysis is also discussed and pays particular attention to the challenges that chlorophyll (and carotenoid) analyses present. Different ionization modes are compared with respect to their sensitivity as well as the the usefulness of MS^n and LC-MS for identification.

4.2. E141(i) and E141(ii)

The Cu-derivatives of E141(i) and E141(ii) are stable to both moderate heat, light and mineral acids but there remains the possibility for a multiplicity of coloured

components in each group. These are likely to be present as a result of the various extraction and purification processes to meet additives specifications of purity and could include native (i.e. non-coppered) chlorophylls, phaeophytins, phaeophorbides, rhodochlorins (free carboxyl form of phaeophorbides) as well as the principal colouring components (Belitz, Grosch and Schierberle, 2004; Coultate, 2002; Hendry, 1992).

Analytical methods for the determination of E141(i) and E141(ii) in foodstuffs, especially the water soluble forms, are very poorly documented even in comparison to methods for chlorophylls and chlorophyllins. Tsunoda et al, (1993), used TLC to separate copper, iron and magnesium derivatives of chlorophylls and chlorophyllins extracted from 36 different vegetables and 5 chewing gums. Vegetables were extracted with ethanol and partitioned against n-butyl acetate after pH adjustment and chewing gum extracted with n-butyl acetate and hot water prior to partition. Flame atomic absorption spectroscopy was used to discriminate between the metals. Up to 4.91 mg/kg copper chlorophylls and 5.22 mg/kg copper chlorophyllins were found in vegetables, whereas copper chlorophyllins were not detected in chewing gums, but between 0.55 and 1.13 m/kg copper chlorophylls were reported. Amakawa et al. (1993) developed a method for the determination of sodium copper chlorophyll in foods (chewing gum, candies, processed seaweeds, processed edible wild plants and chocolate) based upon suspension of the sample in citrate buffer (pH 2.6) and homogenization with ethyl acetate: acetone. This mixture was extracted with 1% aqueous ammonia solution and ethanol was added to the aqueous layer. The extract was analysed using RP-HPLC on a C_{18} column with a mobile phase comprising methanol:water:acetic acid (100:2:00.5) and photodiode array detection at 625nm. Yasuda et al. (1995) identified copper chlorin e4 (CuCe4) as a suitable indicator for the analysis of sodium copper chlorophyllin in foods. The colouring matter was extracted from a small range of samples (boiled bracken, agar-agar and chewing gum) with diethyl ether after pH adjustment (3-4). Following removal of the solvent, the residue was dissolved in methanol and analysed using RP-HPLC on a C_{18} column with a mobile phase comprising methanol: water (97:3) containing 1% acetic acid. A monitoring wavelength of 405 nm was used and the compounds characterised using photodiode array detection.

Inoue et al., prepared and separated the components of copper chlorophyllin, consisting of copper pheophorbide a (CuPP*a*), copper chlorin e6 (CuCe6), copper rhodin g7 (CuRg7) and copper chlorin e4, using semi-preparative RP-HPLC (Inoue et al., 1994). Separation was achieved using a mobile phase of methanol-water (97:3, v/v) containing 1% (v/v) of acetic acid. Linear calibration plots were obtained for copper chlorophyillin in the concentration range of 0-30 ug/ml with UV-VIS detection at 407 or 423 nm. The detection limits of CuPPa, CuCe6, CuRg7 and CuCe4 were 3.5, 1.5, 3.3 and 1.4 ng/ml respectively. The reversed-phase HPLC method proposed was demonstrated to be useful for the determination of the components of sodium copper chlorophyllin in food colour formulations.

Five samples of commercial copper chlorophyllin preparations were analysed by Chernomorsky et al. using gradient elution C_{18} RP-HPLC with methanol/ammonium acetate/acetone mobile phase and photodiode array detection (Chernomorsky et al., 1997). Analysis revealed several significant differences in the porphyrin compositions of the samples and copper iCe4 was identified as the major component in most commercial materials. Copper complexes of Ce6 (i.e. carboxyl-), PP*a* and unidentified porphyrins with either chlorin or non-chlorin type PDA spectra were found in some samples, which eluted within 15 minutes. Almela, Fernandez-Lopez and Roca (2000) used similar chromatographic conditions to screen chlorophyll derivatives produced during the ripening of fruit. In that study, chromatograms were monitored at 660 nm (PDA) and by using fluorescence (Ex = 440 nm, Em = 660 nm). This method was particularly successful in separating a series of compounds exhibiting a broad range of polarities. This causes some problems because the chlorophyllins are dissociated even at neutral pH and can thus interact hydrophobically with the C_{18} stationary phase. The high concentration of ammonium acetate used in the mobile phase was essential for decreasing proton equilibration times, especially for the ionogenic chlorophyllides and phaeophorbides. Inoue et al. used non-aqueous RP-HPLC to separate and characterise several copper (II) chlorophyll derivatives (Inoue et al., 1988). This work was later extended to the analysis of iron (III) derivatives of chlorophyllin using ion-pair RP-HPLC (Nonomura et al., 1996).

Scotter et al, (2005) developed a method for the determination of E141(i) and E141(ii) in foods and beverages using refined analytical procedures from previously reported methods, covering a range of food colour formulations and foods. Samples (1-5 g) were extracted with citrate/phosphate buffer (pH 2.6) and ethyl acetate: acetone (5:1 v/v). The artificial dye Solvent Green 3 was used as internal standard. Samples such as jelly, boiled sweets and jelly sweets were dissolved in warm water prior to extraction. Certain samples such as dried soup mixes and flour confectionery required prior homogenisation in buffer and biscuit samples were defatted with hexane. Following centrifugation, the upper solvent layer was removed and the extraction repeated if necessary. The solvent was removed by gentle blow-drying under nitrogen at <40°C and reconstituted in acetone: methanol solution with sonication prior to HPLC. Extracts were found to be stable for several days at 4-5°C prior to HPLC analysis. For longer-term storage, the vial was flushed with nitrogen and stored at ca. –18°C. The HPLC system comprised a Vydac 201TP54 C_{18} column thermostatically controlled at 25 °C. A gradient elution programme was used; mobile phase A comprised methanol: 1.0M ammomium acetate (80:20, v/v) and mobile phase B comprised methanol:acetone (60:40 (v/v). A gradient elution programme of 100%A to 100%B over 30 minutes (linear) held 30 minutes at 100% B was used with a mobile phase flow rate 1.0 ml.min-1. Dual detection was used (1) Photodiode array at 650 x 40 nm bandwidth with reference channel at 720 x 4 nm bandwidth (2) Fluorimetric at Ex = 400 nm and Em = 640 nm. Qualitative analysis of analyte components was achieved through detailed peak spectra library matching with reference standards over the wavelength range 300-700nm. Cupro analogues of Ce4, Ce6, PP*a*, Chl*a* and Chl*b* were prepared along with rhodin g7 as reference standards. HPLC calibration was achieved by using a standardized, commercially obtainable sodium copper chlorophyllin reference material and results expressed as total sodium copper chlorophyllin equivalents. The mean (n=11) linear regression coefficient was 0.9980.

The method of Scotter et al (2005) was single-laboratory validated and mint jelly containing declared copper chlorophyllin complex was used as an in-house reference material (IHRM). Recoveries of copper chlorophyllins spiked into 8 different foodstuffs at 14.5 mg/kg (except for ice cream at 70 mg/kg) total polar copper chlorophyllins (CuRg7, CuCe6 and CuCe4), were in the range 79-109%, except for jelly confectionery (49%). Recoveries of total non-polar copper chlorophylls (CuPP*a*,

CuChl*a*, CuChl*b* and Cu-PP) were in the range 77-107%, except for jelly confectionery (50%). The coefficient of variation was generally below 12%. The limit of quantitation of the method was 0.7-1.0 mg/kg total copper chlorophylls and chlorophyllins depending upon sample type. The concentration of total copper chlorophylls and chlorophyllins in foods with declared E141 (lime preserve, lime flavoured jelly, jelly confectionery, mint sauce, ice cream, sugar confectionery and soft drink) ranged from < LOQ to 13.0 mg/kg. The majority of E141-containing foods and colour formulations analysed exhibited a multiplicity of components due to the various extraction and purification processes that are used to obtain these colour additives. This was confounded by the presence of overwhelming amounts of native chlorophylls in certain samples (e.g. mint sauce). Food commodities containing significant amounts of emulsifiers (i.e. ice cream), gelatine or fats were problematic during extraction hence further development of extraction regimes was recommended by the authors.

More recently, HPLC with photodiode array and mass spectrometric detection has been used to characterize the components of five different sodium copper chlorophyllin food colorants (Mortensen and Geppel, 2007). The method employed a C_{30} column in order to separate a larger number of compounds than in previous studies, characterized by their absorption and mass spectra. A gradient solvent system comprising mobile phase A (methanol:water:acetic acid 90:10:0.5) and mobile phase B (*tert*-butyl methyl ether:methanol:acetic acid (100:10:0.5) was used: 0-50% B in 30 min, 50-100% B in 10 min, 100% B for 5min and 100-0% B in 5 min at a flow rate of 1.1 mL/min. In the MS analysis, both electrospray and atmospheric pressure chemical ionization modes were used, in positive and negative modes to obtain the maximum amount of information. Mass spectra of component peaks were characterized by a distinctive splitting due to copper and carbon isotopes and a total of sixteen different components were identified but assignment of minor components was complicated. The chromatograms of the five sodium copper chlorophyllin samples showed clear differences in composition and also showed that Cu iCe4 is present in sodium copper chlorophyllin and not Cu Ce4. In samples that were not too degraded, Cu Ce6, Cu Cp6 and Cu iCe4 were the main constituents, while in samples that were more degraded a more complex pattern of components was observed and the amount of porphyrins was higher. Cu rhodins were observed in one sample only.

Overview and recommendations

The chemistry of chlorophyll and its many analogues is complicated and requires the extraction, separation, identification and quantitation of several components. Suitable specific marker compounds may therefore need to be identified in order to make the methods more readily transferable to enforcement laboratories. This in turn may require the preparation of analogues as reference materials. Where standard colour formulations may be used as reference materials, it is important that colorant profiles are known hence access to standards of the main colouring principles of known purity is crucial. Schoefs (2005) discusses the crucial role that pigment standards have in analytical calibration, where availability is often an issue and that preparation may be necessary for which minimum identification criteria are requisite.

Annex III of 94/36/EC permits the addition of E140(i) and (ii) and E141(i) and (ii) *quantum satis* in a small range of foodstuffs i.e. sage Derby cheese, vegetables in vinegar, brine or oil (excluding olives), jam, jellies and marmalades and other similar fruit preparations including low calorie products (EU, 1994). Annex V Parts 1 and 2 permits their use in all listed foodstuffs *quantum satis*. While most available published methods do not provide data on limits of quantitation, they should be capable of achieving adequate sensitivity when using HPLC with photodiode array detection, and especially where chlorophyll analogues and degradation products can be confirmed using LC-MS. Among these, the extraction conditions are generally very similar but are likely to require refinement for the various food matrices likely to be encountered, particularly for highly processed and compound foodstuffs. SPE cleanup techniques offer scope for isolation and concentration of analytes prior to HPLC. Fluorescence detection (FLU) provides a very useful and sensitive way to distinguish between coppered and non-coppered chlorophyll / chlorophyllin analogues.

Few methods have been validated to any useful extent, and only for a limited number sample types. Methods for native and coppered analogues have been developed separately. Methods therefore require development to increase their scope to cover both native and coppered analogues, as well as cover those foods prescribed in the regulations. Conditions for the extraction and cleanup are reasonably straightforward

but are likely to require refinement to accommodate the increased scope. Methods based on HPLC-PDA and HPLC-FLU (Scotter et al, 2005) and LC-MS(MS) (Mortensen and Geppel, 2007) could be considered for use as a basis for any future development and validation since they appear to offer adequate selectivity and sensitivity for the detection and quantitation of the main chlorophyll/copper chlorophyll analogues, and can also used for the identification and measurement of degradation products .

The extraction and analysis conditions for a selection of available methods for native and coppered chlorophyll and chlorophyllin analogues are summarized in Table 2.

Group 5: Caramels E150a-E150d

Caramel usually occurs as a dark brown to black liquid or solid and is a complex mixture of compounds, some of which are in the form of colloidal aggregates. Caramel is manufactured by heating carbohydrates, alone or in the presence of food-grade acids, alkalis, and/or salts, produced from commercially available, food-grade nutritive sweeteners consisting of fructose, dextrose (glucose), invert sugar, sucrose, malt syrup, molasses, and/or starch hydrolysates and fractions thereof. The acids that may be used are food-grade sulfuric, sulfurous, phosphoric, acetic, and citric acids; the alkalis are ammonium, sodium, potassium, and calcium hydroxides; and the salts are ammonium, sodium, and potassium carbonate, bicarbonate, phosphate (including mono- and dibasic), sulfate, and bisulfite. Food-grade antifoaming agents such as polyglycerol esters of fatty acids may be used as processing aids during its manufacture. Caramel is soluble in water and is used to impart a range of brown colours to foods. Caramel colour preparations can be obtained as viscous liquids or in powder exhibiting a range of physicochemical properties suitable for application in a wide range of foods and beverages. Four distinct classes of caramel can be distinguished by the reactants used in their manufacture and by specific identification tests:

- Class I (Plain Caramel, Caustic Caramel, E150a) prepared by heating carbohydrates with or without acids or alkalis; no ammonium or sulfite compounds are used.

- Class II (Caustic Sulfite Caramel, E150b) prepared by heating carbohydrates with or without acids or alkalis in the presence of sulfite compounds; no ammonium compounds are used.

- Class III (Ammonia Caramel, E150c) prepared by heating carbohydrates with or without acids or alkalis in the presence of ammonium compounds; no sulfite compounds are used.

- Class IV (Sulfite Ammonia Caramel, E150d) prepared by heating carbohydrates with or without acids or alkalis in the presence of both sulfite and ammonium compounds.

Commission Directive 2008/128/EC prescribes separate definitions and purity criteria for E150a, E150b, E150c and E150d (EU, 2008). A general specification for caramel colours is prescribed by JECFA, containing detailed specifications depending for each class of caramel (JECFA, 2010). Both specifications prescribe a series of identification tests for classification/colour binding based on spectrophotometry along with various purity tests, which in the JECFA specification include total nitrogen and total sulfur content.

The addition of the reactants affects the chemical composition of the final product, which in turn determines the functional properties and ultimately the range of application. Thus, caramel colour preparations represent a complex mixture of compounds that can be approximately divided into high and low molecular weight fractions (HMW and LMW respectively). The HMW fraction contains the majority of the coloured components (polymeric melanoidins), whereas the LMW fraction contains the majority of solids. The process conditions used and the concentration of reactants within a given class of caramel will therefore determine the qualitative and quantitative composition, and hence the overall colour intensity. It is generally accepted that each caramel molecule carries a net electrical (colloidal) charge formed during processing. Class I caramel, which is processed using the least number of reactants, carries a slightly negative charge. This is an important feature of caramel colour and determines in many applications which product must be used (Kamuf, Nixon and Parker, 2010). Class II and IV caramels, which are produced using sulfites

have strongly negative colloidal charges and Class III, which is produced using only ammonium compounds, have strongly positive colloidal charge. Colloidal charge is highly influenced by pH, so by changing the pH of caramel solutions, the isoelectric point can be identified. Further pH adjustment will cause the charge to switch to the opposite polarity. The charge of Class III is usually positive up to around pH 5 and the isoelectric point will be between 5 and 7 depending upon the product, and will be negative above that. Class IV caramel has a negative charge above pH 2, the isoelectric point will usually be between 0.5 and 2, and be positive below that. Isoelectric points below pH 4.7 can be determined using the gelatin test. These electrical properties have important implications for analysis of caramels, particularly by capillary zone electrophoresis (CZE). HMW caramel fractions can be characterized using electrochemical methods such as electrophoresis, but also by methods based upon the molecular weights of the complex molecules such as gel permeation chromatography and ultrafiltration. HPLC and GC have been used to characterize the LMW fraction but relatively few compounds have been identified. Most analytical studies on caramels have therefore centred on method-driven characterization i.e. 'fingerprinting' of sub fractions.

Burch, Topping and Haines (2007) have recently reviewed the extraction of caramels from complex food matrices. Licht et al (1992 a and b) characterised caramel colour classes and determined whether there were factors that were consistent for caramel colours within a class, regardless of manufacturer and the end use for which a particular preparation had been made. Size-exclusion (SE) HPLC was used to investigate the HMW fractions and HPLC with ultraviolet (UV) or refractive index (RI) detection was used to investigate LMW compounds. Similar 'fingerprints' were observed for caramels within a class. Conversely, Patey et al, (1985) used GC-FID to analyse silylated LMW fractions which revealed variabilities in the compounds detected and their levels. The technique allowed identification of the manufacturer of UK-produced Class III caramels but not of class type. UK-produced Class I and Class IV caramels and Canadian and Japanese-produced Class II caramels each had a distinctive fingerprint unlike UK-produced Class III caramels. The HPLC data reported by Licht et al. (1992 a and b), sowed differences in peak ratios for compounds common to different caramels. Moreover, the relative amounts of compounds determined by peak height were inconsistent between caramel

preparations. The resulting data were used to develop specifications for caramel colours to ensure the comparability within classes and not for classification of caramel colours after they had been incorporated into foods or drinks, or for quantification purposes (Licht et al., 1992c).

There have been many difficulties associated with the development of methods to extract and quantify caramel colours in foods. This has been due largely to the fact that the chemical constituents of caramels remain poorly defined. The AOAC Official method for artificial colours in distilled liquors includes caramel albeit in a non-specific context, and is based on the marsh test in which the presence of a coloured layer is positive for the presence of caramel, synthetic dye or extractive material from uncharred white oak chips (AOAC, 1995).

Boscolo et al. (2002) developed a spectrophotometric method to determine caramel (made from sucrose) in spirits aged in oak casks, based on the differences between the UV-VIS spectra of oak aqueous alcoholic extracts and caramel solutions in the same solvent. The data were treated by 2 different approaches: (1) a plot of caramel concentration versus the ratio of absorbance at 210 and 282 nm, and (2) a partial least squares (PLS) calibration model using the first derivative of the spectral data. Both methodologies were applied to analysis of 159 aged spirit samples. The mean caramel content of several Brazilian sugar cane spirits (cachaga) and all of the whiskies manufactured in the United States were smaller than that of Scottish whiskies and other brandies from several countries. Correlation was good between caramel concentrations for the same sample calculated by the 2 methods. The uncertainties following PLS and the absorbance ratio method were 0.01 and 0.03 g/L, respectively, for a sample containing 0.45 g/L caramel. Treatment of UV-VIS spectra by pattern recognition using hierarchical clustering analysis and principal components analysis allowed discrimination of the samples as a function of their caramel content. It was possible to distinguish U.S. whiskies from other whiskies. Although caramel quantities as low as 0.08 g/L were detected, the true limit of detection was 0.4 g/L based on a maximum value for measurement uncertainty of 10%.

RP-HPLC with PDA detection was used to profile various Class IV (and for comparison, Class III) caramels and to determine caramel colour added to acerola

juice as an adulterant, where a sequence of four distinctive peaks was used to confirm the presence of caramel colour (Ciolino, 1998). Some attempt was made to ascertain the chemical nature of the marker compounds based on their retention times and UV-VIS spectra, relative to known marker compounds 5-hydroxymethylfurfural (5-HMF), 2-acetyl-4-tetrahydroxybutylimidazole (THI) and 4-methylimidazole) 4-MeI. Based on the peak areas obtained for commercial caramel colours, the limit of detection was estimated at 0.02%. While it was suggested that this approach could also be used to identify which caramel colour preparation had been used (and subsequently used as a reference standard), the suspected adulterant of the acerola juice had apparently already been identified hence the results are largely meaningless if the method was intended to identify and quantify a caramel class of unknown origin.

Over the last 20 years, the Food Standards Agency (previously MAFF) has commissioned several studies to develop suitable methods of analysis of caramel colours, relying largely on HPLC and electrophoretic techniques. Initially, the research was focused on the development of methods to differentiate chemically between caramel classes, which has been reasonably successful given that the approach has been empirically based. However, once added to foods and beverages, the analysis becomes more complicated, largely because of the difficulty in distinguishing between components of caramels arising from their manufacture and those produced during the manufacture and cooking of foodstuffs. Coffey and Castle (1994) reported the presence of a UV-absorbing peak in the RP-HPLC trace of Class III caramels which was not present in the traces for Classes I and IV. (Coffey et al., 1997) demonstrated that the area of this peak was found to correlate with nitrogen content of the caramel. Since the nitrogen content of Class III caramels from different manufacturers and different formulations varied over a 2.3-fold range, the variability in the composition of the food additive limited the HPLC method quantitatively to a RSD value of 38%. No false positives and no false negatives were found when the method was tested on a variety of beers, biscuits, gravy powders, savoury spreads, confectionery products and baked goods. The limits of detection for beers and solid foods were 0.1 and 0.3 g/kg respectively. For biscuit samples supplied by manufacturers with a known caramel content, analysis found between 38% and 92% of the caramel concentration added by the manufacturer. For beers, the corresponding values were 34%-78%. Since the analytical recovery for beers and biscuits spiked

with caramel and analysed immediately was typically 100 ± 10%, this was taken as evidence that the marker component is only partially stable during the manufacture, processing and storage of the foods. This postulate was supported indirectly by the results from the analysis of 3-year-old caramels, which contained only 34% of the amount of marker substance compared to fresh caramels, after allowance was made for nitrogen content.

Based on the work reported by Coffey and Castle (1994), Burch et al., (2002) developed a method for the analysis of Class III and IV caramels in various foods (chicken pie, flavoured rice, stock cubes, soup, marinade, gravy powder/granules, sauce, noodles, malt loaf, marmalade, mustard, soft drinks, biscuit and sweet snack). The Class III marker peak was detected in the HPLC chromatograms of a range of caramel preparations from different manufacturers, and a composite calibration curve was constructed from the peak area versus caramel concentration, using a number of different caramel preparations. Good correlation was seen between the size of the marker peak and the concentration of dry matter in the caramel colour preparation. Food samples were extracted by homogenisation of the sample in phosphate buffer, followed by centrifugation and analysis of the supernatant using RP-HPLC with a methanol/pentanesulfonic acid gradient elution and detection at 280nm. Initial results showed variable recoveries (30-271%) thought to be due to sample inhomogeneity but further work, including analysis of blind samples in a comparison exercise with another laboratory showed improved recoveries. It was noted however that samples containing yeast extract gave high (over) recoveries. Further investigation showed that samples containing yeast extract but no added caramel colour contained a peak eluting at the same time as the caramel marker peak. Resolution of Class III and Class IV caramels was achieved using free-flow zone electrophoresis (FFZE). In all cases, the caramel samples were continuously separated into 96 fractions using FFZE and measured by absorbance at 450nm. The reproducibility of the separation was confirmed using a range of authentic commercial caramels. The fractions contained in the Class IV caramel were further analysed using fluorescent scanning to characterize the marker peaks. The performance characteristics of the Class IV method in terms of recovery and precision were determined in five food and drinks products. Spiked recovery rates of 70-115% and 82-152% were obtained using single and composite caramel calibration curves respectively. Sample precision (n=3) was found to be <

20% RSD. A further 15 commercial products were analysed for Class IV caramel to determine typical usage levels and various recovery was reported for 'blind' samples i.e. 22-148%. Burch (2005) developed and validated this method further (see below).

Capillary electrophoresis has been used to distinguish between caramel classes, taking advantage of the differences in charge of the coloured components in different caramel classes (Royle et al., 1998). Using this technique, electrpherograms (E-grams) showed characteristic migration patterns, where the HMW melanoidins (>3000 Da) migrate as a broad peak, whilst LMW Maillard reaction products (<1000 Da) migrate as sharp, well defined peaks. The method was developed for quantitative analysis of Class IV caramels in soft drinks using sodium carbonate buffer at pH 9.5. A peak corresponding to the coloured component was used to quantify caramel colour solids. Analysis of a range of caramels from more than one manufacturer showed that the migration time of the peak was related to the sulfur level in the caramel and a linear relationship between the caramel concentration in water and the detector peak area was observed over the range 0.1 to 10 g/L. Seven non-cola and twelve cola products containing Class IV caramel of high sulfur and high nitrogen content were reported to contain between 0.57 and 0.82 g solids per litre. Non-cola drinks had estimated concentrations of between 0.60 and 2.84 g solids/L. It was proposed that by estimating the added caramel from an unknown source it would be possible to select a caramel for use as a standard that had a similar sulfur composition, thus improving the accuracy. The data were obtained from caramels from two UK manufacturers; hence more data would be required on caramels from other sources before the method could be considered reliable for use as a quantitative method for caramels in foods. In addition, it was seen that some soft drink ingredients could potentially interfere with quantification due to co-migration with the caramel peak. This notwithstanding, the data showed that the narrow range of caramel concentration in the majority of cola drinks suggested that manufacturers were trying to ensure product consistency, whereas the amount of caramel in the other products analysed varied much more between manufacturers, which was indicative of more distinctive products.

Royle and Radcliffe (1999) extended this work to distinguish between Class I, III and IV caramels using CZE at pH 2.5 and 9.5. The majority of the coloured components were shown to be in the HMW fraction (from ultrafiltration at 5000 Da cut off)

fractions in all caramels. The HMW Class I and Class IV caramel peaks were shown to migrate with a negative charge at both PHs but the Class IV caramel showed several sharp peaks from the LMW components compared to a relatively small peak from the Class I caramels. The Class III caramels showed a HMW peak which was positively charged at pH 2.5. The migration time, and hence the charge, of the HMW peak of Class III caramels was shown to be related to the nitrogen content.

Ames, Inns and Nursten (2000) used the CZE method described by Royle and Radcliffe (1999) to quantify Class III caramel in prepared biscuits and other retail foods using the extraction method described by Coffey et al (1997) based on hot water extraction followed by partition with toluene. Biscuits with or without added caramel colour were compared and while the caramel peak could be clearly seen, a similarly broad peak was apparent in biscuits baked without caramel, although at a lower concentration. The broad peak corresponding to the coloured components could not therefore be used for quantification. An alternative peak was assessed for its suitability as a marker peak for quantification, wherefrom good recoveries from spiked biscuit and dessert-mix samples were obtained (87.2-106.6%). When applied to a wider range of samples (dessert mix, gravy browning, stock cube, liquorice, soup and sauce) Class III caramel was detected in all foods for which it was a declared ingredient but but no recovery data were given. It was observed, however that the presence of salts in the food extracts led to changes in the migration times, and the possibility of mis-identification of the peak used for quantification.

Free flow electrophoresis (FFE) has been successfully used as a way of distinguishing Class III and Class IV caramels prior to RP-HPLC analysis (Aulenta, et al., 2001). Based on the identification of a unique marker compound, good correlation (R^2 = 0.99) was observed between Class III caramel dry matter content and chromatographic peak area. A reliable method, based on ion-pair HPLC and UV detection was developed for the quantification of Class III caramels and applied satisfactorily to a range of foods (instant noodles, dried beef risotto, sweet and sour rice, Cajun marinade and malt loaf). Where food the food matrix (minestrone soup, cake bar, meatballs and malt loaf) gave rise to interfering peaks in the HPLC analysis, FFE separation was carried out prior to HPLC. The marker peak used for quantitation was found to give good correlation with the amount of dry matter in commercial

caramel colours, enabling the construction of a single calibration curve for all caramels tested. The marker peak was not found in foods free of caramel colour and recovery data obtained from spiking of these foods over the range 0.2 to 2.0% was 40-145% using HPLC. The recoveries of added caramel (0.2%) to two malt loaf samples analysed using a combination of FFE and HPLC analysis, were 94 and 21%. A preliminary method, based on FFE and UV-VIS spectrophotometry for quantification of Class IV caramel added to foodstuffs was also developed and tested on a smaller range of samples (minestrone soup, vegetable gravy and cake bars). The correlation between caramel dry weight and absorbance at 450nm for four different Class IV caramels was 0.83. Recovery data obtained from spiking of these foods over the range 0.5 – 2.0% was 52-90% for vegetable gravy and cake bar, and 26-28% for minestrone soup.

The CZE method developed by Royle and Radcliffe (1999) was applied with modifications to the analysis of malted grain and roasted barley, but low-roast barley could not be distinguished from Class III caramel (Ames et al 2001; Royle et al., 2002). Using a dynamic sieving (DS) CE approach, with dextran, it was possible to allocate E-grams in to three groups representative of the degree of roast and colour intensity. Class I, III and IV caramels gave differences on running at pH 9.5 alone and Class III caramel gave an E-gram that was distinguishable from that of a high-roast malt. Capillary isoelectric focusing (CIEF) failed to give satisfactory E-grams for Class I and Class IV caramels due to lack of solubility, but allowed different types of Class III caramels to be differentiated. A malt extract was also clearly distinguishable from Class III caramels.

Wood et al, (2002) developed and validated CZE-based methods to determine the levels of Class IV caramels in soft drinks and Class III caramels in foodstuffs. The repeatability (RSD) of the method was assessed by analysing ten soft drink samples (n=12) and was less than 15%. Three samples analysed repeatedly (n=12) on four occasions on two separate CE instruments gave an overall RSD value of <20%. A second instrument gave higher values for caramel content. The same three samples analysed using the same method but on different CE instruments gave significantly different results ($p<0.05$). Five of ten samples of 'in cup' soft drinks gave significantly different results ($P<0.05$) before and after freezing. The presence of the

permitted sweetener Acesulfame K caused significant interference and an effect on recovery rates. Twelve soft drink samples were analysed by two different laboratories using CZE or FFE. CZE generally gave higher caramel contents than those determined by FFE, and samples with caramel contents <2% gave analytical problems with both techniques. Linear regression analysis of the data for samples of known caramel content gave a better correlation for CZE (R^2 = 0.8636) compared to FFE (R^2 = 0.4962). Two commercial samples showed good agreement between laboratories whereas another two did not. A partly developed CZE method for the determination of Class III caramels in foodstuffs was applied to six food samples containing known amounts of added caramel for comparison of results using CZE and FFE. For two samples, the caramel contents determined by CZE were in good agreement with the added amounts but for a further two samples, problems were encountered with extraction of the caramel from the sample matrix. Similarly, the high salt content of the remaining samples affected the electrolytic capacity of the buffer resulting in a large discrepancy in results. This work highlighted a need for further research into sample and analyte stability and interferences from other food ingredients.

The RP-HPLC method for Class III caramels described by Coffey et al (1997), was refined and validated, and applied to the analysis of foods via a ring trial (Burch, 2005). Preliminary analysis of samples of burger, biscuit and cake spiked with caramel at levels of between 0.1 and 5.0% gave recoveries of 91-135% using the original caramel used for spiking to calibrate the method. The variability in recovery was even wider (102-180%) when a combined calibration standard was used i.e. 6 Class III caramels from different manufacturers – which were found to have a wide variability in marker peak concentration (dry weight basis). In the light of this, further improvements to the extraction and HPLC conditions were made. Extraction conditions were optimised with respect to extraction time, extractant, temperature, pH, protein precipitation, defatting, partition and SPE cleanup. However, no significant improvements were observed. The HPLC method was refined to allow for greater column re-equilibration time and more consistency in marker peak retention time to ameliorate the effects of coeluting peaks. Internal validation of the refined procedure gave recoveries from biscuits spiked at 0.5% and 5.0% added caramel of 94–95% and 90.5-99.6% respectively. For burgers spiked at 0.1 and 1.5%, the recoveries were 106-108% and 104% respectively.

Six laboratories undertook a ring trial to determine the levels of Class III caramel spiked into biscuits at 0, 0.5, 2.0 and 5.0%, and in burger and cake at 0, 0.1, 0.5, and 1.5%. The results indicated clearly that the data reported by one laboratory was more variable than the data reported by the other laboratories. Overall, average recoveries were good with over-recovery observed only for the lowest caramel concentrations in the burger and cake samples. The method was reported to work well for the burger samples, considering both repeatability and reproducibility data. Biscuit and cake samples, however, gave far more variable results. In general with an HPLC method, greater variability might be expected from extraction rather than replicate HPLC injections, but results from the ring trial did not show this. This could be explained by the fact that the marker peak eluted at the beginning of the HPLC run, and was not always completely resolved from interferences. This may go some way to explaining why the cake and biscuit samples gave more variable results than the burger. Moreover, the cake and biscuit samples were baked, and are therefore likely to contain a wide range of potentially interfering compounds produced during heating, whereas the burger sample was analysed raw.

Characterisation of the marker peak was achieved using LC-MS with a modified mobile phase. Initial trials were carried out using a fraction obtained by collecting the marker peak after separation by HPLC and using tandem UV monitoring at 280nm. One main peak was seen in the UV trace of the fraction when analysed by LC-MS. The full scan mass spectrum of this peak in the positive ion mode had a mass ion at 305 m/z, which was assigned as the $[M+H]^+$ ion, equating to a molecular weight of the marker compound of 304. The negative ion full scan mass spectrum showed a predominate ion at 363 m/z, which corresponded to an acetic acid adduct of a compound with molecular weight 304 also. The whole caramel standard was analysed using the same LC-MS conditions which revealed at least six peaks in the chromatogram monitored by UV. The mass spectrum of the peak eluting at 2.38 minutes was found to match that of the marker compound, in both positive and negative ion mode. The mass range 304-306 was used selected ion monitoring (SIM) in subsequent work. Calibration graph obtained using the area of the marker peak obtained using SIM showed considerable non-linearity at higher concentrations, an effect not seen when using UV monitoring, but was linear up to 0.1 mg/ml caramel. It

was therefore proposed that LC-MS offered the potential for quantifying caramel colour in solution.

Reverse-phase HPLC with UV-VIS and/or photodiode array detection is the most common chromatographic technique used for the characterization of caramel classes and the determination of caramels in foods and beverages. Electrophoretic techniques have greatly enhanced caramel analysis capabilities, especially in conjunction with HPLC. However, the specialized instrumentation is not as widespread as HPLC, especially with respect to CIEF, DSCE and FFE techniques. FFE in conjunction appears to work well but recoveries from spiked foodstuffs are variable. LC-MS has had only very limited application but has shed some light on the identity of the LMW marker compound found in Class III caramels.

Existing methods for caramel analysis are empirically based; relying on the measurement of an unknown marker compound and correlating this with the nitrogen or sulfur content. This has been a perennial issue in caramel analysis thus a degree of scientific innovation is required to address this. The other main limiting factors for quantitative analysis are the large variability in caramel composition between manufacturers and evidence for the instability of caramel marker compounds. Several modern analytical chemistry research techniques exist that have the potential to provide more chemical information about the LMW and HMW fractions of caramel. These include modern HPLC stationary phases designed for the separation of highly polar compounds using high-aqueous mobile phases, multiple reaction monitoring by LC-MS/MS and high resolution MS techniques such as time-of-flight (TOF) to identify marker compounds and provide more detail of component profiles, and nuclear magnetic resonance (NMR) spectroscopy. All of these complementary techniques have the potential to provide greater enhancement of analytical capability for the identification and measurement of caramels in foodstuffs. The information provided from applying such research tools may then be used to develop better methods of analysis for caramels in foodstuffs that are applicable in a routine enforcement situation. For example, Monajjemzadeh et al (2009) have used LC-MS/MS to characterize the LMW Maillard reaction products of lactose and Baclofen.

Overview and recommendations

The use of caramel colours is of great importance to the food industry. In the UK for example, caramels account for approximately 90% by weight of the total colouring agents added to food. Of the four classes available, Classes I and IV are used for beverages (whiskies and soft drinks respectively), use of Class II in the UK is negligible, and Class III is used in a wide range of non-beverage foods and in beers. In Annexes III and V of Directive 94/36/EC, caramel colours may be used *quantum satis* in a wide range of foods and beverages (EU, 1994). Current extraction protocols only go some way to providing sufficient scope to cover all of the foodstuffs listed in 94/36/EC hence more development is required, especially clean-up techniques for the removal of interfering components particularly those arising from the presence of yeast extract or those formed during food processing/cooking.

Methods for the determination of caramels in a variety of foodstuffs remain empirically based and limited by variations in between caramel products and stability of analytes. In this context, conditions for the extraction and cleanup are sample dependent and require refinement to widen method scope with subsequent validation, to include the wide range of foodstuffs permitted to contain caramel. Sample extraction protocols and quantitative HPLC and CZE methods are reasonably well established and have been validated for a small number of different sample types with mixed success. In this context, published methods appear adequately sensitive for the levels of caramel added for colouring purposes but for the detection in foodstuffs that are not permitted to contain caramel, sensitivity may be an issue. It is clear that the identification of specific marker compounds for qualitative and quantitative analysis of caramels in foodstuffs is requisite. The methods described by Coffey et al (1997), Royle and Radcliffe (1999), Ames et al (2000), Aulenta et al (2001), Wood et al (2002) and Burch (2005) could be used as a basis for any future development of extraction protocols. Both HPLC and CZE analysis should be considered as a choice of separation and measurement technique since they will provide greater accessibility for enforcement laboratories.

The extraction and analysis conditions for a selection of available methods for beet red are summarized in Table 2.

Group 6: Carotenoids

Carotenoids E160a, E160b E160c, E160d, E160e, E160f, E161b, E161g.

Because of their chemical similarity and the fact that most published methods for carotenoids usually include several members of the class in their scope, the first section covers E160a(i) mixed carotenes, E160a(ii) β-carotene, E160c paprika extract, E160d lycopene, E160e β-apo-8′-carotenal, E160f ethyl ester of β-apo-8′-carotenoic acid, E161b lutein and E161g canthaxanthin. Annatto (E160b) is covered separately.

Carotenoids are responsible for the yellow, orange and red colours of many plants and animals and are used extensively as natural and nature-identical colouring materials in foodstuffs. The major representative carotenoid is β-carotene and like most other carotenoids is formed by lnkage of C_5 isoprenoid units. Most carotenoids contain 40 carbon atoms and can be classified into carotenes, which are hydrocarbons and their oxygenated analogues the xanthophylls. The structure of *trans*-β-carotene is shown in Figure 1, VI. Carotenes tend to be soluble in apolar solvents such as hexane, while xanthophylls are more freely soluble in polar solvents such as methanol and ethanol. Carotenoids are largely unstable, especially in isolation, to light, heat and oxygen so special precautions are necessary during handling. The isoprenoid structure of carotenoids is manifested in a series of conjugated diene bonds that confer useful spectral properties, therefore carotenoids exhibit characteristic absorption spectra in the UV-VIS range that have diagnostic features (such as *cis-trans* isomerism), which are useful for analytical detection and measurement. Such properties are however dependent upon physicochemical factors such as choice of solvent where shifts in λmax and extinction coefficient may occur. A significant effort has been made by the food industry to use carotenoids as colouring materials in foods not only as a replacement for artificial colours but also because of their associated health benefits. Consequently, there are a very large number of published articles available on the extraction and analysis of carotenoids for purposes of identifying new carotenoids, identifying new or better source materials, determining the nutritional profile of foods including *cis*- and *trans*- isomers, studying the health consequences of carotenoid consumption and regulating the use of added carotenoids as food colorants (e.g. Lea, 1988; Schiedt and Liaaen-Jensen, 1995; Lacker, Strohschein and Albert, 1999; Delgado-Vargas, Jiménez and Paredes-López, 2000; Feltl, Pacáková, Štulík and

Volka, 2005; de Quirós and Costa, 2006; Řezanka, Olšovská, Sobotka and Sigler, 2009).

E160a(i) mixed carotenes and E160a(ii) β-carotene

Mixed carotenes are obtained by solvent extraction of natural strains of edible plants, carrots, vegetable oils, grass, alfalfa (Lucerne) and nettle (EU, 2008). β-Carotene is the major carotenoid constituent with minor amounts of α-carotene, γ-carotene, lutein, zeaxanthin and β-cryptoxanthin which may be present. Mixed carotenes may also be produced from natural strains of the algae *Dunaliella salina.* β-Carotene may be produced synthetically, where the *trans-* isomer predominates but diluted and stabilized preparations may have different *cis-/trans-* isomer ratios, and may be obtained by fermentation of the fungus *Blakeslea trispora* mainly as the *trans-* isomer containing ca. 3% of minor carotenoids. Commission Directive 2008/128/EC prescribes separate definitions and purity criteria for E160a(i) and E160a(ii), which include definitions of the source material(s) and the solvents permitted for extraction, the identification and the minimum content of the colouring material measured by spectrophotometry in cyclohexane using a prescribed extinction coefficient between 440 and 457nm (EU, 2008). The results are expressed as β-carotene equivalent. A number of separate specifications for similar products are prescribed by JECFA, depending upon the production method (e.g. JECFA, 2010a,b), in which the prescribed assay methods are similarly based on spectrophotometry.

E160c parika extract, capsanthin, capsorubin

Parika is obtained by solvent extyraction of natural strains of paprika, which consists of the ground fruit pods, with or without seeds, of *Capsicum annuum* L. The major colouring principles are capsanthin and capsorubin but a wide variety of other colouring compounds is known to be present. Commission Directive 2008/128/EC prescribes definitions of the source material(s) and the solvents permitted for extraction, the identification and the minimum content of the colouring material measured by spectrophotometry in acetone using a prescribed extinction coefficient at 462nm. Two separate specifications for paprika oleoresin and paprika extract are

prescribed by JECFA (JECFA, 2010c,d), in which the prescribed assay methods are similarly based on spectrophotometry (oleoresin) and HPLC (extract).

E160d Lycopene

Lycopene is obtained from solvent extraction of the natural starins of red tomatoes (*Lycopersicon esculentum* L.). The major colouring principle is lycopene and minor amounts of other carotenoid pigments may be present. Commission Directive 2008/128/EC prescribes definitions of the source material(s) and the solvents permitted for extraction, the identification and the minimum content of the colouring material measured by spectrophotometry in hexane using a prescribed extinction coefficient at 472nm. Three separate specifications for lycopene extract are prescribed by JECFA (e.g. JECFA, 2010e) depending upon the source material, in which the prescribed assay methods are similarly based on HPLC.

E160e β-apo-8′-carotenal

β-apo-8′-carotenal is a synthetic carotenoid. The major colouring principle is the *trans*- isomer but minor amounts of other carotenoids may be present and diluted and stabilized preparations may have different *cis-/trans*- isomer ratios. Commission Directive 2008/128/EC prescribes a definition and identification procedure, and the minimum content of the colouring material measured by spectrophotometry in cyclohexane using a prescribed extinction coefficient at ca 460-462nm. The specification prescribed by JECFA (JECFA, 2010f) lists an assay method similarly based on spectrophotometry.

E160f ethyl ester of β-apo-8′-carotenoic acid

The ethyl ester of β-apo-8′-carotenoic acid is a synthetic carotenoid. The major colouring principle is the *trans*- isomer but minor amounts of other carotenoids may be present and diluted and stabilized preparations may have different *cis-/trans*- isomer ratios. Commission Directive 2008/128/EC prescribes a definition and identification procedure, and the minimum content of the colouring material measured by spectrophotometry in cyclohexane using a prescribed extinction coefficient at ca

449nm. The specification prescribed by JECFA (JECFA, 2010g) lists an assay method similarly based on spectrophotometry.

E161b lutein

Lutein is obtained by solvent extraction of natural strains of edible fruits and plants, grass, alfalfa (lucerne) and *Tagetes erecta*. The major colouring principle consists of carotenoids of which lutien and its fatty acid esters but minor amounts of other carotenes may be present. Commission Directive 2008/128/EC prescribes a definition and identification procedure, and the minimum content of the colouring material measured by spectrophotometry in chloroform/ethanol or in hexane/ethanol/acetone using a prescribed extinction coefficient at ca 445nm. JECFA prescribed separate specifications for lutein from *Tagetes erecta* (JECFA, 2010h) in which he assay method is based on spectrophotometer and *Tagetes* extract (JECFA, 2010i) in which he assay method is based on HPLC.

E161g canthaxanthin

Canthaxanthin is a synthetic carotenoid. The major colouring principle is the *trans*-isomer but minor amounts of other carotenoids may be present and diluted and stabilized preparations may have different *cis-/trans*- isomer ratios. Commission Directive 2008/128/EC prescribes a definition and identification procedure, and the minimum content of the colouring material measured by spectrophotometry in chloroform, cyclohexane or petroleum ether using a prescribed extinction coefficient at ca 485, 468-472 and 646-467nm respectively. The specification prescribed by JECFA (JECFA, 2010j) lists a simple assay method similarly based on spectrophotometry.

General extraction procedures

There is no standard extraction procedure for carotenoids due to the wide variety of food products and animal samples containing them, and the range of different carotenoids that may be found. This is made more complicated when considering the range of different permitted carotenoid food colours that may be added to a wide range of different processed foods and beverages.

The strong light absorption properties of carotenoids allows microgramme quantities to be visible to the naked eye, which can be seen readily in solution and during chromatography, and facilitates isolation and monitoring of purification steps (Schiedt and Liaaen-Jensen, 1995). The polyene chain is largely responsible for the instability of carotenoids and is susceptible to oxidation with air or peroxides, addition of electrophiles including H^+ and Lewis acids, and *cis-trans* (*Z/E*) isomerization caused by heat, light or other agents. Appropriate precautions should be taken to minimize the detrimental effects of such agents such as the prudent use of antioxidants, carrying out anlayses in subdued light or wrapping containers in aluminium foil, and purging with nitrogen or argon to exclude air. Heat should be avoided whenever possible, so when solvent removal is necessary vacuum-assisted rotary evaporation at low temperature (<40°C) or blow-drying under nitrogen should be used. Carotenoids generally have good solubility in acetone or acetone/methanol mixtures, which because they are miscible with water, facilitate extraction of carotenoids from biological materials that contain water – otherwise the principle of like-for-like solvents such as hexane, diethyl ether and ethyl acetate applies. Halogenated solvents such as chloroform and dichloromethane are used for extraction of carotenoids, usually in combination with other solvents, but traces of HCl may be present that may have an adverse effect on carotenoid stability especially *cis-trans* isomerization. It is recommended that analytical, spectrophotometric or HPLC grade chemicals and solvents are used, and materials such as adsorbents and drying agents must be of satisfactory purity.

Carotenoids should be extracted from samples as rapidly as possible but if immediate extraction is not possible, storage at -18°C or below is recommended. This also applies to sample extracts and carotenoid standards, where storage under an inert atmosphere is recommended. Food samples are usually homogenized immediately prior to analysis or in some cases, the sample and extracting solvent are homogenized together. Antioxidants such as butylated hydroxytoluene (BHT) are added at this stage as well as any acid-neutralizing agents such as tris buffer, and calcium or magnesium carbonate (Eder, 2000). The initial homogenate is usually filtered under vacuum and the extraction repeated on the residue until no more colour is extracted, whereupon the filtrate is partitioned from the water-miscible solvent such as diethyl ether or

hexane into a water-imiscible solvent by the addition of saturated NaCl solution. This procedure effects a suitable clean up of the extract and effectively removes co-extracted water, but it is not often sufficient. Solid phase extraction (SPE) is then often used to clean up and concentrate extracts prior to instrumental analysis. Reverse-phase materials are commonly used but other adsorbent materials such as alumina, silica, magnesium oxide, magnesium and calcium carbonate, calcium hydroxide and diatomaceous earth have been used (Lea, 1988; Eder, 2000; Oliver and Palou, 2000; Feltl et al, 2005). Shen et al (2009) have briefly investigated options for the solid phase extraction of carotenoids, based on the isolation performance of various phases for β-carotene and lutein. Four different phases (C_{18}, C_{30}, diol and silica) were compared using a common frontal analysis technique. The C_{18} and C_{30} phases showed high retention for both carotenoids whereas the diol and silica phases only showed good retention for lutein, which was dependent upon loading solvent polarity, the comparative hydrophobicity of the phases and the presence of oxygen in the carotenoid molecular structure. The method was optimized for analysis of serum and breast milk (after protein precipitation) using a mixed solvent system comprising isopropanol:ethyl acetate:water (1:1:1). Recovery, accuracy and precision compared well with liquid-liquid extraction. Moreover, it was rapid and required no chloroform.

Soxhlet extraction, sonication and other techniques have been replaced by microwave-assisited extraction which can shorten analysis time and improve extraction efficacy (Feltl et al, 2005). Compared to traditional extraction procedures, microwave-assisted extraction is reported to be simple, not limited by solvent selectivity, can circumvent the need to preconcentrate and avoid contamination from solvent impurities.

Supercritical fluid extraction (SFE) is a rapid and selective method that lends itself to automation (Barth et al, 1995; Pfander and Niggli, 1995; Delgado-vargas et al, 2000; Gnayfeed et al, 2001; Schoefs 2004, 2005). However, despite these advantages SFE equipment is not commonplace in enforcement laboratories.

Saponification

Traditionally, saponification (alkaline hydrolysis) has been widely used to facilitate carotenoid isolation. It is particularly effective for removing colourless contaminating lipid material and for destroying chlorophyll. However, there are several methods in the literature that describe methods for the simultaneous determination of carotenoids and chlorophylls, which have been reviewed by Schoefs (2002, 2003, 2005. Saponification also helps to solubilise large quantities of other food components such as proteins and carbohydrates which would otherwise interfere with the extraction and analysis. Saponification will hydrolyse carotenoid esters and it is therefore to be avoided when attempting to determine esterified carotenes such as ethyl ester of β-apo-8′-carotenoic acid (E160f) unless the objective is to measure the free acid. The saponification procedure is usually carried out by the addition of ethanolic or methanolic potassium hydroxide to give an overall KOH concentration of between 5 and 10% in the extraction mixture, usually in the presence of an antioxidant such as ascorbyl palmitate. Various temperature regimes are used for the saponifaction, depending upon the required speed of analysis or on the requirement for minimal carotenoid degradation. These regimes may range from refluxing the extract under a stream of nitrogen for 5-30 minutes, to low temperature (4-25°C) agitation under an inert atmosphere overnight. The choice of regime may also depend upon the sample composition. Saponification is usually followed immediately by liquid-liquid partition into diethyl ether or a mixture of diethyl ether and hexane (or petroleum spirit) to isolate the unsaponifiable fraction prior to solvent removal and further clean up, specially to remove sterols which are often present in meat and dairy products, and some fruits. The extract is then washed several times with water to remove residual KOH.

It is important that the formation of artefacts is minimized during extraction, saponification and work-up e.g multiple *cis*- isomers and oxidation products. Careful monitoring by separation methods such as thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) is therefore necessary to recognize any effects, and to identify and measure the products. de Quirós and Costa, (2006) have summarized different saponification conditions in various samples and recommended that as a general rule, samples with low fat content should be saponified under mild conditions and high-fat samples under stronger conditions. The authors cite the work

of Fernandez et al (2000) who compared the alkali saponification with enzymatic hydrolysis for the determination of the total carotenoid composition in palm oil and obtained higher results with the enzymatic process. Conversely, Scotter et al (2003) reprted improved recoveries of β-carotene and β-apo-8′-carotenal added to high-fat foodstuffs when using enzymatic (lipase) hydrolysis compared to saponification. Saponification has been reported to lead to significant carotenoid losses of 12.6 +/- 0.9% (Biehler et al, 2010; see below).

Spectrophotometry

Spectrophotometric analysis has traditionally been used for the quantitative determination of carotenoids. The amount of carotenoid present is calculated by reference to a specific absorption coefficient, as prescribed in 2008/128/EC and the various JECFA specifications. In industry, specific absorbance at 1% concentration in a 1cm cuvette ($E_{1cm,1\%}$) at a specified wavelength (usually λmax) is generally used rather than the molar extinction coefficient. The $E_{1cm,1\%}$) values for some common carotenoids have been published (Britton, 1992). For example, the EU spectrophotometric method of assay for mixed carotenes is based on absorbance measurement under the conditions prescribed under for assay and identification (2008/128/EC). Quantitative analysis is achieved by reference to the $E_{1cm,1\%}$ value of 2500 at approximately 440-457nm in cyclohexane. The value given in the EU specification is widely accepted for analysis of carotenes of unknown composition or for mixtures of carotenes, with the results expressed in terms of the main component which in this case is β-carotene (Scotter, 1997).

Because of the inherent variation in the carotene content of mixed carotene formulations, it is not possible to obtain by experimentation a more meaningful value for the specific absorbance. This accepted, there are several ways in which the spectrophotometric assay procedure can be improved. The wide range in λmax values given in the specification is necessary because of the variation in carotene content as well as isomerisation and solvent effects. The position of the main absorption band of carotenoids is dependent upon the refractive index of the solvent used hence any shifts in λmax observed upon changing solvent depends upon the solvent polarizability (Britton, 1995). In practice, λmax values for any given

carotenoid are similar in hexane, diethyl ether, ethanol, methanol and acetonitrile, but pronounced and consistent λmax shifts may be observed in other solvents such as acetone (2-6 nm), chloroform and dichloromethane (10-20 nm) and benzene (18-24 nm) (Britton, 1995). The carotene content (as % β-carotene) of three mixed carotene samples was determined according to the procedure prescribed in the EU specification (Scotter, 1997). The results compared favourably with those obtained by HPLC and with the manufacturer's figures.

Biehler et al (2010) have recently compared three published spectrophotometric methods for the determination of carotenoids in frequently consumed fruits and vegetables. The methods were based on a rapid extraction protocol and spectrophotometric measurements to determine the total amount carotenoids present in fruits and vegetables (n = 28), either with or without chlorophyll. Two published methods were compared with a newly developed method based on the average molar absorption coefficient (135310 $Lcm^{-1}mol^{-1}$) and wavelength (450 nm in acetone), for 5 predominant carotenoid species (β-carotene, zeaxanthin, lycopene, lutein, β-cryptoxanthin) and the results compared to HPLC. To avoid overestimating carotenoid concentrations due to chlorophyll A and B presence, the effect of saponification was studied for all methods. Overall, saponification led to significant carotenoid losses (12.6 +/- 0.9%). The three spectrophotometric methods and the HPLC method yielded 5.1 +/- 0.4 mg/100 g, 4.6 +/- 0.5 mg/100 g, 4.3 +/- 1 0.5 mg/100 g, and 4.2 +/- 0.5 mg/100 g total carotenoids respectively. One spectrophotometric method gave significantly higher mean concentrations compared to all other methods ($P < 0.001$), while the other two spectrophotometric methods were not significantly different and highly correlated compared to HPLC ($R^2 > 0.95$). The authors concluded that while rapid screening methods therefore desired, their accuracy varies depending on the carotenoid profile and the matrix of the plant food and method selection is therefore important.

While spectrophotometry is useful for assaying the total carotenoid content of a sample and provides spectral information which aids the identification of carotenoids and their geometric isomers, the technique is not specific. This is exacerbated by the fact that accurate carotenoid content of sample extracts containing several different

carotenoids and their isomers is affected by the solvent(s) used, which in turn affects the position and absortivity of λmax values. Thus for more accurate and precise measurement of carotenoids in food extracts, separation techniques such as HPLC coupled to sensitive and selective detection systems are favoured.

Separation techniques

Suitable techniques are required to separate carotenoids from the food extract and to purify individual carotenoid components. The strategy employed depends largely on the quantity and nature of the sample material available, the properties of the target carotenoid analytes, the nature of the sample matrix interferences, and the qualitative and quantitative information required. Partition and adsorption techniques for carotenoid extraction discussed above notwithstanding, chromatographic separation techniques have a long and successful application to carotenoids. Due to advances in adsorbent technologies, thin-layer chromatography (TLC) still remains in common use not only because of its simplicity and relatively low cost, but also because it is very often used to separate carotenoids from crude extracts prior to analysis using another method, such as HPLC (Isaksen and Francis, 1986; Schiedt, 1995; Delgado-Vargas et al, 2000). More recently, high-performance TLC has been used to detect lycopene added to alcoholic and non-alcoholic beverages (Oliver and Palou, 2000). The separating power of modern HPLC techniques coupled to highly selective and sensitive detection systems such as photodiode array (PDA) and mass spectrometry (MS) means that TLC has been largely superseded as an end-determination technique.

HPLC systems

HPLC is recognized as the ideal technique for the separation, identification and quantitation of carotenoids in that it possesses greater speed, sensitivity, resolution and reproducibility than traditional techniques such as spectrophotometry and TLC. While a number of published articles on carotenoid analysis by normal phase HPLC are available (Eder, 2000), the vast majority of HPLC methods for carotenoids are carried out by reverser-phase (RP) utilizing a wide range of bonded-phase chemistries. Separated peaks can be monitored at several different wavelengths in the UV-VIS range with simultaneous acquisition of peak spectra for identification purposes using PDA. Similarly, HPLC with MS detection allows identification and

selective detection of carotenoids via their characteristic fragmentation properties (see below). The problems associated with the analysis of carotenoids in foods by HPLC have been discussed (Scott, 1992) in which the importance of column choice and the potential for interaction between carotenoids and metal surfaces and mobile phases were addressed.

RP-HPLC solvent systems are usually based on a mixture of polar and non-polar solvents with or without water, depending upon the separation required. Isocratic elution is usually adequate for separation of a small number of target analytes but gradient elution with two or more solvent systems is often required for the separation of large numbers of carotenoids covering a wide range of polarities. Reverse-phase HPLC will elute the more polar xanthophylls first followed by the carotenes. There are a great number of specific applications available, however solvent compositions given in the literature are often so precise that reproducibility is sometimes difficult to achieve. According to Eder (2000), the commonly used stationary phases are those with C_{18} (ODS) bonded chains, the performances of which are influenced by the extent of end-capping and the carbon loading. Eder cites the work of Bushway (1985) and Epler et al (1992) who compared various RP (and normal phase) methods and found that C_{18} columns provided the most efficient separation of carotenoids, the latter under standard conditions. The cis- and trans- isomers of □-carotene and lutein (as well as several other carotenoids) in ethanolic extracts of bean leaves have been successfully separated using C18 RP-HPLC using gradient elution with mixtures of acetonitrile, methanol and dichloromethane, and PDA detection (Schoefs et al., 1995, Darko et al, 2000). More recently, RP columns with up to C_{30} bonded chains have been used, particularly for the separation of *cis-trans* isomers (Lacker et al, 1999; Delgado-Vargas et al, 2000). It is important at this stage to note that losses of carotenoids, particularly lycopene, may be observed when in contact with stainless steel column frit materials (Scott, 1992; Craft et al, 1992). This can be solved by the use of polyethylether ketone (PEEK) alloyed with Teflon frit materials (Konings and Roomans, 1997).

There is no doubt that PDA detectors have added a powerful dimension to the detection and measurement of carotenoids in HPLC analysis, reflected in the large number of literature references reporting their use. Carotenoids absorb very strongly

in the visible region between 400-500nm, with *cis*- isomers exhibiting an additional absorption band around 320nm. The spectra of carotenoids are therefore very characteristic and provide detailed structural information by the position and relative intensity of absorption bands, which can be used for the simultaneous determination of HPLC peak purity (Britton, 1995; Scotter et al, 1994). PDA detectors are relatively cheap and easy to operate due to advances in data processing technology. According to Feltl et al (2005), typical characteristics of quantitative determination of β-carotene in olive oil containing 2.75 mg/kg are RSD of <5% and a correlation coefficient of R^2=0.999 over the calibration range 0.5 to 5 ug/mL. The sensitivity of the method was reported as limit of determination (LOD) 5.5ng and limit of quantitation (LOQ) 31.0ng.

The method developed by Scotter (1997) used to determine the carotene content of three commercially available mixed carotenes formulations is an example of the typical HPLC conditions used. The system comprised a reverse-phase column (Vydac 201TP, 250 x 4.6 mm) which contained a 300Å pore diameter, 5 um particle size silica, polymerically modified with C_{18}. This was used with an isocratic mobile phase consisting of (A) 5% (v/v) 1,1-dichloroethane in acetonitrile, (B) 5% (v/v) 1,1-dichloroethane in methanol and (C) water premixed in the ratio 49:48:3 (A:B:C). The flow rate was 1 ml/min and the column was maintained at a temperature of 35°C. PDA detection was used with a monitoring wavelength of 450 nm at 50 nm bandwidth to optimise the detection of carotenes. Under these conditions it was possible to separate α- and β-carotenes as well as other β-carotene isomers, β-cryptoxanthin and lutein within 20 minutes. *Trans*-β-carotene assayed spectrophotometrically and found to be 91% pure, was used as a reference standard. Standard solutions were prepared over the range 0-40 mg/l and detector response was found to be linear over this range (R^2 = 0.995). Solutions of three mixed carotene samples were prepared in 1:1 acetone:methanol at a level of between ca. 0.02-1%.

The main advantage of HPLC with MS detection is that it enables not only the analyte quantification, but also the elucidation of its structure on the basis of the molecular mass and of fragmentation. The separated carotenoid peaks are usually monitored through the total ion current response (LC-MS) in the first instance. Tandem MS (LC-

MS/MS) is used to monitor specific ion fragments and multiple reaction monitoring for greater specificity and sensitivity (Van Breemen, 1996, 1997). Most of the methods applied to carotenoids use the atmospheric pressure chemical ionization (APcI) mode due to the absence of protonation sites in carotenoids (Feltl et al 2005). Řezanka et al (2009) have comprehensively reviewed the use of APcI-MS with HPLC and other separation techniques for the identification of carotenoids, which supplies significant detail on analytical methodologies. Isotopically labelled analogues (^{13}C or ^{2}H) are often used as internal standards but they are expensive and difficult to obtain for certain carotenoids. The sensitivity of electrospray ionization (ESI) modes in MS detection is generally two orders of magnitude higher than UV-VIS detection (Feltl et al (2005), who report a detection limit of 0.7 pMol for β-carotene, which approximates to 0.5ng). APcI in positive and negative modes gives detection limits of 1-13 pMol and has a broader linear dynamic range than ESI; over at least 3 orders of magnitude. Naturally, the sensitivity of LC-MS(MS) depends upon the nature of the carotenoid(s) being determined, the sample matrix and the sample workup regime used. Careri, Elviri and Mangia (1999) investigated LC-ESI-MS for the analysis of β-carotene, lutein, canthaxanthin and other carotenoids. Operating in the positive ion mode over the mass range m/z 500-560, the effects on the formation of the molecular ion species or adduct ions and the MS response were studied. Detection limits were estimated to be in the range 0.1-1.0ng and the dynamic range established between one and two orders of magnitude were achieved using RP-HPLC columns and data acquisition in SIM mode. While LC-MS(MS) equipment is expensive, it is finding increased use in laboratories and especially for analyte confirmation. For food quality and/or safety enforcement purposes it is more likely to be utilized in investigative work or in method development, since PDA detection provides adequate sensitivity.

Additional information on the structure of carotenoid (and chlorophyll) analogues can also be obtained using NMR (Schoefs, 2004). For example, For example, Valverde and This (2008) used ^{1}H NMR (1D and 2D) for quantitative determination of photosynthetic pigments from green beans and reported that it provides more structural information than UV/VIS spectroscopy. Twenty-eight discrete chemical shift assignments were reported to give a measurement precision comparable to other methods but with higher discriminatory power compared to UV/VIS spectroscopy

which can only determine total carotenoids. While NMR is a powerful research tool, it is not likely to find common use in enforcement laboratories due to high capital costs. Other detection modes such as infra-red spectroscopy and circular dichroism have been discussed by Schoefs (2004) but are essentially outside the scope of this review.

Availability of carotenoid standards

One of the main issues surrounding the analysis of carotenoids is the diversity, geometric isomerism and availability of standard materials. Their inherent instability requires storage under inert conditions (typically under an atmosphere of argon or nitrogen at -80°C) with regular purity checking using HPLC. Scott et al (1996) have emphasized the importance of certified materials in analyses of carotenoids in vegetables in an interlaboratory study (see below). While most of the main carotenoids are available commercially as analytical standards, they are costly. For quantitative analysis, β-apo-8′-carotenal or echinenone are occasionally used as internal standards.

Analysis of food and beverages for carotenoids

Several authors have reviewed extraction methods for carotenoids in foodstuffs consequently revealing a large number of available procedures (e.g. Lea, 1988; Eder, 2000; Delgado-Vargas et al, 2000;Oliver and Palou, 2000; de Quirós and Costa, 2006). Feltl et al (2005) have reviewed the reliability of carotenoid analyses with the emphasis placed on modern methods and the most recent references, but with no systematic survey of applications. Almost all of the carotenoid separations of interest to food analysts have been carried out on relatively clean and undegraded extracts prepared from natural foodstuffs which contain relatively high levels of pigment. There are also several studies in which the effects of cooking and other degradative steps have been assessed for their role in causing isomerization and producing other breakdown products. There are comparatively very few methods available on the determination of carotenoids in processed foods and beverages, and even fewer on analysis of added carotenoid colours. It is clear therefore that suitable published

methods will require adaptation to cover the analytical scope of permitted carotenoid colouring materials, particularly with respect to sample extraction and cleanup.

A selection of relevant methods for single and multiple carotenoids discussed within the scope of this review are detailed below.

A small number of literature references on the validation of carotenoid analysis in foods are available. In the study by Scott et al (1996), a reference material was prepared from a mixture of vegetables selected for their content of one or two predominant carotenoids: sweetcorn, (lutein), tomatoes (lycopene) and carrots (α- and β-carotene). The trial was designed to assess the likely problems associated with the chromatography (HPLC) system, carotenoid standardization and extraction. Through several phases, various aspects of the methodology were studied: spectrophotometer calibration, absorbance measurement of β-carotene standard with prescribed solvent, λmax and extinction coefficient, and chromatographic purity of β-carotene standard. This was then applied to an extract of the reference material. The results from 17 participating laboratories suggested that the effect of the chromatographic system is probably not a major variable and standardization of the carotenoid solution was not a significant problem. However, there was greater variability in lycopene calibration and measurement. Overall, the results suggested that the preparation of the carotenoid extract may account for 13% of the total variance of 23%.

Konings and Roomans (1997) evaluated and validated a HPLC-PDA method for the analysis of lutein, zeaxanthin, α-carotene, β-carotene, lycopene and β-cryptoxanthin in carrot, spinach, tomatoes, corn (canned) and tangerines. Analysis was carried out on freeze-dried samples by extraction into methanol:tetrahydrofuran (1:1) at 0°C followed by partition into petroleum ether after the addition of 10% NaCl solution. Ethyl-β-apo-8′-carotenoate was used as internal standard. When β-cryptoxanthin was suspected as being present, sample extracts were saponified for 2 hours with 10% methanolic KOH prior to partition. The purity of carotenoid standard stock solutions determined by HPLC ranged from 94-100%. The response of the HPLC system was linear over the range 0-5 ug/mL except for lycopene (0-3.5 ug/mL) due to its reduced solubility in the HPLC injection solvent. The detection limit for individual

carotenoids was ca. 0.1 ug/mL for standard solutions, which corresponded to ca. 1ug/100g in samples. Mean (n=10) recovery figures obtained from spiking tomatoes (without saponification) ranged from 93-107% while the mean recovery of the internal standard was 96% (n=4). Repeatability standard deviations (RSD_r) over the carotenoid concentration range 0.05-13.0 mg/100g ranged from 1.9 to 4.9%. For *cis*-β-carotene in carrot, *trans*- and *cis*-β-carotene in spinach and *cis*-lycopene in tomato detected around the detection limit, the reproducibility standard deviation (RSD_R) ranged from 10.5 to 24.1%.

Seventeen laboratories participated in a collaborative study for the analysis of ten products (cereal, infant formula, carrots, mixed vegetable juice, baby food squash and carotene capsule) for carotenes (as β-carotene) and retinol by RP-HPLC (Bueno, 1997). The test materials were saponified and extracted with petroleum ether, with BHT added as antioxidant. The extracts were analysed for carotenes by RP-HPLC using a C_{18} reversed-phase column with a mobile phase of acetonitrile:methylene chloride:methanol:water (70:20:8:2, v/v). Carotenes were detected by UV-VIS at 450 nm/436 nm. The within laboratory repeatability coefficients of variation ranged from 3.46-15.65%, and the reproducibility (among laboratories) coefficients of variation ranged from 5.34-15.77%. Invalid data was identified from participants who did not follow the protocol with respect to correct test portion weight.

Taungbodhitham et al (1998) evaluated six extraction methods for the analysis of carotenoids in tomato juice, carrot and spinach. The use of double extraction, each with 35 ml of ethanol:hexane mixture (4:3 by volume) with added $MgCO_3$, resulted in good recoveries of carotenoids (lycopene 96%, α-carotene 102% and β-carotene 93-100%). RP-HPLC with UV-VIS detection was used and quantitation achieved using β-apo-8′-carotenal internal standard. Coefficients of variation (RSD%) conducted on different days were 5% and 7% for lycopene and β-carotene respectively. The method was applied to carrot and spinach as representative samples of root and leafy vegetables, where the average recoveries of carotenoids added to canned tomato juice, carrot and spinach were: 101, 99.8 and 101% for α-carotene (12.4, 24.8, 49.6 and 99.2 ug/10mL of added α-carotene); and 98.1, 99.7 and 96.1 percent for β-carotene (25.5, 50.9, 101 and 201 ug/10mL of added β-carotene). The similar recoveries

obtained over the stated concentration ranges confirm that the application of the extraction method was unaffected by differences in matrix composition of the samples.

An analytical method using RP-HPLC with PDA detection was developed and applied to the determination of the permitted food colour additives β-carotene and β-apo-8′-carotenal in foods and beverages (Scotter et al, 2003). The scope of previously reported methods was broadened to cover a wide range of retail foods and enzymatic (lipase) hydrolysis was used in place of saponification for high-fat samples. Quantitative results (> 0.1 mg/kg) are given for the major colour principals *trans*-β-apo-8´-carotenal and *trans*-β-carotene. Semi-quantitative results were given for the various *cis*-isomers of each colorant for which authentic reference standards were not available. The method was used successfully for the analysis of a wide range of processed foods and beverages with differing fat content without the need for saponification, except for moderate to high-fat foodstuffs containing significant levels of emulsifiers, for which it was limited. The limits of detection and quantitation of the method were 0.01mg/kg and 0.1mg/kg respectively. Analyte response was linear over the range 0-50 mg/L. The method was developed using two validation matrices based on vegetable, protein and carbohydrate based mixture (infant food), and a fat-based material (half fat butter spread), spiked with *trans*-β-apo-8´-carotenal at 100 and 8 mg/kg respectively. Soft drink spiked at 10 mg/kg with both β-carotene and β-apo-8-carotenal gave mean recovery figures of 86.3 and 91.7% respectively, with corresponding RSD values of 11.4 and 5.6% (n=3). Comminuted meat product (pâté) spiked at levels of 2, 5, 10 and 40 mg/kg with β-carotene and β-apo-8´-carotenal gave recoveries of between 81 and 88% (RSD 3.1%). The recovery figures for ice cream spiked at 20 mg/kg were 80% and 76% (n=2) for β-carotene and β-apo-8´-carotenal respectively, while for cheese spiked at a level of 20 mg/kg for each carotenoid the recovery figures averaged 109% and 86% (n=4) for β-carotene and β-apo-8´-carotenal respectively. The recovery rate for β-carotene was thought to be enhanced by the presence of a significant level of naturally occurring β-carotene. The results from this study suggested that β-apo-8'-carotenal does not have widespread use in the UK. None of the samples exhibited total β-carotene content greater than 20 mg/kg and none of the high-fat samples and only 1 of the 17 low-fat/beverage samples contained

total β–carotene at levels less than 0.1 mg/kg. The total β-carotene contents of the low-fat/beverage samples ranged from 0.4 ± 0.03 to 8.4 ± 0.71 mg/kg, and the total β-carotene contents of the high-fat samples ranged from 0.1 ± 0.01 (jelly confectionery) to 18.5 ± 0.98 mg/kg (processed cheese).

Accelerated solvent extraction (ASE) has been compared with manual solvent extraction to determine several food colouring carotenoids capsanthin, lutein, canthaxanthin, β-apo-8´-carotenal, β-apo-8´-carotenoic acid ethyl ester, β-carotene, bixin, norbixin and lycopene in beverages, pudding mix, cereals, cookies and sausage (Breithaupt, 2004). Reverse-phase HPLC with a C_{30} column and photodiode array detection at 450nm successfully separated the carotenoids from one another but no attempt was made to distinguish *cis*- and *trans*- isomers. For extraction, a manual process as well as ASE was applied using mixed solvents (MeOH:EtOAc:pet spirit) followed by partition. Saponification was not used unless samples were known to contain oleoresin. Echinenone was used as internal standard and the calibration curves for the carotenoids were found to be linear over the range 0.5 to 25.0 mg/L (R^2 >0.999). Average recoveries for all analytes ranged from 88.7-103.3% (manual extraction) and 91.0-99.6% (ASE), with exception of norbixin using ASE (67.4%). Limits of quantitation ranged from 0.53-0.79 mg/L. The levels of carotenoids determined in beverages ranged from 1.1 to 27.8 mg/L β-carotene and 0.4-5.5 mg/L β-apo-8´-carotenal, and in pudding mixes from 19.7 to 39.3 mg/kg β-carotene. The β-carotene and capsanthin contents of cereals ranged from 17.7 to18.5 mg/kg and 1.6 to5.6 mg/kg respectively.The lutein content of cookies was in the range 2.5 to 4.49 mg/kg, while for sausages between 6.4 and 6.7 mg/kg canthaxanthin were found. For unequivocal identification, the mass spectra of all analytes were recorded using LC-(APcI)MS in positive mode, by monitoring the $[M+H]^+$, $[M+H-H_2O]^+$, $[M+H-CH_3OH]^+$ and $[M+H-C_2H_5OH]^+$ ions where appropriate. The ASE method was recommended to monitor both non-permitted application of carotenoid food colours and for the compliance of food within legal limits. Schlatterer and Breithaupt (2006) used similar conditions to determine the carotenoids including β-apo-8´-carotenoic acid ethyl ester in commercial egg yolks and showed that it was the most stable of the carotenoids analysed.

Sérino et al (2009) recently validated a rapid microextraction technique for the analysis of the four tomato carotenoids lutein, lycopene, β-carotene and phytoene using isocratic RP-HPLC with PDA detection. Microextraction was performed at subambient temperature in the presence of NaCl solution, hexane, DCM and ethyl acetate on fresh tomato powder prepare after treatment with liquid nitrogen. This was followed by agitation and centrifugation with no evaporation step. Extracts were stable over several days at -20°C. The method showed very good correlation of precision and accuracy with a reference method for all carotenoids except lutein, which showed a negative bias with the reference method. Linear regression coefficients were all close to unity. The similarity of results obtained with internal and external standardization showed an absence of any significant matrix effects, and recoveries of added carotenoids quantified by standard addition technique were reported to be 100%.

Dias et al (2008) have validated a RP-HPLC PDA method for the analysis of α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin in tomatoes and have estimated the measurement uncertainty. Solvent extraction was followed by centrifugation and partition into petroleum ether and solvent evaporation. BHT was added as antioxidant and β-apo-8´-carotenal used as internal standard. The linearity of calibration between 0.05 and 4.0 ug/mL gave correlation coefficients of $R^2 > 0.9985$. the detection limits and limits of quantification ranged between 0.57 and 0.91 ug/100g, and between 1.74 and 2.52 ug/100g respectively. The method bias was estimated from analysis of certified reference materials with satisfactory z-scores obtained throughout. Mean recovery values, based on addition of β-apo-8´-carotenal internal standard were 93.7% (n=48).

Other notable publications are: the detection of lycopene, canthaxanthin, β-apo-8´-carotenal and bixin in products derived from red pepper using TLC and RP-HPLC (Mínuez-Mosquera et al, 1995); measurement of capsanthin in food as an indicator for the presence of paprika following saponification, partition, SPE cleanup with measurement by RP-HPLC (Hayashi et al, 2001); the distribution of canthaxanthin and astaxanthin in rainbow trout and atlantic salmon (Page and Davies, 2007); an improved extraction procedure for the determination of β-carotene and lutein (plus

zeaxanthin and β-cryptoxanthin) in cereal products corn grain, semolina and flour using RP-HPLC (Burkhardt and Bohm, 2007); and an enzymatic treatment to improve extraction of capsaicinoids and carotenoids from chilli fruits (Salgado-Roman et al, 2008). Zhang et al (2008) describe a method for the determination of canthaxanthin in animal feed by isocratic RP-HPLC-PDA with a linear range of 1.0-20.0 mg/L and detection limit of 0.28 mg/kg. Recoveries of spiked feed were in the range 96-112%. Hou et al (2010) have recently reported a highly sensitive method for the determination of canthaxanthin and Sudan dyes in animal feeds using ultra performance HPLC with PDA detection at 500nm. The sample was extracted with acetonitrile and cleaned up using C_{18} SPE. Detector response was linear (R^2=0.9993) over the calibration range 0.1 to 1.0 ug/mL and the limit of quantitation was 0.02 mg/kg. Feedstuff fortified at 0.2, 0.4 and 0.8 mg/kg canthaxanthin gave mean (n=15) recoveries of 88.4, 88.6 and 85.7% respectively, and inter day RSD values of < 7.2%.

Overview and recommendations

Annex III of 94/36/EC permits the addition of E160a(i) and E160a(ii) to a range of foodstuffs at, and in sausages, pâtés and terrines up to a maximum level of 20 mg/kg (EU, 1994). Paprika extract (E160c) is approved for use *quantum satis* in a similar number of foodstuffs except butter and other spreads, and is permitted in sausages, pâtés and terrines up to a maximum level of 10 mg/kg. Lycopene (E160d) and lutein (E161b) are only pemitted in jams, jellies and marmalades up to a maximum limit of 100 mg/kg, whereas both β-apo-8′-carotenal (E160e) and the ethyl ester of β-apo-8′-carotenoic acid (E160g) are not permitted under Annex III.

Annex V Parts 1 and 2 permits the use of (carotenes) and paprika extract in all listed foodstuffs *quantum satis*, whereas lycopene, β-apo-8′-carotenal, ethyl ester of β-apo-8′-carotenoic acid and lutein are permitted in all foodstuffs over the range 50-500 mg/kg, except for edible cheese rind and casings at *quantum satis.* Canthaxanthin (E161g) has only very restricted usage under Annex IV, where it is permitted up to a maximum level of 15 mg/kg in Saucisses de Strasbourg. Canthaxanthin is however permitted in animal feed for farmed salmon and trout and for poultry at up to 80 mg/kg.

While most available published methods do not provide data on limits of quantitation, most should be capable of achieving adequate sensitivity when using HPLC with photodiode array detection for all permitted carotenoids and their geometric isomers. Among these, the extraction conditions are generally very similar but will require refinement for the various food matrices likely to be encountered, particularly for highly processed and compound foodstuffs and particularly for high fat foods where saponification or enzymatic hydrolysis will be necessary. The fact that many methods are capable of determining mixtures of carotenoids is clearly an advantage. SPE cleanup techniques offer scope for isolation and concentration of analytes prior to HPLC. Since the vast majority of available methods for the extraction and analysis of carotenes are focused on the determination of specific carotenoids from a fruit, vegetable and algal sources, there is relatively little information available on methods for their determination in foodstuffs containing added carotenoids, particularly for the ethyl ester of β-apo-8′-carotenoic acid, for which only one application was found and for canthaxanthin, which was limited to analysis of fish. It is not clear therefore, to what extent the presence of carotenoids present naturally in a foodstuff will affect quantitative measurement, or whether there will be a need to discriminate between added carotenoids and their naturally occurring analogues in a given foodstuff. It is presumed that regulatory compliance in terms of added levels is not an issue since most are permitted *quantum satis*. However, since the addition of carotenoids to foodstuffs satisfies a technological need, it is likely that any present in such foods will be added. This applies particularly to those with more restricted use i.e. β-apo-8′-carotenal, ethyl ester of β-apo-8′-carotenoic acid and canthaxanthin. It is also crucial that proper consideration is given to the availability, preparation, storage and purity assessment of carotenoid reference standards and also to the preparation and use of CRMs and/or IHVMs.

Canthaxanthin may be used in feed for poultry and for farmed salmon and trout at up to 80 mg/kg. Capsanthin, β-apo-8′-carotenal, ethyl ester of β-apo-8′-carotenoic acid, lutein are all permitted to be added to poultry feed up to the same level. According to the FSA (2010), the European Commission's Scientific Committee on Animal Nutrition (SCAN) were of the view that the acceptable daily intake (ADI) of 0 – 0.03 mg per kg body weight per person per day for canthaxanthin might be currently

exceeded by high consumers of salmonid and/or poultry products. The method described by Hou et al (2010) for the determination of canthaxanthin in animal feeds is more than adequate for measuring canthaxanthin at and below the maximum permitted level and is likely to be readily adaptable to the analysis of foods.

Apart from β-apo-8′-carotenal, ethyl ester of β-apo-8′-carotenoic acid and canthaxanthin, for which a small number of methods were identified, the use of carotenoids to colour foodstuffs is reasonably widespread. The distribution of the different carotenoids in fruit and vegetable source materials is wide and complicated by the occurrence of geometric isomers. Most of the published analytical methods relate to their determination in source materials or for nutritional purposes, rather than as added colourings but several allow the determination of multiple analytes. Solvent extraction is generally used with some form of selective clean-up such as partition and/or SPE followed by reverse-phase HPLC. Depending upon the fat content of the sample, saponification or enzymatic hydrolysis is required. RP-HPLC with PDA detection is used for quantitation and to spectrally characterise the pigments. Most methods demonstrate sufficient sensitivity but only relatively few methods have been validated. There is a clear need for development and validation of analytical methods for the determination of added carotenoids in the wide range of processed foods in which they are permitted. Several methods are available for the determination of canthaxanthin, β-apo-8′-carotenal and the ethyl ester of β-apo-8′-carotenoic acid in animal feeds, which follow similar extraction and measurement protocols. The following points are suggested for future consideration:

- methods are developed and single-laboratory validated for the determination of paprika extract (capsanthin and capsorubin) the ethyl ester of β-apo-8′-carotenoic acid and canthaxanthin for the foodstuffs in which they are permitted,
- methods for the other carotenoids such as those reported by Konings and Roomans (1997), Taungbodhitham et al (1998), Scotter et al (2003), Breithaupt (2004) and Hou et al (2010), are considered for use as a basis for refinement and single-laboratory validated for the foodstuffs in which they are permitted, and

- all methods should be subjected to full validation by collaborative trial, which should include protocols for analyte standardisation

The extraction and analysis conditions for a selection of available methods for carotenoids are summarized in Table 2.

Annatto E160b

Annatto is a natural colouring agent obtained from the outer coats of the seeds of the tropical shrub *Bixa orellana.* Annatto and its extracts are designated collectively as E160b. The major colouring component of annatto is confirmed as the apo-carotenoid 9'-*cis*-bixin, the monomethyl ester of the dicarboxylic acid 9'-*cis*-norbixin, commonly referred to as *cis*-bixin. The structures of 9′-cis bixin/norbixin are shown in Figure 1, VII. 9'-*Cis*-bixin is soluble in most polar organic solvents to which it imparts an orange colour but is largely insoluble in vegetable oil. It may be readily converted to the all-*trans* isomer due to its instability in the isolated form in solution. *Trans*-bixin is the more stable isomer and has similar properties to the *cis*-isomer but exhibits a red colour in solution and is soluble in vegetable oil. Commercially, isomerisation is achieved by heating a suspension of the *cis*-isomer in oil to 130°C *in vacuo.* The water-soluble analogue 9'-*cis*-norbixin can be isolated from annatto seeds by agitation in aqueous alkali at <70° C or formed by alkaline hydrolysis of *cis*-bixin to give either the sodium or potassium salt. The dicarboxylic acid is soluble in polar solvents to which it imparts an orange colour. 9'-*Cis*-norbixin is only sparingly soluble in chloroform and 0.1M sodium hydroxide (Preston and Rickard, 1980). Under extraction conditions, 9'-*cis*-bixin undergoes isomerization to produce oil solutions containing approximately 0.2 - 0.5% of pigment comprising a mixture of all-*trans*- and 9'-*cis*-bixin in variable proportions and characteristic degradation products, dependent upon extraction temperature and time. While it is reported that 80% of the carotenoids in the annatto seed coat comprise bixin (Preston and Rickard, 1980; Lauro, 1991), traces of bixin diesters may be found (Mercadante *et al*., 1997).

Commission Directive 2008/128/EC prescribes separate definitions and purity criteria for (i) solvent-extracted bixin and norbixin, (ii) alkali-extracted annatto and (iii) oil-extracted annatto (EU, 2008). Solvent-extracted bixin and norbixin formulations are

often referred to as indirectly-extracted annatto formulations, whereas alkali- and oil-extracted annatto are termed directly-extracted. The purity specifications include definition of the source material(s) and the solvents permitted for extraction, the identification and the minimum content of the colouring material (measured by spectrophotometry). Twelve separate specifications for annatto colour are prescribed by JECFA, depending upon the production method (JECFA, 2010), in which the prescribed assay methods for both bixin and norbixin are based on spectrophotometry.

Historically, chloroform has been used as solvent for the spectrophotometric analysis of bixin and dilute sodium hydroxide (ca. 0.1M) for norbixin (Scotter, 2009). Absorbance measurements at the two most intense spectral peaks are used for quantitative analysis, since interference from yellow decomposition products may be problematic. In practice, the spectrophotometric determination of annatto (as bixin or norbixin) is somewhat confused by the use of conflicting extinction coefficients. This has been discussed in detail and the published extinction coefficients for norbixin and bixin summarized and compared to highlight disparities (Levy and Rivadeneira, 2000). Depending upon the extinction coefficient used, large errors might be incurred and propose a practical conversion factor to correlate the relative absorbances at the two peak maxima. This is based upon the increase in absorbance observed upon hydrolysis of bixin to norbixin at constant concentration – thus proving that the extinction value for norbixin must be higher than that for bixin, which was also reported (Smith et al., 1983). Furthermore, from data recorded by the authors from more than 1000 spectrophotometric measurements of different samples of bixin before and after hydrolysis, the difference between the extinction values of bixin and norbixin was reported to be of the order of 6%. When compared with a value of 3208 reported for pure norbixin, this equates to an extinction coefficient for bixin of 3016, which concurs with the values reported for purified bixin in chloroform (Scotter et al., 1994). However, these extinction values to not agree with those adopted for colour purity specifications by the European Union or the FAO/WHO , largely due to misassumptions made regarding solvent effects. The discrepancy in published extinction values might be traced back to the 'erroneous' coefficient reported by Reith and Gielen (1971) that has been used subsequently as a reference value by various other workers. Serious doubt is expressed over the validity of the extinction values for norbixin in aqueous alkaline solution at 453nm (2850) and 482nm (2550).

The chemistry and analysis of annatto food colour has been recently comprehensively reviewed (Scotter, 2009) which is summarized briefly here. The qualitative and quantitative analytical aspects of annatto extraction methods published prior to 1976 have been reviewed briefly (Aparnathi and Sharma, 1991). Other notable methods include those developed for the analysis of foods and beverages using spectroscopic and chromatographic techniques, including whey solids (Hammond, Chang and Reinhold, 1973); meats (McNeal, 1976); milk and ice-cream (AOAC, 1980); drinks and syrups, butter, margarine, yoghurt, cheese and pastries (Corradi and Micheli, (1981); margarine, cheese and boiled sweets (Smith *et al*., 1983) and cheese (Luf and Brandl, 1988).

Other workers have developed refined methods for the extraction of annatto from high-fat foods, dairy products and candy (Lancaster and Lawrence, 1995) and fruit beverages, yoghurt and candies (Lancaster and Lawrence, 1996); red peppers (Mínguez-Mosquera, Hornero-Méndez and Garrido-Fernández, 1995); commercial seasonings comprising mixtures of corn meal and powdered annatto seeds or annatto extract (Haas and Vinha, 1995); cheese and milk products (Bareth, Strohmar and Kitzelmann, 2002) and a wide range of food commodities (Scotter *et al*., 2002) where solvent extraction regimes were developed for five specific sample matrices; extruded corn snack products (Rios and Mercadante, 2004); beverages, pudding mix, cereals, cookies and sausage (Breithaupt, 2004).

More recently, a method for the determination of norbixin and bixin in meat products using HPLC-PDA and LC-MS has been reported (Noppe *et al*., 2009). Samples were extracted into acetonitrile using vortex mixing and centrifugation. HPLC-PDA analysis was carried out using a reverse-phase system with detection at 458 and 486nm. Mass spectrometric detection was carried out using multiple mass spectra (MS^n) in both positive and negative ion modes at m/z 379 and m/z 395 for norbixin and bixin respectively. The limits of determination for HPLC-PDA and LC-MS were 0.5 mg/kg and 1 mg/kg respectively. Sausage meat samples spiked with norbixin and bixin mixture at levels of between 0.5 and 4 mg/kg gave recoveries of between 99 and 102%. The method was validated using procedures described in Commission Decision 2002/657/EC (EU, 2002).

While annatto is permitted for use in food commodities such as savoury snack products, coated nuts, extruded products and flavoured breakfast cereals, it is not permitted for use in spices. However, amongst other non-permitted dyes bixin was detected in 18 of 893 samples of spices, sauces and oils by UK enforcement laboratories during 2005-2006 as part of the UK Imported Food Programme (Food Standards Agency, 2006). This has led directly to a need for analytical methods capable of detecting very low levels of annatto in food ingredients and commodities in which it is not permitted, driven not only by the enforcement of regulations on a national scale (disseminated through the EU Rapid Alert System; EU 2008) but also by the need for the food manufacturing industry to ensure compliance, especially in a proactive manner and through the adoption of a 'zero tolerance' approach as applied to the monitoring of illegal dyes such as the Sudan Red group. Established HPLC methods capable of detecting bixin or norbixin at ca. 0.1 mg/kg in samples using UV-VIS or diode-array technology are not sufficiently sensitive. LC-MS/MS methodology is the obvious candidate but sufficiently detailed methods in peer-reviewed publications have not been forthcoming to date, although bixin isomers have been separated characterized using C30 RP-HPLC with PDA and APcI-MS in positive mode with capillary HPLC-NMR to aid structural assignment (Rehbein et al., 2007) . Nevertheless, LC-MS/MS is capable of detecting bixin at ca. 0.1 mg/kg in certain commodities, but this is heavily dependent upon the degree of signal suppression caused by matrix effects (Fera, 2010). This can give rise to false negative results using a screening approach, which in turn identifies a need for suitable extract clean up regimes, and guarding against ion suppression by using the method of standard addition

Overview and recommendations

Annexes III and IV of 94/36/EC prescribe maximum limits of between 10 and 50 mg/kg of annatto in a specified range of different foods. Most modern published methods are able to achieve limits of quantitation of ca. 0.5 mg/kg using HPLC with photodiode array detection. Extraction conditions are very sample-dependent (Scotter et al., 2002; Rios and Mercadante, 2004; Breithaupt, 2004) and may need refinement if recoveries are low. LC-MS(MS) may offer enhanced selectivity and sensitivity but

there are very few methods available and which have yet to be validated, especially for norbixin.

Methods for the determination of annatto in a variety of foodstuffs are reasonably well established and have been validated for a number of different sample types. Conditions for the extraction and cleanup are sample dependent and require refinement to widen their scope to include all of the foodstuffs covered by EU regulations. The methods reported by Scotter etal (2002), Breithaupt, (2004) and Noppe (2009) for example, could be considered as a basis for future method development and validation. Published methods for annatto are adequately sensitive for the levels of annatto added for colouring purposes and for detection in foodstuffs that are not permitted to contain annatto but HPLC methods must be capable of detecting and quantifying all of the main bixin and norbixin isomers. It is also necessary to have access to standards of the main colouring principles of known purity.

The extraction and analysis conditions for a selection of available methods for annatto are summarized in Table 2.

Group 7: Beet red E162

Beet red is obtained from the roots of natural strains of red beets (*Beta vulgaris* L. var. *rubra*) by pressing crushed beet as press juice or by aqueous extraction of shredded beet roots and subsequent enrichment of the active principle (EU, 2008). The colour is composed of different pigments all belonging to the class betalaine (or betalain) which areall very water soluble. The main colouring principle consists of betacyanins (red) of which betanin accounts for 75-95%. Minor amounts of yellow betaxanthins (mainly vulgaxanthins) and degradation products of betalaines (light brown) may be present. The structure of the betalain chromophore may be described as a protonated 1,7-diazaheptamethrin system. Betaxanthins and betacyanins are distinguished by substitution on the dihydropyridine moiety by specific R and R′ groups. The red betacyanins have substituents that extend the conjugation of the system, whereas the yellow betaxanthins do not. All betacyanins are glycosylated, where in betanin from *Beta vulgaris* a single □-glucose substituent is found. In betaxanthins, the cyclodopa

unit is displaced by either an amine or an amino acid. The substituents which characterise vulgaxanthins I and II from Beta vulgaris are glutamine and glutamic acid respectively (Figure 1, IVa-IVb). Besides the colour pigments the juice from *Beta vulgaris* consists of sugars, salts and/or proteins naturally occurring in red beets. The solution may be concentrated and some products may be refined to remove most of the sugars, salts and proteins. The betanine content in extracts of beetroot will suffer a progressive degradation which is accelerated by raising the pH, temperature and water activity; it is expected that all commercial products will lose their colour and alter their shade according to the conditions of storage (JECFA, 2010).

Commission Directive 2008/128/EC prescribes the identification and the minimum content of the colouring material measured by spectrophotometry (EU, 2008). The JECFA specification (JECFA, 2010) prescribes an assay procedure based on spectrophotometric measurement at 530nm and pH 5. Betalaines have complex amphoteric structures. Betanin and its C_{15} epimer isobetanin, are glycosides of the free aglycones betanidin and isobetanidin respectively. These are purplish-red in colour with λmax at 535nm. The yellow coloured vulgaxanthins exhibit a λmax value at ca. 480nm. It is important that analytical methods for the determination of beet red colour encompass all of these colouring principles. Both betacyanins and betaxanthins are water soluble, are insoluble in organic solvents and are very susceptible to degradation due to heat, light, oxygen and pH change (Azeredo, 2009). Special precautions must therefore be taken during extraction and analysis of beet red colour.

Over the past 50 years, there has been considerable interest in the pigment composition of red beets and other *Centrospermae*, including many studies on extraction, colorant content and stability of more than 50 betalain colour principles from a wide variety of plant sources. This is reflected in the large number of scientific articles available, however apart from studies focussing on beet extracts and subsequent colour formulations, very few articles could be found describing methods of extraction and analysis of added beet colour in foods and beverages.

Plant materials are usually extracted with water or water-based solvents such as water:0.1% HCl and citric acid-phosphate buffer (Eder, 2000). In some cases, Celite

filter aid may be added to improve clarification and ascorbic acid is often used to inhibit polyphenoloxidase activity. Other extraction procedures have employed aqueous alcoholic mixtures e.g. ethanol:water (20% to 50%), and 60% and 80% aqueous methanol. However betacyanins can be precipitated by a slight acidification with HCl or with acidified ethanol (0.4 to 1%) (Delgado-Vargas, Jiménez and Paredes-López, 2000; Kujala, Loponen and Pihlaja, 2001). Betalian extraction with ethanol:HCl (1:99) is reported to give higher extraction rates than water but the latter provided more stable extracts Garciá Barrera et al (1998). The initial homogenate is clarified by either centrifugation or filtration but the crude extract may contain many potentially interfering substances hence additional purification steps are occasionally required such as gel filtration, ion chromatography or solid-phase adsorption. Azeredo (2009) has recently reviewed methods of betalain extraction from various plant sources, in which he cites the work of Castellar et al (2006), who reported that water extracted higher levels of pigments from *Opuntia* fruits than ethanol:water. Since ca. 80% of beet juice solids consist of fermentable carbohydrates and nitrogenoud compounds, fermentation of beet extracts can reduce free sugars thereby increasing the betacyanin content (Pourrat et al., 1988) and heat treatment to deactivate enzymes has been used to avoid betalain degradation (Delgado-Vargas et al., 2000). It has also been shown that betaxanthin pigments in methanolic extracts of juices from Opuntia fruits are fairly heat stable, especially in the presence of ascorbic acid at pH 3.5 as monitored by spectrophotometry at 271 and 482nm (El Gharras et al, 2008). Azeredo et al (2009) studied the effects of pH, solvent-to-sample ratio, solvent temperature and grinding time on the efficiency of betacyanin extraction from beetroots. The most adequate extraction conditions were pH 3.0, solvent:sample ratio of 5:1, solvent temperature 70°C and grinding time 2 min.

A two phase extraction procedure for the extraction of betalains from red beet revealed that the purity of the extract was dependent upon tie-line length, phase volume ratio, neutral salt content and pH (Chethana et al., 2007). Polyethylene glycol (PEG) 6000 with ammonium sulfate was found to be the most suitable system for purification, where 70-75% of the betalains partitioned into the upper phase and 80-90% of the sugars partitioned into the lower phase. The PEG was separated from the betalains by chloroform/aqueous extraction. This procedure may be of use in the purification of food extracts, especially where significant amounts of sugars and other

water-soluble materials may be present. López et al (2009) used pulsed electric fields (PEF) at different intensities to improve the yield of betanine from beetroot discs, with subsequent release into media of different pH and temperatures, with pH 3.5 at 30°C giving the highest yield. Such a process however would not lend itself to routine application in an enforcement laboratory.

Isolation and purification of betalains prior to qualitative and quantitative analysis is required not only to remove potentially interfering sample co extractives but also to facilitate the production of reference materials. The purification of crude betalain extracts is usually accomplished by chromatographic or electrophoretic methods, thereby allowing separation and quantification of individual and total betalains (Eder, 2000). A two-step procedure has commonly been used where the first step is either ion chromatography on cation exchange resins e.g. Dowex 50W-X2, H+ form, or gel filtration with sephadex G-25. The second step is carried out by adsorption chromatography on polyamide and/or Polyclar AT (polyvinylpyrrolidone).

Very few literature references are available on the extraction of added beetroot red colour from foodstuffs. Henning (1983), cites several examples (in German) where beet red has been extracted from red wine brawn, milk products, fruit products and other foods using similar isolation methods. In the simplest case, the betanin is adsorbed directly onto polyamide powder from an aqueous extract of the sample, washed with water and the betanin desorbed using an acidic eluant (e.g. methanol:formic acid 60:40 v/v). For other sample types such as sausage, separate additional steps are required to separate out the protein and to remove the fat. The author used these procedures as a basis for the development of an extraction method for betanin in milk products, fruit juices and products, and meat products. Betanin was extracted from the sample by homogenization with water acidified with a few drops of acetic acid, and the homogenate warmed to 40°C, filtered through paper and then through a Sep-Pak C_{18} cartridge. Betanin was eluted from the cartridge with methanol:water (1:3), and the eluate concentrated to a few drops at 35-40°C on a rotary evaporator piror to analysis by TLC (see below). Egginger (1985) describes a simple test for the presence of betanin in cooked and raw sausage based on direct extraction into pyridine. However, this method cannot discriminate betanin from anthocyanins so the author recommends subsequent analysis using a quantitative

method. Brockmann (1998) reported a revised version of Henning's method for sausage which included a defatting stage using petroleum ether prior to aqueous acetic acid extraction, RP-SPE cleanup and TLC analysis.

The traditional methods for the quantitative determination of betacyanins and betaxanthins in beetroot and beet red colour formulations have been based on spectrophotometry. The procedure described by Nilsson (1970) and by Knuthsen (1981) has formed the basis of most spectrophotometric methods and involves dissolution of the sample in pH 5.0 phosphate buffer, with centrifugation if necessary. Measurement of the sample solution is carried out against a buffer blank and the colour content is calculated on the basis of the maximum absorption at ca. 530 nm using the specific absorbance of betanin (1120). The results are expressed as '% red colour', but whilst this expression may be somewhat ambiguous it is the industry-accepted standard (Scotter, 1997). Stintzing, Trichterborn and Carle (2006) describe a modified spectrophotometric method for the characterization of betalains in anthocyanin-betalain food colouring mixtures. The betalains were quantified at pH 6.5 at 600nm and the values obtained expressed as betanin (ε = 60,000 L/mol cm at 538nm).

As far back as 1981, Schwartz, Hildenbrand and von Elbe (1981) compared the spectrophotometric method with HPLC to quantify betacyanins from fresh, blanched and canned beets. Pigment solutions at pH 4.0, 5.0 and 6.0 were heat treated under nitrogen and analysed for pigment losses. Quantitative determinations of pigment in all purified and undegraded samples compared well when analysed by either method. Discrepancies between results of the two procedures occurred and increased up to 15% with extended heat treatment. The differences were attributed to the formation of degradation products or interfering substances, hence HPLC was preferred when interfering substances are present and demonstrates the limitations of the spectrophotometric method. The Nilsson spectrophotometric method was developed solely for the purpose of studying quantitative pigment differences among beetroot varieties and is applicable and accurate for such purposes. If degradation products are believed to be present in the sample, separation of these from the pigment by HPLC should be the method of choice.

Although TLC has been used to identify red betalains in beet extracts and specific foods e.g. beet red colour formulations (Knuthsen, 1981) milk products, fruit mixtures, fruit juices, fruit syrups and meat products (Henning, 1983) cooked and raw sausage (Egginger, 1985 and Brockmann, 1998), its use is not widespread due to the low Rf values obtained for the colour principles. A preparative system employing cellulose plates and two different mobile phases (isopropanol:ethanol:water:acetic acid at 6:7:6:1 and at 11:4:4:1 (v/v)) has been described by Bilyk (1981) where betalain mobility was enhanced by the presence of acid. Streck et al. (1993) used a similar system comprising diethyaminocellulose plates and isopropanol:water:acetic acid at 13:4:1 (v/v). Henning (1983) used reverse phase solid-phase extraction to isolate betanin from food extracts (see above), which was then subjected to TLC on cellulose, with butanol:formic acid:H_2O (10:3:3) as developing solvent. The pigment was identified by comparison with pure betanin and by spectrophotometry of an aqueous solution of the isolated pigment in the range 400 to 600 nm, and displayed a characteristic λmax at 532nm. Three samples of sausage spiked with beet red colorant at 1, 2 and 3 g/kg were analysed in a ring trial using the TLC method described by Brockmann (1998). Of the 15 results returned for potential beet red colour detection, two were false positives. Electrophoretic techniques have also been used for betalain analysis including paper electrophoresis and capillary zone electrophoresis, where the latter permitted the separation of betanin, isobetanin and their corresponding aglycones (Delgado-Vargas et al., 2000).

HPLC (reverse phase) has indeed become the method of choice for the separation, identification and quantitation of beet pigments and there are many literature references available, however very few of them have been developed for the determination of added beet colour to processed foods. Vincent and Scholz (1978) used ion-pairing reagent (tetrabutylammonium phosphate, TBA) in aqueous methanol at pH 7.5 to give non-polar unprotonated complexes and separation of betacyanins and betaxanthins from red beets. Two different wavelengths were used to monitor selectively the red and yellow pigments (538 and 476nm). The configurational isomers of the principal betacyanins, betanin and betanidin, were resolved using gradient elution and quantitative measurement using prepared standards showed good linearity of response. Recoveries of gelatin deessert spiked with ca. 2mg betanin were

95-99%. Schwartz and von Elbe (1980) employed an alternative approach using methanolic phosphoric acid at pH 2.75 in ion-supression mode but the elution order was similar for both methods. Schwartz et al. (1981) compared the quantitative data obtained from the spectrophotometric and HPLC determination of betacyanins (see above).

The HPLC procedure described by Schwartz and von Elbe (1980), Schwartz et al., (1981) and subsequently modified by Pourrat et al., (1988) has been developed for the quantitative determination of individual betacyanin and betaxanthin pigments in commercial beet red formulations (Scotter, 1997). The final system comprised a C_8/C_{18} reverse-phase column (HiChrom HRPB), a mobile phase of (A) 0.05M KH2PO4 at pH 2.75 and (B) methanol under the following linear gradient conditions: 0% to 30% B in A over 30 minutes at a flow rate of 1 ml/min and a temperature of 35°C. Photodiode array detection was used with monitoring wavelengths bands of (i) 535 nm at 10 nm bandwidth for red betacyanins and (ii) 475 nm at 10 nm bandwidth for yellow betaxanthins. Four commercial beet colour formulations were analysed and good separation of the betacyanins was achieved within 30 minutes under these conditions. Several other red/yellow coloured components were detected at lower levels. Since no authentic betacyanin or betaxanthin reference standards were available, component peaks were tentatively identified by comparison of their relative retention times to literature values and their spectrophotometric characteristics (Knuthsen, 1981; Pourrat et al., 1988). Analysis of a fresh beet extract gave similar results. This method showed suitable scope for its recommendation as an alternative assay procedure, providing authentic reference materials are used for calibration.

A large number of articles have been published on the determination (i.e. separation and identification) of betalaine analogues in a range of plant sources in addition to beetroot, employing relatively modern separation and detection systems, notably PDA and MS. Among these are the RP-HPLC methods described by Kujala et al (2001), Stintzing et al (2006) and Kugler et al (2007) which all used C_{18} columns and gradient elution with acetonitrile:formic acid: water mobile phase, PDA MS detection. Electrospray ionization in positive mode is favoured for the detection of the $[M+H]^+$ ions of betanin and isobetanin at m/z 389. Stinzing et al (2006) reported no fragmentation data whereas Kugler (2007) reported fragmentation of betanin at m/z

345 (35%) and 150 (100%), and for isobetanin at m/z 345 (100%) and 150 (54%). Vulgaxanthin I $[M+H]^+$ was readily detected at m/z 340.

Overview and recommendations

Annex III of 94/36/EC prescribes maximum limits of between 200 mg/kg (fruit-flavoured breakfast cereals) and *quantum satis*(vegetables in vinegar, brine or oil (except olives); jam, jellies and marmalades and other similar fruit preparations including low calorie products; and sausages, pâtés and terrines (EU, 1994). Annex V Part 2 allows all foodstuffs specified to contain E162 *quantum satis*. Most published methods do not provide useful limits of quantitation and very little validation has been carried out. It is reasonably safe to assume however, that RP-HPLC analysis with diode-array and MS detection will provide sufficient sensitivity and selectivity. Methods will require full validation for the range of foodstuffs permitted under 94/36/EC.

While extraction methods for beetroot and other plant sources of betalaines are fairly well established, there a very few useful methods available and they do not cover the scope of foods permitted to contain E162. Apart from a very small number of literature references on the analysis of milk products, fruit products and meat products, methods for the determination of E162 in a variety of foodstuffs are poorly established and have not been validated for the range of different sample types permitted to contain it. Conditions for the extraction and cleanup of E162 are relatively simple and based on aqueous extraction with a defatting stage, and chromatographic and SPE clean up techniques have been reported. However, steps to minimise analyte degradation are required. Analytical methods will also require suitable markers to identify pigment changes during food processing and storage. Betanin, isobetanin and vulgaxanthin I are the principle colours found in red beet so their ratios will be indicative of stability. Identification of the main degradation product betalamic acid will be useful as well as knowledge of the pigment pattern of betalaine source material order to characterise any processing-induced changes. Measurement of the main colour principles from red beet (betanin, isobetanin and vulgaxanthin I) may not give the true level of total added beet red colour. Robust analytical HPLC-DAD-MS techniques permit monitoring of changes induced by processing; however the lack of commercially available reference materials

complicates analyses, especially with respect to quantitative determination. Continuing studies are therefore required to substantiate the optimum parameters for routine application.

Published methods currently do not provide sufficient data to assess analytical sensitivity for detection of E162 in foodstuffs but several RP-HPLC methods with UV-VIS (PDA) and MS detection, are likely to be amenable to application by enforcement laboratories. The development of analytical procedures based on aqueous alcohol extraction with adsorption / SPE cleanup and measurement by HPLC-DAD be such as those described by Scotter (1997), Kujala et al (2001) and Stintzing et al (2006) are candidates for deveopment. LC-MS(MS) as confirmatory technique should facilitate development (e.g as described by Kugler et al (2007)), since the scope of the method should cover not only the main colour principles but also potential betalaine degradation products. Procedures for the extraction, purification and characterisation of reference materials should also require development.

The extraction and analysis conditions for a selection of available methods for beet red are summarized in Table 2.

Group 8: Anthocyanins E163

Anthocyanins are red, purple and blue pigments that occur naturally in a wide variety of flowers, fruit and vegetables. In common with other polyphenolic substances, anthocyanins occur in nature as glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium (or flavylium) salts, i.e. the aglycones (anthocyanidins). Differences between individual anthocyanins are the number of hydroxyl groups and their degree of methylation, the nature and number of sugars attached to the molecule and the position of the attachment, and the nature and number of aliphatic or aromatic acids attached to the sugars in the molecule. More than 500 individual anthocyanins have been identified (Andersen and Jordheim, 2006). Of the known naturally-occurring aglycones, six occur most frequently in plants. These are pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. The sugars most commonly bonded to these are glucose, galactose, rhamnose and arabinose. Combinations of these four monosaccharides as di- and tri-

saccharides may also be similarly bonded. The structures of the 6 aglycones are given in Figure 2a. Glycosylation confers increased structural stability and water solubility to the parent aglycone. The most commonly found glysoside classes are 3-monosides, 3-biosides, 3,5-diglycosides and 3,7-diglycosides. Glycosylation of the 3′-, 4′- and 5′-hydroxyl group however, have been demonstrated. In many cases, the sugar residues are acetylated by p-coumaric, caffeic, ferulic, sinapic, p-hydroxybenzoic, malonic, oxalic, mailc, succinic or acetic acids. Methoxyl substituents are found at the 3′ and 5′ positions and, less frequently, at positions 7 and 5. A free hydroxyl group at one of the 5, 7 or 4′ positions is essential for generating the in situ colours responsible for plant pigmentation, which arises mainly from the loss of an acidic hydroxyl hydrogen from the flavylium structure. In acidic solutions, four anthocyanin species exist in equilibrium, the quinoidal base, the flavylium cation, the pseudo base and the chalcone as shown in Figure 2b (Mazza, Cacace and Kay, 2004).

The pigmentation of plants is rarely due to a single anthocyanin, and several food sources of anthocyanins contain significant levels. These include grape, blackcurrant, elderberry, cranberry and cherry, the aqueous extract of roselle (the calyces of hibiscus) and of red cabbage. Grapes alone contain at least four to over sixteen separate anthocyanins, depending upon the variety. The potential number of anthocyanins present in a fruit or vegetable extract would make the preparation of a full range of reference materials difficult and expensive. The most commonly occurring anthocyanins are based on cyanidin, followed by pelargonidin, peonidin and

delphinidin, then by petunidin and malvidin (Jackman and Smith, 1992). In terms of glycoside distribution, 3-glycosides occur approximately 2.5 times as often as 3,5-diglycosides, the most ubiquitous anthocyanin being cyanidin-3-glucoside. Table 1 lists the major anthocyanins to be found in selected plant sources used for the production of food-grade anthocyanin colouring preparations. If the main anthocyanin of the extract is known or can be identified, the total anthocyanin content may then be expressed in terms of the major component. Only one purified reference standard is then required for quantitation.

Commission Directive 2008/128/EC defines anthocyanins as colorants obtained by extraction with sulfited water, acidified water, carbon dioxide, methanol or ethanol from the natural strains of vegetables and edible fruits. Anthocyanins contain common components of the source material, namely anthocyanin, organic acids, tannins, sugars, minerals etc., but not necessarily in the same proportions as found in the source material. The method of assay in the specifications is based on spectrophotometric measurement at pH 3.0 between 515 and 535nm, with reference to a prescribed extinction coefficient.

For identification purposes, separate absorption maxima are prescribed for the six main aglycones in 0.01% HCl. JECFA prescribe separate specifications for blackcurrant extract (JECFA, 2010a) and grape skin extract (JECFA, 2010b) in which specific anthocyanins are defined for each extract. The prescribed assay methods for both extracts are based on spectrophotometric measurement of colour intensity at pH 3.0 and λmax values of 520nm (blackcurrant) and 525nm (grape skin).

It is possible to extract colour from any of the above raw materials but for economic reasons, grape skins, a by-product of the wine industry, are the most common source. However, EU legislation permits the use of sources that include the concentrated juice of blackcurrant, strawberry, cranberry, elderberry, cherry and red cabbage. Other less familiar sources include radish and black carrots. Application of anthocyanins is limited generally to acidic foods i.e. around pH 4 or below because blue-grey colour changes occur at higher pH values. These includes soft drinks, fruit preserves (usually fresh/frozen rather than sulfited/canned), sugar confectionery (particularly high

boiling/pectin jellies), dairy products (acidic products such as yoghurt), frozen products (water ice ca. pH 3) dry mixes (acidic dessert mixes and drinks powders) and alcoholic drinks. Anthocyanin stability is an important issue, the major factors being pH, heat, light, oxygen, proteins/enzymes, sulfites and nucleophilic agents. The various conditions encountered in food processing and storage have a very large impact on the anthocyanin content and profile (and therefore overall colour) of foods coloured with anthocyanins. Many reports have been published on the effects of methods of processing and storage on colour characteristics and the issue is therefore very complex. However this issue is outside the remit of this review. According to Lea (1988), much of the literature on food colours is concerned not so much with the role of natural pigments as food additives, as with the composition and analysis of those pigments which are inherently present in foodstuffs. This is certainly the case with anthocyanins.

Anthocyanins are generally more soluble in water than in non-polar solvents, but depending upon the media conditions, anthocyanins could be soluble at a pH value where the molecule is unionized. These characteristics aid in the extraction of anthocyanins and separation of anthocyanins (Delgado-Vargas, Jiménez and Paredes-López, 2000). Acidic (pH ca. 1.5) conditions are generally preferred for the extraction of anthocyanins in order to minimize pH effects on the flavylium equilibria. Conventional methods of anthocyanin extraction usually employ dilute hydrochloric acid methanol (ca. 1%). However, there are alternative extraction schemes available which avoid the use of acid, in particular organic acids, in which the formation of formyl and acetyl artefacts has been demonstrated (Bakker and Timberlake, 1985). Anthocyanins are heat-sensitive so high temperatures must be avoided during extraction and concentration i.e. <30°C. Qualitative analysis generally involves extraction with a weakly acidified alcoholic solvent, followed by concentration under vacuum, purification (using solid phase extraction) and separation of the pigments. The most commonly used solvents are methanol, ethanol or acetone, at compositions of 70-80% in water (Mazza, Cacace and Kay, 2004). Moreover, quantitative extraction using homogenization often requires and adequate solvent-to-sample ratio but two or more extractions of the sample residue with fresh solvent may be required. Liquid plant products such as juices, syrups and wines require very little sample preparation.

Purification methods for anthocyanin extracts are generally nonselective and result in extract solutions containing large amounts of undesirable co-extractives such as sugars, acids, amino acids and proteins that require removal. Partitioning of extracts with ethyl acetate has been shown to remove interferences prior to LC-MS analysis (Giusti et al, 1999). However, consideration should be given to the potential loss of less polar materials that would otherwise contribute to the total anthocyanin colour content, such as aglycones. Solid phase extraction (SPE) techniques have been used successfully for the isolation, cleanup and concentration of anthocyanins. Giusti et al., (1999) used C_{18} SPE to isolate and purify anthocyanin extracts which involved loading the extract onto the SPE resin and washing with acidified water followed by ethyl acetate, and eluting the anthocyanins with acidified methanol. C_{18} SPE has also been used to clean up cherry extracts (Chandra, Rana and Li, 2001). Shah and Chapman (2009), used mixed mode cation exchange SPE to purify anthocyanins from tulip extracts in 50:50 methanol:water with 0.1% formic acid. The extract was diluted with an equal volume of 0.1% formic acid and loaded onto the cartridge. The cartridge was washed with 0.1% formic acid solution, then with methanol to remove flavanoid glycosides. The anthocyanins were eluted with 50:50 methanol:pH 6 phosphate buffer. Sephadex has also been used for the initial purification of crude anthocyanin extracts (Lee and Hong, 1992; da Costa, Horton and Margolis, 2000) and Ling et al (2009) have used hydrophilic-lipophilic balanced (HLB) SPE to extract anthocyanins from human tissue homogenates.

Hydrolysis of the anthocyanins to the parent aglycones is commonly undertaken to aid identification of the major anthocyanin peaks (Scotter, 1997). In general, the HCl concentration in each sample extract is adjusted to 2M, placed in screw-capped vial and heated on a water bath at 90°C for ca. 1 hour. The cooled hydrolysate is loaded onto a C_{18} solid-phase adsorption cartridge and washed with water. The pigments are eluted with the minimum volume of 1% in methanol, filtered and analysed. This procedure is useful for obtaining the aglycone profile of an extract and may be used quantitatively when results are expressed as the major aglycone. Moreover, analytical methods become much more simplified if the number of analytes is reduced to ca. 6 well characterized compounds for which standards are available or can be readily

purified. The major drawback here is that the aglycones are generally less stable than the anthocyanins.

There are a large number of published articles on analytical methods for anthocyanins, as well as several detailed reviews (Francis, 1982; Lea, 1988; Jackman and Smith, 1992; Lee and Hong, 1992; Delgado-Vergas, Jiménez and Paredes-López, 2000; Eder, 2000; da Costa, Horton and Margolis, 2000; Merken and Beecher, 2000). However, these have been concerned with optimization of extraction, separation of individual anthocyanins, qualitative characterization, identification and structural elucidation rather than with the determination of anthocyanin colours added to foodstuffs. Mazza, Cacace and Kay (2004), have reviewed comprehensively the technologically advanced methods available for the analysis of anthocyanins in plants, as well as the application of these methods to biological fluids which may have future relevance to the development of methods for their determination in fish and meat products.

Classically, anthocyanin mixtures are analysed quantitatively by spectrophotometric methods, where absorbance measurements at a single low pH value are proportional to total concentration. However, estimates of the concentration of individual anthocyanins in a mixture can only be obtained with prior knowledge of the proportions of the individual pigments therein. Absolute concentrations may then be estimated by the use of weighted average absortivities and absorbance measurement at weighted average wavelengths (Jackman and Smith, 1992). The single pH measurement approach is subject to interference by co-extractives and brown-coloured degradation products and cannot be used in their presence, hence these methods are not suitable for the specific identification and determination of anthocyanins. Differential or subtractive absorption methods may be used to determine total anthocyanin concentration in those samples that contain interfering substances (Jackman et al., 1987; Francis, 1989). The differential method relies on the structural transformations of anthocyanin chromophores as a function of pH. The difference in absorbance at two pH values and same wavelength (e.g. λmax of the anthocyanin) is assumed to give a measure of the anthocyanin concentration since absorption due to interfering substances cancels in the subtraction. The

spectrophotometric characteristics of interfering brown (degradation) products are generally not altered with changes in pH. The advantage of this method is that it can be used to calculate an anthocyanin degradation index , defined as the ratio of the total anthocyanin determined at a single pH to that determined by the differential method.

The value of the extinction coefficient ($E_{1cm/1\%}$ = 300 at pH 3.0) given in 2008/128/EC is an industry-derived average value (EU, 2008). According to Jackman and Smith (1992), most anthocyanin extracts (derived from grape skins) have a colour strength (i.e. absorbance per gramme) of between 150 and 300 which equates to 0.5% and 1% anthocyanin colour respectively. The JECFA colour value is empirically-derived and its use is therefore purely for comparative purposes. It remains in the JECFA specification for anthocyanins as a stop-gap and the specification allows for the use of other suitable assay methods. Scotter (1997) found that whilst the correlation between the single and differential pH methods for EC colour content of anthocyanin colour formulations was good (R^2=0.993), the disparity found in the absolute values, especially the higher ones, required further investigation. As expected, the EC colour content correlated well with the JECFA colour value (R^2=0.999) at single pH measurement. In the absence of detailed supportive data on the validity of the differential pH method, the single pH method as described in the EC specification was recommended. However, it is vitally important that certain precautions are taken with the measurement. Because of the structural transformations undergone by anthocyanins in solution at different pH values, prepared solutions must be given adequate time (ca. 1 hour) to equilibrate before absorbance readings are taken otherwise erroneous results may be expected. Furthermore, since co-pigmentation and self-association effects cause deviations from Beer's Law, leading to inaccurate estimates of total anthocyanin concentration, anthocyanin solutions should be prepared such that the measured absorbance falls within the range ca. 0.2 to 0.6 AU.

Lee, Rennaker and Wrolstad (2008) found a high correlation ($R \geq 0.925$, $p \leq 0.05$) between the differential pH method and two HPLC methods for the analysis of seven juice samples containing an array of different anthocyanins. In general, the total anthocyanin content was greater when expressed as malvidin-glucoside rather than cyaniding-glucoside, despite the method used. The authors recommended the use of

the pH differential spectrophotometric method for laboratories that do not have suitable HPLC capability and highlighted the importance of reporting the standard used to express the results.

Anthocyanins have been analysed by a number of separation techniques including paper, thin-layer and column chromatographic methods, and capillary electrophoresis. Nowadays HPLC is the method of choice because it is fast, sensitive and quantitative. However, absolute peak retention times can often vary between workers even when identical conditions are used. For peak identification and qualitative evaluation of chromatograms, the use of pure anthocyanin standards is recommended (Eder, 2000). As for other natural colouring mnaterials, this is complicated by the general lack of availability of pure anthocyanin standards but these are just becoming more available commercially to researchers. Also, there are a number of commonly-available materials for which the anthocyanin contents have been thoroughly characterized that could be used as reference materials. If a special anthocyanin standard is not commercially available, the preparative isolation of pure anthocyanins is necessary, for which several published methods are available. Alternatively, it is common practice to use only one anthocyanin (surrogate) standard e.g. malvidin-3-glucoside, and to quantify all other anthocyanin peaks using it. However, due to differences in absorbances and absorbance maxima this will incur quantitative errors. Even with a powerful separation method such as HPLC, it would be difficult to calculate the exact colour content without having prior knowledge of the anthocyanin profile of the extract (this is of course valid for other pigments).

Many HPLC methods for the determination of anthocyanins and anthocyanidins are available in the literature and almost all of them are based on reverse-phase columns. In combination with photodiode array and mass spectrometric techniques, structural characterization of the pigments is readily achievable. The vast majority of HPLC separations of anthocyanins are carried out using reverse-phase systems using gradient elution, especially for anthocyanins that are structurally similar. Most of the solvent systems use binary gradient elution with methanol or acetonitrile (and/or occasionally acetone) as organic modifiers. The solvent systems almost always include an aqueous acid (formic or acetic) or acidic buffer e.g. phosphate, in order to ensure that the flavylium cationic form predominates at a pH of <2. Modern HPLC

column materials are capable of withstanding low pH mobile phases hence column stability is not a significant issue. Detection is usually carried out using UV-VIS/PDA detectors in the wavelength range 510-546nm. Spectral acquisition using PDA is often used to elucidate structures (Hong and Wrolstad, 1990a,b; Hebrero et al., 1989), where the A440/Aλmax ratio has been commonly used to characterise 3-glycosides and the 3,5-diglycosides. Moreover, the acetylated anthocyanins generally exhibit similar absorption characteristics to the parent anthocyanins plus those of the acylating acids i.e. increased absorption in the 300-325 nm region. For the separation of aglycones, isocratic conditions are often preferred due to the relative simplicity of aglycone occurrence and because the retention of the aglycones is correlated with compound polarity, the elution order being delphinidin < cyanidin < petunidin < pelargonidin < peonidin < malvidin (Lee and Hong, 1992). A detection limit of between 0.5 and 1.0 mg/L anthocyanin can be assumed generally.

The determination of free anthocyanins by liquid chromatography was investigated to improve a method for quality control of these natural products found in eight red fruit juices, concentrated juices and syrups (black currant, elderberry, sour cherry, strawberry, grape, blueberry, raspberry, and red currant) (Goiffon, Mouly and Gaydou, 1999). Among the various experimental possibilities, an isocratic simple method using water, acetonitrile and formic acid as eluting mixture was chosen. Results obtained by nine laboratories concerning the determination of the main anthocyanins contained in strawberry (cyanidin-3-glucoside, 3.9-10.6%; pelargonidin-3-glucoside, 89-95%; and pelargonidin-3-arabinoside, 3.1-3.9%), raspberry (cyanidin-3-glucoside, 16-17% and cyanidine-3-sophoroside, 78-81%), elderberry (cyanidin-3-sambubioside-5-glucoside, 13.4%; cyanidine-3-sambubioside, 47.8% and cyanidin-3-glucoside, 38.6%) and black currant (cyanidin-3-glucoside, 3.9-6.9%; cyanidin-3-rutinoside, 29-39%; delphinidin-3-glucoside, 14-16% and delphinidin-3-rutinoside, 41-52%) juices allowed this new protocol to be studied and tested for reliability using the repeatability and reproducibility criteria. Both the RSD_r and RSD_R values were variable and attributed to the dependency of the chromatographic efficiency on the apparatus used, particularly the column, and the variability of analyte retention times.

An HPLC system based on that described by Hong and Wrolstad, (1990a) was evaluated for the analysis of the anthocyanin colour preparations and their corresponding acid hydrolysates i.e. for the parent aglycones (Scotter, 1997). The final system comprised an HRPB C_8/C_{18} column (250 x 4.6 mm, 5 um). This was used with a gradient mobile phase consisting of (A) 10% (v/v) aqueous formic acid and (B) acetonitrile. The linear gradient profile was 10% to 20% B in A over 30 minutes. The flow rate was 1 ml/min and the column was maintained at a temperature of 35°C. Photodiode array detection was used with a monitoring wavelength of 530 nm at 20 nm bandwidth to optimise the detection of anthocyanins and aglycones. Under these conditions the aglycones were separated within 25 minutes, except petunidin which was not available commercially. This system was also used for the separation of the anthocyanins, where the main peaks in the chromatograms obtained from grape skin and elderberry extracts were identified by their retention and spectrophotometric characteristics, and found to be consistent with literature values (Jackman and Smith, 1992; Hong and Wrolstad, 1990a,b).

Hebrero, et al., (1989) confirmed that the elution order of anthocyanins in RP-HPLC is closely related to their polarity, which is directly related to the degree of hydroxylation and methoxylation of aglycones, which aids the identification of unknown anthocyanins. On this basis, the unknown peak observed to elute between cyanidin and peonidin in the hydrolysate of grape skin extract was been tentatively identified as petunidin (Scotter, 1997). As verified in Table 1, which shows that petunidin-3-glucoside is a major component of grape skin anthocyanins. The chromatogram obtained from the hydrolysate of extract elderberry anthocyanin concentrate showed almost total hydrolysis of the anthocyanins to cyanidin which is in keeping with the known components of elderberries cyanidin-3-glucoside and cyanidin-3-sambubioside. In fact, cyanidin anthocyanins are reported to account for 100% of the total pigments in elderberry juices and concentrates (Hong and Wrolstad, 1990b).

Prior to ca. 2000, there were very few published reports on the use of LC-MS for analysis and characterization of anthocyanins. Giusti et al (1999) used direct sample injection into electrospray and tandem MS systems to characterize anthocyanin

fractions obtained from HPLC separation of vegetable extracts. The positive charge of anthocyanins favoured fast and effective ES-MS detection of intact molecular ions and tandem MS provided clear and characteristic fragmentation patterns. da Costa et al (2000) have briefly reviewed MS techniques used for analysis of anthocyanins and also report an LC-MS method based on atmospheric pressure chemical ionization (APcI) for the analysis of blackcurrant anthocyanins (3-glucosides and 3-rutinosides of cyanidin and delphinidin). The molecular ion $[M]^+$ and mass fragments corresponding to the successive loss of sugar residues $[M-146]^+$ and $[M-146-162]^+$ were detected. By increasing the fragmentor voltage the aglycone fragments produced an additional series of ions, the most important of which was a retro-Diels-Alder fragment that permitted confirmation of the identity of the aglycone.

Chandra, Rana and Li (2001) developed and validated a method for the identification and quantification of individual and total anthocyanins in raw plant materials by LC-MS with positive electrospray ionization to detect $[M]^+$ ions. Separated anthocyanins were calculated individually against a single commercially available external standard (cyanidin-3-glucoside hydrochloride) and expressed as its equivalents. The amounts of each anthocyanin were then multiplied by a molecular weight correction factor to afford their specific quantities. The method was validated using cherry and elderberry anthocyanin extracts based on cyanidin being the predominant aglycone in both and in combination with PDA detection. Mazza, Cacace and Kay (2004), discuss in detail other MS techniques for anthocyanin analysis such as fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI) and time-of-flight (TOF) that have proven to be very useful for structural elucidation but which are not used routinely. In addition, there are several publications that report on the use of LC-MS in specific applications. Among these are methods employing LC-MS/MS such as that described by Tian et al (2005) for anthocyanin screening of the precursor ions of the six common aglycones as well as common neutral loss and selected reaction monitoring (SRM) for identification of specific anthocyanins in raspberries, blueberries and grapes. When combined with PDA detection, these techniques represent an important tool for systematic identification and characterization of anthocyanins in commercial biological samples. HPLC-PDA-MS analysis was used by Stintzing, Trichterborn and Carle (2006) to characterize anthocyanin-betalain food

colouring mixtures and by Shah and Chapman (2009) for the analysis of tulip anthocyanins.

Ling et al (2009) have developed and validated an LC-MS/MS method for the quantification of four anthocyanins in a bioadhesive black raspberry gel and biological fluids. While this method was applied to non-food samples, the validation data show that the technique is very sensitive with limits of quantitation in the range 1 to 5 ng/mL. The within- and between-run RSD_R values at quality control concentrations of 1, 5, 50 and 500 ng/mL were all < 18.3%, with recoveries in the range 90.7-119.3% depending upon sample type.

Capillary zone electrophoresis (CZE) has found limited use in the analysis of anthocyanins, due in part to the instability of anthocyanins in basic media and because the predominant ion species does not absorb light around 500nm at basic pH, and at pH 8, the response of CZE is reported to be 87-fold lower than HPLC at pH 1.8 (da Costa et al., 2000). The authors cite several CZE-based methods; analysis of anthocyanins in blackcurrant juice at pH 1.8 (da Costa et al., 1998), anthocyanins using cationic surfactant at pH 2.1 (Bicard et al., 1999) and elderberry pigments used to colour candy, juice and jelly (Watanabe et al, 1998). Other CZE applications include analysis of bilberry (Ichiyanagi et al, 2000) and peonidin and cyanidin in cranberries (Watson, Bushway and Bushway, 2004). The separation, resolution and peak shapes of the anthocyanins are critically influenced by the pH of the running buffer and the presence of an organic solvent. According to Mazza, Cacace and Kay (2004), the use of CZE under acidic conditions can significantly increase peak resolution and improve detection limits, but does not offer the separation of complex samples that can be achieved with HPLC.

The use of NMR for structural elucidation of anthocyanins has been reviewed by Schoefs (2004) but is largely outside the scope of this review.

Overview and recommendations

Annex III of 94/36/EC permits the addition of E163 to fruit flavoured breakfast cereals up to a maximum limit of 200 mg/kg and at *quantum satis* in a small range of foodstuffs i.e. red marbled cheese, Americano, vegetables in vinegar, brine or oil

(excluding olives), jam, jellies and marmalades and other similar fruit preparations including low calorie products (EU, 1994). Annex V Parts 1 and 2 permits their use in all listed foodstuffs *quantum satis*. While most available published methods do not provide data on limits of quantitation, most should be capable of achieving adequate sensitivity when using HPLC with photodiode array detection, and especially where anthocyanin analogues including the aglycones can be confirmed using LC-MS(MS). Among these, the extraction conditions are generally very similar but will require refinement for the various food matrices likely to be encountered, particularly for highly processed and compound foodstuffs. SPE cleanup techniques offer scope for isolation and concentration of analytes prior to HPLC. Since the vast majority of available methods for the extraction and analysis of anthocyanins are focused on the isolation, purification and identification of specific analogues from a wide range of food sources, there is very little information available on methods for their determination in foodstuffs containing added anthocyanins. It is not clear therefore, to what extent the presence of anthocyanins present naturally in a foodstuff will affect quantitative measurement. It is not clear whether there will be a need to discriminate between added (E163) and naturally occurring anthocyanins in a given foodstuff. It is presumed that regulatory compliance in terms of added levels is not an issue since E163 is permitted *quantum satis*. However, since the addition of E163 to foodstuffs satisfies a technological need, it is likely that any anthocyanins present in such foods will be added.

Though generally limited to acidic foods, the use of anthocyanins to colour foodstuffs is reasonably widespread. The distribution of the different anthocyanins in fruit and vegetable source materials is wide and complex but all are based on the 6 parent aglycones. Acidic solvent extraction is generally used with some form of selective clean-up followed by reverse-phase HPLC. Diode-array detection is used to monitor and to spectrally characterise the pigments. The intact anthocyanins and parent aglycones can be analysed by RP-HPLC with PDA and/or MS(MS) detection with sufficient sensitivity but there is very little evidence of validated methods. There is a clear need for development and validation of analytical methods for the determination of added anthocyanins in processed foods and in order to facilitate the transfer of technology, methods need to be kept as simple as possible. Two optional strategies

require consideration, which must be developed with sufficient scope to cover the range of foodstuffs permitted to contain added E163:

(i) Development of methods to characterise and measure intact anthocyanins. For spectrophotometric measurement, results are non-specific and can be expressed in terms of the major anthocyanin, but it is important to select the most appropriate standard. Determination of individual anthocyanins using HPLC is recommended since it will provide a greater amount of detailed data on anthocyanin profiles. However, anthocyanin reference standards are not widely available so where necessary, these will need to be prepared and /or surrogate standards would have to be used.

(ii) Development of methods to measure the parent aglycones and relate this to overall anthocyanin content. This option is technologically easier to achieve but data will however be relatively limited. It may be possible to devise a procedure for relating the aglycone content to the anthocyanin content for instance by expressing each aglycone as the most common anthocyanin e.g. the 3-glucoside. All of the aglycones may be obtained or purified as reference materials but they are relatively less stable than the anthocyanins.

The methods described by Goiffon et al (1999), Tian et al (2005), Lee et al (2005) and Ling et al (2009) could be considered for use as a basis for any future development.

The extraction and analysis conditions for a selection of available methods for anthocyanins are summarized in Table 2.

Group 9: Other colours

Vegetable carbon E153

Vegetable carbon (often referred to as vegetable carbon black) is produced by the carbonization of vegetable material such as wood, cellulose residues, peat and coconut and other shells (EU, 2008). The raw material is carbonized at high temperatures and consists essentially of finely divided carbon. It may contain minor amounts of nitrogen, hydrogen and oxygen, and some moisture may be absorbed after manufacture. It is an odourless and tasteless black powder, which is insoluble in water

and organic solvents. By comparison, carbon black is a material used for industrial applications is produced by the incomplete combustion of heavy petroleum products such as coal tar, ethylene cracking tar, and a small amount from vegetable oil.

No method of assay is prescribed in the EU purity criteria (EU, 2008). However, the JECFA specification for vegetable carbon prescribes a method in which the total carbon is measured in a dried (120°C for 4 hours) sample by one of several methods or commercial instruments for carbon analysis, such as instruments for C, H, O determinations or combustion / gravimetric carbon analysis (JECFA, 2010). Vegetable carbon is not soot or black carbon ('carbon black'), which are the two most common generic terms applied to various unwanted carbonaceous by-products resulting from the incomplete combustion of carbon-containing materials. (ICBA, 2004). Soot and black carbon also contain large quantities of dichloromethane- and toluene-extractable materials, and can exhibit an ash content of 50% or more.

No literature references could be found on the analysis of vegetable carbon food colouring or on the extraction or analysis of foodstuffs for added vegetable carbon. By far the greatest use of carbon (carbon black) is as a pigment and reinforcement in rubber and plastic products and a small number of articles describing methods used for the characterization or analysis of carbon black from the perspective of its industrial applications have been identified. Kiattanavith and Hummel (1993) describe a method for the determination of carbon black filler in natural rubber vulcanizates by olefin metathesis degradation using a $WCl_6/(C_2H_5)_3Al_2Cl_3$ catalyst . Essentially, natural rubber vulcanizate samples were Soxhlet extracted and dried, and reacted with 1-octene and catalyst under controlled conditions for ca. 48h. The carbon black particles were separated from the reaction mixture by repeated centrifugation and solvent washing and dried prior to gravimetric determination. Solvent density was reported to be a critical factor in analyte recovery. It is very doubtful that this approach would lend itself to the analysis of foodstuffs, given the complexity of possible sample types likely to be encountered.

Oxygen and air were used as purge gasses for the thermogravimetric determination of carbon black in high-density polyethylene (Faud, Ismail and Moh, 1997). The authors highlighted the care required when selecting the purge gas and highly specialized

instrumentation was required. It is very doubtful that this technique could be used to determine small amounts of vegetable carbon in food samples since it would not be possible to discriminate between carbon added as a colorant and the carbon originating from charring of the sample constituents.

Leisen, Breidt and Kelm (1999) used solid state (magic angle spinning (MAS)) ^{1}H nuclear magnetic resonance (NMR) relaxation to study cured natural rubbers with different carbon black fillers. The studies allowed confirmation of the mechanisms concerned with the interactions between elastomeric networks and active filling materials i.e. the reinforcing effect of the carbon black. While the method was reported to work well, the carbon black contents of samples studied ranged between 35 and 65%, which is far in excess of what might be expected in a food sample even though E153 is permitted *quantum satis* in a wide range of foodstuffs. Moreover, the technique requires highly specialized and expensive NMR equipment and MAS probe, and is very unlikely to be applicable to quantitative analysis in complex food systems. In a similar vein, electron spin resonance (ESR) has been used to characterize vegetable carbon materials by studying the paramagnetic characteristics of carbons produced by low temperature carbonization (Kravchenko et al., 2001).

Saxena, Gilmour and Hays (2008) have developed a method based on Ficoll (a hydrophyllic polysaccharide) density gradient centrifugation and elemental carbon analysis using thermal optical transmittance to isolate and measure carbon black (and diesel exhaust) particles ingested by cultured cells. Microscopy was also used to observe and characterize the particles *in vivo*. The cell pellets obtained after trypsinization (with EDTA) and centrifugation were resuspended in RPMI medium and recentrifuged. The free carbon black settled at the bottom of the Ficoll layer whereas the cells with ingested carbon black were observed at the Ficoll-RPMI interface, which were harvested and washed before being heated (100°C) in sodium dodecy sulfate (SDS) solution. The ingested carbon black is insoluble in hot SDS solution and was isolated by high speed centrifugation, facilitated by the addition of silica. Following washing and suspension in saline, the extracted carbon black was dried on a quartz filter prior to carbon analysis. A two-stage carbon analysis regime was used; volatile organic carbon was released by stepwise heating to ca. 850°C in a helium atmosphere. This was first oxidised to carbon dioxide using a manganese

dioxide catalyst and secondly reduced to methane using nickel hydride, whereupon it was measured using a flame ionization detector (FID). The non-volatile elemental carbon due to carbon black was determined subsequently following stepwise heating in a helium/oxygen atmosphere to ca. 900°C, oxidation/reduction and FID detection.

Overview and recommendations

No methods were identified for the determination of added E153 to foodstuffs. Of the non-food methods for carbon black analysis reviewed here, the Ficoll density gradient centrifugation with elemental carbon analysis method appears to have the greatest potential for development as a technique for the extraction and estimation of carbon black in foodstuffs, especially when coupled to microscopy. However, the method was developed specifically for samples of lung epithelial cells and alveolar macrophages *in vitro*, which are comparatively simple matrices compared to compound foods. It is therefore not likely to lend itself to facile transfer to enforcement laboratories. Vegetable carbon is permitted for use in Morbier cheese under Annex III and in all foodstuffs under Annex V parts 1 and 2 of Directive 94/36/EC, all at *quantum satis* (EU, 1994). What it is not clear is the range of levels at which vegetable carbon is used in foodstuffs, so it is difficult to assess the qualitative and quantitative suitability of the Saxena, Gilmour and Hays (2008) method, or indeed its potential for development into a robust analytical method for foods that can be readily applied in enforcement laboratories.

It is questionable whether investment in research to develop methods for E153 in foods is worthwhile, given its small application range, that no decision on its ADI has been made by EFSA and that the JECFA ADI is 'acceptable'. Conversely, the EU specification for E153 does however contain a purity specification for polyaromatic hydrocarbons, which is defined as the result of a semi-quantitative comparative assay.

The extraction and analysis conditions for a selection of methods that could be potentially adapted for analysis of foods for vegetable carbon are summarized in Table 2.

Calcium carbonate E170

Calcium carbonate ($CaCO_3$) is the product obtained from ground limestone or by the precipitation of calcium ions with carbonate ions (EU, 2008). It is a white crystalline or amorphous, odourless and tasteless powder that is practically insoluble in water and alcohol. It dissolves with effervescence in diluted acetic acid and mineral acids. No method of assay is prescribed in the EU purity criteria for E170 (EU, 2008). However, the JECFA specification for calcium carbonate prescribes a method based on titrimetric procedure following acidification (JECFA, 2010).

The calcium content of foodstuffs varies considerably and may be enhanced by the presence of other additives or ingredients containing Ca. Chalk, a natural form of $CaCO_3$, has historically been used to adulterate flour and methods for its determination have been based on the measurement of total Ca or by release of carbon dioxide (Kirk and Sawyer, 1991). In the latter, the evolved CO_2 may be determined volumetrically following acidification with HCl and capture in a Chittick apparatus. Alternatively, the CO_2 may either be adsorbed onto active trap and determined gravimetrically, or absorbed into barium hydroxide solution and back-titrated against oxalic acid. Ion chromatography (IC) can be used for the determination of carbonate but it is limited by the fact that one of the more common eluants in suppressed IC is bicarbonate-carbonate based (Tarter, 1987). However, carbonate has been determined in beer and wine by IC using conductivity detection (Haddad and Jackson, 1990).

Total Ca is usually determined after ashing by precipitation as calcium oxalate and titration with potassium permanganate or by a colorimetric procedure with chloranilic acid (Kirk and Sawyer, 1991). Atomic absorption spectroscopy (AAS) and atomic emission spectroscopy (AES) are the most common techniques used for the determination of total Ca. In AES the spectra of multi-elemental samples can be very congested, and spectral separation of nearby atomic transitions requires a high-resolution spectrometer. Excitation processes such as inductively-coupled plasma (ICP) can greatly reduce molecular interferences. The 422.7nm line width is used for the detection of Ca in AAS. Samples are commonly digested in concentrated HNO_3. Over 20 years ago Salvador, de la Guardia and Mauri (1988) described an AAS method for Ca in food slurries (cocoa, milk, oyster tissue) and obtained similar results when comparing dry ashing with wet digestion, and discussed the influence of sample particle size. More recently, Ca and other metallic ions have been determined

simultaneously in foods using AAS in which the detection limits depended upon sample type and wavelengths used, where for Ca this was 1.9mg/kg fresh sample (Gottelt et al., 1996). Calcium contents of cattle liver and kidney, maize, lucerne and grass ranged from 37.5-14,820 mg/kg. Standard reference materials were used to validate the method comprising mussel tissue, bovine liver and wholemeal flour.

Julshamn, Maage and Wallin (1998) reported the results of a collaborative study involving 11 laboratories on the determination of Ca (and Mg) in foods by AAS after wet microwave digestion. The method was tested on 5 food samples (apple, milk powder, minced fish, wheat bran and chocolate) and 2 composite diet samples ranging in Ca concentration 290±15 to 3900±200 mg/kg. Repeatability and reproducibility of the results was very good indicating that the method is suitable for determining Ca at levels in excess of 4000 mg/kg dry matter, provided the HNO_3 concentration is carefully controlled. ICP-mass spectrometry (ICP-MS) has been used for the determination of trace amounts of calcium in foodstuffs (Fera, 2010). Typically, 0.5g (dry) to 3g (fresh) of sample is digested in nitric aid using quartz high-pressure closed vessels and microwave heating prior to ICP-MS. Detection limits are generally in the range 0.1 to 1 mg/kg, which is adequate for the determination of Ca added as a food colorant but the method is not specific to $CaCO_3$.

Other analytical techniques that have been used for the determination of Ca in foods include capillary zone electrophoresis for vegetables following crushing and extraction into boiling water (Fukushi et al., 1997), and spectrophotometry at 470 and 630nm based on the formation of a Ca-alizarin (1,2-dihydroxyanthraquinone) complex following ashing of the sample and acid digestion (Gao, 2002). The detection limit for the Gao method was 0.035 µg/mL provided that masking agents were used and mean (n=6) recoveries of Ca from vegetables and other foodstuffs spiked at 20 and 5 mg/kg respectively were 108 and 101% respectively. More recently, a biosensor has been reported for the determination of Ca in milk and water using a catalase enzyme electrode (Akyilmaz and Kozgus, 2009). Although reported as a rapid and sensitive method, the range of test samples was very small, however mean (n=5) recoveries of Ca spiked at 120 – 170 mg/L) into 3 different milk samples ranged from 98.2 to 99.2% and compared well with a titrimetric procedure. Ion

chromatography has been used for the determination of $CaCO_3$ in snail shells (White et al., 2007) but this technique does not appear to have been applied significantly to the analysis of foodstuffs but has been used for brine (Dionex, 2010) and a very limited number of foods and beverages (Haddad and Jackson, 1990).

Overview and recommendations

Calcium carbonate does not have widespread use in the food industry as a colouring material. E170 is permitted under Annex V Parts 1 and 2 of 94/36/EC in a wide range of foodstuffs *quantum satis* (EU, 1994). Calcium is present naturally in foods at a variety of different levels and the levels required to achieve the required additive (colouring) effect of $CaCO_3$ are expected to be high. There are very few specific methods available for the determination of calcium carbonate in foodstuffs and they are largely outdated. The most commonly used analytical techniques for determining the $CaCO_3$ contents of foods rely on the measurement of total calcium by atomic spectroscopy using flame, ICP or other related techniques following acid digestion of the sample, all of which should give adequate sensitivity and be readily available to enforcement laboratories, since any added calcium is likely to overwhelm any calcium present naturally. Carbonate can be determined by measurement of evolved CO_2 using a number of techniques but has only been applied to a very limited sample range. AAS/ICP-MS methods such as those described by Julshamn et al (1998), and Fera (2010) appear to be the most promising candidates for method development, but must be validated for the range of different foodstuffs permitted to contain calcium carbonate.

The extraction and analysis conditions for a selection of methods that could be potentially adapted for analysis of foods for calcium carbonate are summarized in Table 2.

Titanium dioxide E171

Titanium dioxide (TiO_2) consists essentially of pure anatase and or rutile which may be coated with small amounts of alumina and/or silica to improve the technological properties (EU. 2008). TiO_2 is insoluble in water and organic solvents and dissolves slowly in hydrofluoric acid and in hot concentrated sulfuric acid. No method of assay is prescribed in the EU purity criteria for TiO_2. However, the JECFA specification for

TiO_2 prescribes a method based on a titrimetric procedure following digestion in sulfuric acid and reduction and complex formation with hydrochloric acid and aluminium (JECFA, 2010). Titanium dioxide is highly stable to heat, light, oxygen and pH making it unaffected by almost any food processing.

Historically, the determination of TiO_2 was based on the spectrophotometric procedure described by Njaa (1961) in which samples of poultry digesta were ashed and dissolved in a mixture of sulfuric acid and hydrogen peroxide to give a yellow complex determined at 410nm. The method prescribed by the AOAC (Leone, 1973) for the determination of TiO_2 in cheese follows a similar procedure and was used as the basis for a refined procedure described by Short et al (1996) for the determination of titanium dioxide added as an inert marker in chicken digestibility studies. Three spiked diet samples (750, 500 and 250g/kg) analysed for Ti gave recoveries of 98.7, 99.5 and 99.7% respectively. Mean (n=2) Ti concentration in 18 digesta samples ranged from 18.93 to 54.95 mg/g.

However, there are very few literature references available for the determination of TiO_2 in foods. Hamano et al (1990) described a colorimetric procedure for the determination of micro amounts (10 – 100 mg/kg) of TiO_2 in processed cheese, chocolate and chewing gum. Approximately 0.5 – 2g of sample was taken, depending upon the sugar content, and digested by refluxing in a mixture of sulfuric and perchloric acids with ammonium sulfate. After dilution with water, the digest was treated with ascorbic acid to remove interference from Fe^{3+} and the colour developed using a solution of diantipyrylmethane in dilute HCl. The absorbance at 390nm was measured against a blank solution after allowing the colour to develop for ca. 10 minutes at ambient temperature. The HCl concentration was reported to be a critical factor for colour development. Despite the safety concerns surrounding the use of concentrated perchloric acid, the authors preferred the wet ashing procedure to dry ashing because it was faster and the risk of losses through volatilization were eliminated. Using H_2SO_4 alone resulted in significantly lower recoveries of TiO_2 spiked into white chocolate, whereas recoveries were much higher in the presence of ammonium sulfate. No explanation was offered for this effect. Kani et al (1986) compared the platinum and porcelain crucibles for ashing of food samples (chocolate,

cheese, chewing gum and jelly beans) prior to colorimetric measurement and reported that the much cheaper porecelain crucibles gave acceptable results.

Calibration was linear over the range 0.2 – 10 mg/kg TiO_2 which gave a limit of determination equivalent to ca. 5 mg/kg when 2g of sample was digested and diluted to 50mL. Percentage recovery values (n=3) of TiO_2 from spiking experiments ranged from 92.1±2.3 to 94.9±3.1 at 10 mg/kg and from 97.4±2.4 to 99.3±2.6 at 100 mg/kg for two different samples of processed cheese. For two samples of white chocolate, the percentage recoveries at 10 mg/kg and 100 mg/kg spike levels ranged from 93.7±2.6 to 93.8±1.8 and from 99.5±2.8 to 99.8±1.9 respectively. No recovery experiments were carried out on chewing gum but no TiO_2 was detected in a single retail sample. Similarly, TiO_2 was not detected in two retail samples of processed cheese, whereas levels of 15±0.4, 50±2.1 and 3200±85 mg/kg were reported for samples of chocolate. The authors estimated that up to 20 samples could be analysed in ca. 6 hours using this method.

More recently, Myers et al (2004) used a similar procedure to that described by Short et al (1996) later refined by Titgemeyer et al (2001) for the analysis of feed and faecal samples developed to be more rapid and to give greater accuracy. Calibration showed a high degree of linearity (r^2 = 0.999, RSD 1.3 – 2.4%) and sample background interference was very low. Recoveries of TiO_2 spiked into three different sample types gave recoveries of between 96.7 and 98.5% even in the presence of chromium, a potential interference (spike levels not given). The average time for complete analysis was reported to be ca. 4.5 hours.

Inductively coupled plasma optical emission spectroscopy (ICPOES) has been used to determine TiO_2 in foods (Lomer et al., 2000). Samples (500mg) were digested in 10mL concentrated H_2SO_4 at 250°C for 1 hour then diluted to 5.9M H_2SO_4 before ICPOES at 336.121nm. Emission intensity was suppressed by H_2SO_4 so standards were acid concentration matched. To assess accuracy, different amounts of TiO_2 embedded in gelatine (0.5 – 12mg) were used as a control samples and a standard reference material was used to determine accuracy. The detection limit for titanium was 5.5±2.0 ug/L and calibration was linear below 5 mg/L. The mean recovery of titanium from the gelatine control samples was 95±9.2% (n=14) and from the

reference material was 95±11.8% 9 (n=12). Twenty-five different retail foodstuffs (including confectionery, cheeses, chewing gum, sauces and dressings, mustard and beverage whiteners) were analysed using the developed method of which 13 contained no TiO_2 above the detection limit. Large differences in the levels of TiO_2 were found in the remaining 12 products that declared TiO_2 as an additive, ranging from 0.045 to 0.782%. Good agreement was found between the analytical results and the levels added by manufacturers where declared (n=4).

Boguhn et al., (2009) compared the colorimetric procedure with ICPOES following an established acid-based sample hydrolysis regime used for the former. Diets for various species of livestock and their ileal digesta and faeces were analysed and spike levels ranged from 0 to 50 g/kg TiO_2. The authors concluded that supplemented TiO_2 can be determined successfully in these samples using either method but the calibration and recovery figures indicate that the ICPOES method gives a higher analytical accuracy. A range of other related spectrometric techniques can be used for the determination of elemental titanium and other trace elements in food, which could be used instead of ICPOES (Caroli, 2007).

Overview and recommendations

E171 is permitted under Annex V Parts 1 and 2 of 94/36/EC in a wide range of foodstuffs *quantum satis* (EU, 1994). Titanium is present naturally in foods at only low mg/kg levels whereas the levels required to achieve the required additive (colouring) effect are expected to be significantly higher. There are very few methods available for the determination of titanium dioxide in foodstuffs but they tend to cover the small range of applications of E171. However, both the colorimetric and ICPOES methods prescribed for the analysis of animal feeds, digesta and faeces, and a restricted range of foods offer promising potential for adaptation, especially to related elemental spectroscopic techniques.

The TiO_2 contents of foods reported by Lomer et al (2000) showed a wide range of concentration hence analytical methods must be able to demonstrate an appropriate level of sensitivity. While the capital investment in ICPOES instrumentation is costly compared to colorimetry, the former is most often used for multi element analysis. ICP-mass spectrometry (ICP-MS) has been used for the determination of trace

amounts of titanium in foodstuffs (Fera, 2010). Typically, 0.5g (dry) to 3g (fresh) of sample is digested in nitric aid using quartz high-pressure closed vessels and microwave heating prior to ICP-MS. Detection limits are generally in the range 1 to 10 mg/kg, which is adequate for the determination of titanium added as a food colorant but the method is not specific to TiO_2.

Adequately sensitive methods such as that described by Hamano et al (1990) could be considered as a basis for method development, since this technique appears to provide greater accessibility for enforcement laboratories. It will require validation for the range of different foodstuffs permitted to contain titanium dioxide, and is likely to require improved selectivity.

The extraction and analysis conditions for a selection of methods for analysis of foods for titanium dioxide are summarized in Table 2.

Iron oxides and hydroxides E172

Iron oxides (and hydroxides) are produced synthetically and consist essentially of anhydrous and/or hydrated iron oxides (EU, 2008). The range of hues includes yellows, reds, browns and blacks. Food quality iron oxides are primarily distinguished from technical grades by the comparatively low levels of contamination by other metals. This is achieved by the selection and control of the source of the iron and/or by the extent of chemical purification during the manufacturing process. Iron oxides are insoluble in water and organic solvents but are soluble in concentrated mineral acids. No method of assay is prescribed in the EU purity criteria for E172 (EU, 2008). However, the JECFA specification for iron oxides prescribes a method based on a titrimetric procedure following digestion in hydrochloric acid and hydrogen peroxide and iodometric titration (JECFA, 2010).

While there are numerous literature references to the determination of iron in foodstuffs, none could be identified that were specific to iron oxides/hydroxides. A small number of articles were identified describing the analysis of various other substrates for iron oxides. Abramov et al (1982) determined the iron (II) and iron (III) oxide content of glasses using redox titrimetry based on acid digestion followed by the formation of a complex between iron (III) in the presence of sodium vanadate and sulfosalicylic acid. The level of Fe_2O_3 determined in glass was ca. 0.08% and interference was observed from other transition metals, fluoride and phosphate. Iron oxide has been determined in compact cosmetic products using neutron activation where a limit of quantitation of 45 µg Fe was reported (Kanias, 1987) and in finely dispersed sludges at levels approaching 35% w/w using complexiometric titration (Kostousova and Osokina, 1989). Kanai (1990) used ion chromatography to determine the iron (II) and (III) oxide content of geological materials and also applied to the analysis of sedimentary rocks containing large amounts of organic carbon. The sample was dissolved in 50% H_2SO_4 and 26M hydrofluoric acid, heated at 200°C and diluted with boric acid masking solution prior to ion chromatography. Post-column reaction with pyridylazo resorcinol formed a red complex with Fe(II) and Fe(III) that was detected at 520nm. Divalent copper, manganese and zinc ions were separated from the analytes but showed potential for interference, especially if the pH was not

controlled rigorously. Analysis of geological reference materials gave recoveries of 89.3 – 104.6% over the concentration ranges 0.37 - 4.18% (Fe(III)) and 0.32 – 7.87% (Fe(II)).

X-ray fluorescence has been used for the determination of iron(III) oxide in cement (Bosch Reig et al., 1998), while the iron oxide content of soils has been carried out using flow-injection analysis with inductively coupled plasma (ICP)-atomic emission spectrometry (AES)(Gong et al., 2000) and with diffuse reflectance spectroscopy (Richter et al., 2009). A simple and selective solid phase extraction and flame absorption spectroscopic method has been used for the determination of trace amounts of iron(III) in water, in which the analyte was recovered (>99%) with a preconcentration factor of >166. Various cationic interferences had no effect on recovery and the limit of detection was 0.63 ng/mL (Khayatian and Pouzesh, 2007).

The determination of non-specific iron in foodstuffs such as flour and has been historically carried out using the Pringle method, which requires the sample to first be ashed and digested in HCl/HNO_3 and determined colorimetrically with o-phenanthroline at 520nm (Kirk and Sawyer, 1991). Recently, a sensitive spectrophotometric method for the trace determination of iron and other elements in food, milk and tea using a novel bis-azo dye has been reported Sharma and Singh (2009). Samples were ashed and digested in $HNO_3/HClO_4/HCl$ and buffered before reacting with 2,6-bis(1-hydroxy-2-naphthylazo)pyridine (PBN) and measuring the absorption of the resultant complex at 550nm. Recoveries of Fe spiked in to 7 foods over the range 14.0-63.0 mg/kg was in the range 99.3-100.8%. The corresponding relative standard deviation values ranged from 0.26-1.15% indication good accuracy. The presence of other interfering metals were masked allowing the method to be highly selective for iron however, EDTA, fluoride, oxalate, citrate, tartrate and cyanide interfered significantly at low Fe levels. The results were comparable to those found by Atomic absorption spectroscopy (AAS).

AAS, atomic emission spectrometry (AES) and mass spectrometry are nowadays often used as the end-determination methods after ashing and digestion. There are many literature references for the application of these techniques to the determination of trace levels of iron (and other elements) in foodstuffs such as the comprehensive

work by Cubadda (2007). The limits of determination achievable by the various techniques described are significantly lower than would be required for the determination of iron added as oxide for colouring effect hence sensitivity would not be an issue since E172 is permitted under Annex V Parts 1 and 2 of 94/36/EC in a wide range of foodstuffs *quantum satis*. ICP-mass spectrometry (ICP-MS) is used for the routine determination of trace amounts of iron in foodstuffs (Fera, 2010). Typically, 0.5g (dry) to 3g (fresh) of sample is digested in nitric aid using quartz high-pressure closed vessels and microwave heating prior to ICP-MS. Detection limits are generally in the range 0.1 to 1 mg/kg, which is adequate for the determination of iron added as a food colorant but the method is not specific to iron oxides.

Dos Santos et al (2009) used high resolution continuum source electrothermal (HRCSE) AAS with direct solid sampling for the determination of iron (and cadmium) in grain products (flour, bread and biscuit). While the method showed good analytical performance in terms of selectivity, sensitivity (LOQ 1.7 mg/kg Fe), accuracy and precision (using CRMs), all experiments were carried out using a prototype instrument. A single laboratory validation and ring trial has recently been undertaken to determine nine nutritional elements including iron in food products by ICP-AES after wet microwave digestion (Poitevin et al., 2009). Sample digestion (HNO_3/H_2O_2/HCl, depending upon the system) was performed with either closed or open vessel systems and thorough validation was undertaken. The robustness and efficiency of the method was proved through a successful ring trial for 13 certified in-house reference materials. The method was judged to be cost-efficient, time saving, accurate and fit for purpose according to rigorous internationally recognised quality criteria. The ten study materials included six reference materials comprising sterilized cream, baking chocolate, whole egg powder, baby food composite, corn bran, non-fat milk powder, and four in-house test materials comprising infant cereals with milk powder, chocolate milk powder, dietetic milk powder and pet food. The mean (n=9) iron contents of five reference materials tested using the method (peanut butter, chocolate milk powder, infant cereal, dietetic milk powder and wheat gluten) were 16.4±0.8, 169.9±4.8, 80.4±1.2, 81.1±1.0 and 54.3±6.8 mg/kg respectively. The corresponding RSD_r and RSD_R values ranged from 1.5 to 5.0%, and from 4.1 to 14.9% respectively. The calculated z-scores ranged from -2.2 to 0.2 respectively.

Overview and recommendations

E172 is permitted under Annex V Parts 1 and 2 of 94/36/EC in a wide range of foodstuffs *quantum satis*.No methods were identified for the determination of added E172 to foodstuffs whereas numerous methods are available for total Fe. While a small number of methods are available for the determination of iron oxides in non-food matrices, it is not likely that they will be sufficiently adaptable for routine analysis of foodstuffs due to sample complexity and/or instrument availability and cost. Both spectrophotometric and especially AAS/ICP-AES and ICP-MS methods such as those described by Poitevin et al (2009) and Fera (2010) could be considered for development for total Fe determination in foods as they offer the most potential for adaptation to measuring added iron oxides. These methods have adequate sensitivity but will require validation for the range of foods permitted to contain iron oxides.

Since they share common features for sample digestion, AAS and ICP-MS methods also have the potential to be developed for the simultaneous determination of total Ca, Ti and Fe, hence the development of multi-element methods should be considered.

Synopsis

In undertaking the literature search and subsequent review, several aspects on the current state of knowledge of the extraction and analysis methods for natural colouring materials in foods have become apparent. For those colouring materials with relatively widespread occurrence and use such as the carotenoids and anthocyanins, there are a large number of analytical methods available. However, most of these are concerned either with the identification and measurement of the colours in source materials, or for measurement of levels for nutritional purposes (carotenoids, riboflavin) or colouring effect/application (anthocyanins). However, the importance of methods developed for the analysis of source materials for all of the permitted colouring materials should not be underestimated, since in most cases many either constitute foodstuffs or similar biological matrices. The exceptions to this are clearly the inorganic colouring materials and caramel. While several methods are available that include enzyme pre-treatment of samples prior to extraction to increase extraction efficacy, the technique is not widespread but has the potential for application to complex foodstuffs. Sowbhagya and Chitra (2010) have recently

reviewed the enzyme-assisted extraction of colorants (and flavourings) and compared the technique with conventional extraction procedures. Although enzyme-assisited extraction was not applied to any complex food systems, examples of application to the extraction of carotenoids from marigold, chilli, tomato and carrot, chlorophyll from alfalfa and anthocyanins from blackcurrants are discussed.

The importance that pigment standards have in analytical calibration highlighted by Schoefs (2005) cannot be overemphasised and applies to all colour classes. Availability of colour standards of known purity is often an issue and preparation may be necessary for which minimum identification criteria are requisite. Moreover, while colour formulations may be used as secondary reference materials, it is equally important that colorant profiles are known hence access to standards of the main colouring principles of known purity is crucial. In some cases (e.g. caramel), suitable specific marker compounds may therefore need to be identified in order to make the methods more readily transferable to enforcement laboratories. This in turn may require the preparation of analogues as reference materials. The spectrophotometric determination of natural colours can be confused by the use of conflicting extinction coefficients found in the literature. For example, the published extinction coefficients for annatto have been summarized and compared to highlight disparities (Levy and Rivadeneira, 2000). This issue however applies to all natural colour standards since, depending upon the extinction coefficient used, large errors might be incurred. Moreover, published extinction values to not always agree with those adopted for colour purity specifications by the EU or JECFA , largely due to misassumptions made regarding solvent effects. The discrepancy in published extinction values might be traced back to the 'erroneous' coefficients that have been used subsequently as a reference value by various other workers.

Not all of the carotenoids within those permitted for use in foods are covered to the same extent; there are many publications on methods of analysis for the carotenes,

lycopene, lutein and paprika extract (capsanthin and capsorubin), whereas there are relatively very few for β-apo-8′-carotenal, ethyl ester of β-apo-8′-carotenoic acid and canthaxanthin. Some of the methods for the latter group have been developed for the analysis of animal feed but it is likely that these will be readily adapted to the analysis of foods. One significant advantage is that many carotenoid methods possess sufficient scope for the simultaneous analysis of several carotenoids. The amount of information on annatto mirrors the amount of research undertaken in recent years, much of which has been undertaken by the UK Government, in response to safety concerns. It is also clear that for annatto and the other carotenoids, identification and measurement of all geometric isomers is important and many methods are capable of separating and measuring them.

Due to the complexity of anthocyanin chemistry, the analysis of anthocyanins is likewise complicated. However, there are many literature methods available for their identification and measurement but very few have been developed specifically for processed foodstuffs. Two possible analytical strategies are proposed, based either on the analysis of the intact anthocyanins, or on the production and measurement of the parent aglycones. The chlorophylls/chlorophyllins are a similarly complex group of pigments but a number of methods have been developed for their analysis in recent years. These require broadening in scope to cover all foodstuffs permitted under the regulations with concomitant validation.

Although a few applications to food are available, methods for curcumin analysis are largely restricted to biomatrices other than food, which reflects the interest in its nutritional properties. Some of these have been reasonably well validated but require adaptation to the analysis of foods and must encompass all three curcuminoid analogues. While there are relatively few available methods for the determination of cochineal in foods, the methodologies are reasonably well established for source materials but these require further development, broadening in scope and bringing up to date. A similar situation exists for beet red but methods for its analysis are poorly established. Moreover, it is important that not only the main beet colour principles but also degradation products are included in the method scope.

Given that caramels have the greatest use by far as food colours, their chemistry is not well understood and is further complicated by their classification into four types. As a consequence, unlike all of the other natural colours (excluding vegetable carbon), methods for the determination of caramels are empirically based; reliant on the measurement of unidentified but characteristic marker peak. A reasonable number of well defined but limited methods are available, most of which have been produced under the auspices of the UK Government. There is a clear need for the application of new analytical technologies to the development of methods for caramel.

Because of its nutritional importance, there is a large amount of information available on the analysis of riboflavin (vitamin B_2), for which a number of validated methods are available. Conversely, there are very few methods available on the determination of titanium oxide in foods, and none for the direct determination of calcium carbonate and iron oxides in foods. Methods based on elemental analysis are likely to be the best approach for these colours but they will require development and validation, with a view to the use of multi-element techniques such as AAS, ICP-AES and ICP-MS. Techniques such as particle induced x-ray emission allow a sensitive, multi-elementary and non-destructive analysis of biological samples compared to the more conventional techniques of AAS, ICP-AES and ICP-MS (Bertrand, Weber and Schoefs, 2003). However, while this technique is unlikely to be in common use in food analysis laboratories it does offer comparative sensitivity, may be hyphenated to chromatographic analysers and adapted to microscopic analysis. No literature references could be found for the determination of vegetable carbon in foods and only a small number of methods could be found for the analysis of industrial materials, one of which may have sufficient scope for development.

Apart from those for calcium carbonate, titanium dioxide, iron oxides and vegetable carbon, and to a lesser extent caramels, methods for the extraction and measurement of natural colours in foods or similar matrices possess adequate sensitivity and selectivity – at least as a basis for further development. All require availability of

reference standards, some of which are either difficult to obtain and/or require extraction or synthesis. Not all have been developed and validated to the same extent; some require fundamental development (e.g. $CaCO_3$, TiO_2, Fe_2O_3, vegetable carbon and possibly caramel) whereas others require method refinement/adaptation and single-laboratory validation (e.g. β-apo-8′-carotenal, ethyl ester of β-apo-8′-carotenoic acid and canthaxanthin, curcumin, cochineal, beet red, chlorophylls, anthocyanins). Some methods require only refinement prior to full validation by collaborative trial (carotenes, lycopene, lutein, paprika extract, riboflavin). Most methods require broadening in scope to encompass all foodstuffs permitted to contain them, especially in the light of developments in colour formulation technology and nanotechnology in particular.

Methods available for the detection and measurement of natural colours in foods and beverages present as EU-permitted food additives have been assessed with a view to their potential use in a regulatory context. Knowledge gaps are clearly evident hence analytical methods for certain food colours require fundamental development whereas others require widening in scope and validation to various degrees. A summary of the attributes (applications, extraction, analysis, validation, sensitivity) is provided in Table 2 so that the reader can compare methods at a glance and focus on those attributes of the methods pertinent to their fitness for purpose. The data has been evaluated in order to inform and direct future research in this area with special consideration given to validated analytical techniques and their applicability for use in surveillance and in an enforcement role.

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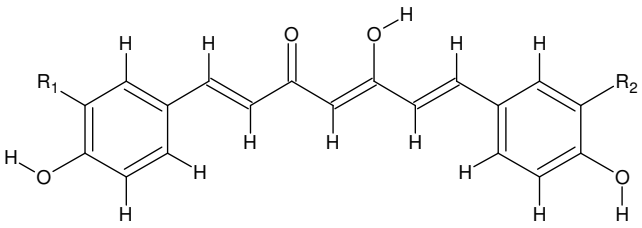
Table 1. Major anthocyanins from selected plant sources.

Plant source	Botanical name	Major anthocyanins[a]
Grape	*Vitis vinifera*	Mv-3-glu, Pn-3-glu, Pt-3-glu, Dp-3-glu, Cy-3-glu (free and acetylated)
Concord grape	*Vitis labrusca* var. Concord	Dp-3-glu,
Elderberry	*Sambucus nigra*	Cy 3-glu, 3-sam and 3-sam-5-glu
Blackcurrant	*Ribes nigrum*	Cy and Dp 3-glu, 3,5-diglu and 3-rut
Red cabbage	*Brassica oleraceae*	Cy-3-sop-5-glu (acetylated)
Strawberry	*Fragaria* spp.	Pg and Cy 3-glu

[a. Major pigments underlined. Cy=cyanidin; Pg=Pelargonidin; Pn=peonidin; Dp=delphinidin; Pt=petunidin; Mv=malvidin]



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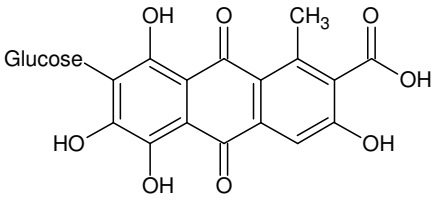

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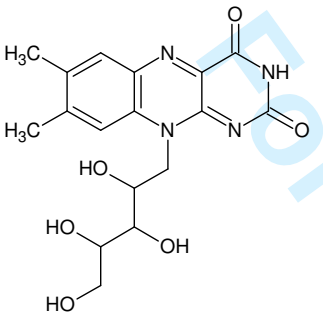

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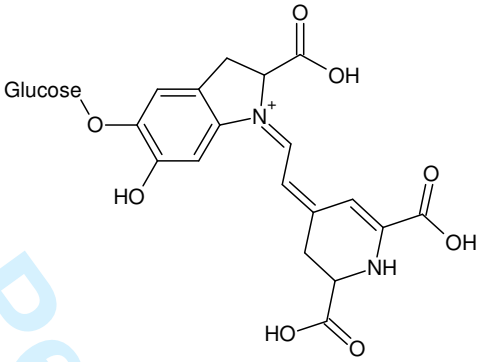

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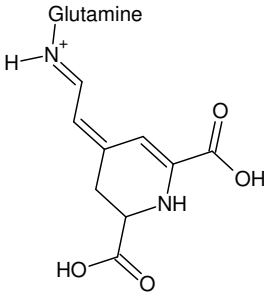

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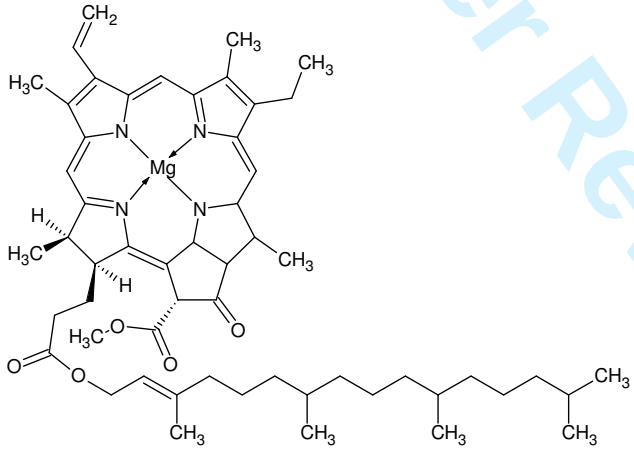

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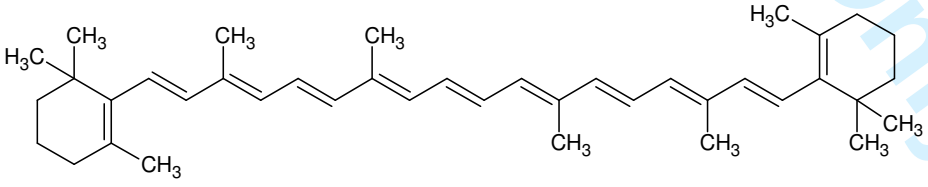

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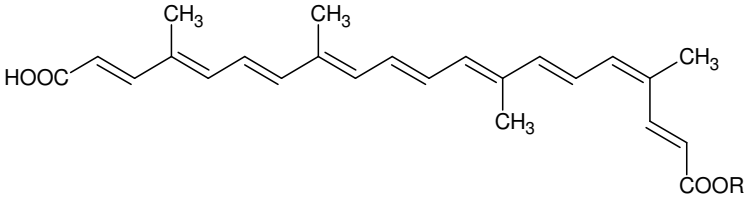
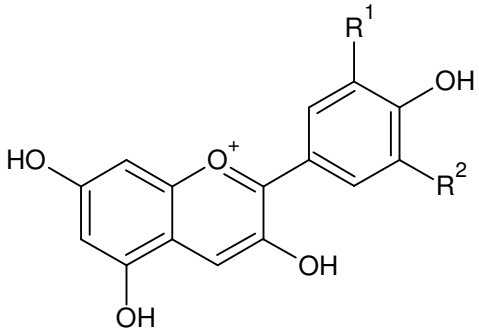


VII

2a



2b