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Review

High-performance liquid chromatography of food colours and its relevance in forensic chemistry

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Abstract

The forensic interest in the use of food colours is related to health rights and safeguard of the consumer, as established in national legislations. The regulations related to food dyes consider the health of human beings and the adulteration of foodstuffs and economic needs connected with quantitative restrictions on imports.

Efficient analytical methods are required for evaluating toxicity and authenticity or adulteration, in order (a) to determine whether there are synthetic dyes present in foods and whether they are permitted, (b) to determine the levels, (c) to confirm the absence of added dyes in foods where they are not declared and (d) to check on the stability of dyes during processing and storage.

Recent HPLC methods for the identification and determination of natural and synthetic dyes and of carcinogenic amines contained as impurities in synthetic dyes are reviewed.

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1. Introduction

1.1 The use of colour in food

What does a green-coloured fruit-jelly taste of? Most people perceive a mint taste, even if the jelly is orange, lemon or strawberry flavoured. Many wine consumers, if blindfolded, cannot distinguish between red and white wine. These and other tests [1] can show how the colour of food is so important as to organoleptically dominate the flavour. The enjoyment of food is so strictly affected by its appearance that an unappetizing colour was proved to adversely affect digestion. People are so used to buying food with a well coloured aspect that, when recently [1] a leading food firm in the UK put on the market canned garden peas and strawberry jams without added colours, sales decreased dramatically and were slowly restored only when colours were again added.

The addition of dyes replaces natural colours destroyed during processing conditions, gives the expected colour to otherwise colourless food or drinks, enhances natural colour and assures uniformity from batch to batch [1,2].

It would be a mistake to believe that colouring food is a choice of our industrial and consumer society. The practice probably predates the written record. Colours naturally present in vegetables, animals and minerals were used. Plinius the Senior (23–79 B.C.) reports that in Rome in the days of Roman Empire (3rd and 2nd centuries B.C.) wines were coloured with fruit berries and white earth was added to bread. In Athens "meat inspectors" overlooked the forbidden addition of a colour [1,2]. In the twelfth century precious rosy and violet sugars were imported into England from Alexandria [1] and an edict of 1396 in Paris banned colour addition to butter. The intensity of the colour and its stability were the only properties of the dye considered and no attention was paid to secondary, often toxic, aspects. The first attempts to draw public opinion against the indiscriminate use of colour in food came in 1820 from Accum and in 1850 from Hassal, who first proposed tests to evaluate dangerous adulterations and denounced a series of widely diffused toxic applications. Brazil woods, husks, elderberry and bilberry were employed in the manufacture of the so-called "genuine" old Port wine. Tea leaves were coloured with verdigris (copper acetate) and Gloucester cheese with red lead and pickles were boiled with a halfpenny to make them green. Confectionery was coloured with lead chromate, cinnabar [mercury(II) sulphide] or prussian blue mixed with lead chromate.

With the advent of dyestuff manufacture (dating from about 1856), new colours were synthesized and many of them were found to be suitable for food. The introduction into food preparations of the new synthetic colours had great success not only because of the generally greater efficiency and stability to light, temperature and redox agents but also because synthetic colours were believed to be safer. In recent times, with the rediscovery of "natural things",

the opposite holds, even though the use of natural colours cannot guarantee the absence of toxic side effects.

When natural and synthetic dyes began to be suspected of toxicity, many actions were undertaken in different countries. Positive lists (containing only the permitted colours) were preferred to forbidden lists (which indirectly permitted the use of all the dyes not included). These lists are continuously updated with the exclusion or inclusion of different dyes. More information about the use of additives and colours in food and in packaging, food chemistry and food safety can be found in specific publications [1–9]. Let us consider some regulations that are useful to know when planning a chemical analysis devoted to the identification and/or determination in food of toxic or adulterating dyes.

1.2. Forensic implications

Food colours are of forensic interest because of their connection with health rights, as established in national legislations [10–12].

Human health protection includes legislation concerning the use of colourants and safeguards for consumers. Every regulation related to food dyes considers, first, the health of human beings and second adulteration of foodstuffs and economic needs, especially connected with quantitative restrictions on imports.

The disparities between national legislations increase the difficulties in achieving a real free market and a greater uniformity within the community. Therefore such problems do not ease the difficulties of the European Economic Community (EEC), one of whose aims is to harmonize practice among the member countries. It is necessary to prepare a positive list that can be accepted by the largest number of countries and that contains the allowed colours and the purity requirements for food, in addition to the permitted divergences.

In the EEC, the colourants used in foodstuffs are regulated by directives, updated in the last few years, stating that: (a) member countries may authorize the movements of the foodstuffs containing only the permitted colourants listed in the directives; (b) the use of the allowed colourants cannot be completely forbidden; (c) if health protection may be at risk by using allowed dyes in the foodstuffs, each member country can withdraw, for a specific period, the authorization to trade the products, informing the other members and the Commission, to update the positive list; (d) the directives specify the transitory period allowed to conform with the national laws with the Community directives; (e) colourants of natural origin, used in the preparation of foodstuffs, must be excluded from the directives concerning the colourants because of their aromatic, tasty and nutritional properties; (f) listed colourants can be marketed only if regularly labelled, indicating the contained food dyes; in other words, consumers must be protected by adequate labelling; (g) imports within the EEC, applying Community regulations, cannot be forbidden only because of inadequate advertizing. The health authority checks the production and marketing of foodstuffs, executing inspections during processing, and confiscation in factories and stores. If necessary, the confiscated foodstuffs can be destroyed by health authority.

The national rules concerning foods and requiring registration in the positive list of food colourants must also be applied to imported products; this is because even if they are lawfully manufactured and marketed in another member country, there may be uncertainties of scientific research in that particular state or there may not be Community harmonization. Applying the first-mentioned regulation to imported products produced and widely consumed in other member states, national authorities, according to EEC Article 36, based on the principle of proportionality, have to authorize the use of colourant additives if justified by a real need or a nutritional need or in the light of eating habits, considering the possible interaction of one colourant with another or their cumulative or global effect. Therefore, the power of a member state to prohibit imports of products from other member States should be restricted to what is necessary to attain the legitimate aim of protecting health, also taking account of international scientific

research, particularly that of the Community committees.

In the same way, national authorities have to evaluate the risk linked to overcoming the acceptable daily intake of additives contained in foodstuffs, paying attention to eating habits. The absence of Community harmonization shows that European legislation has been very careful about the potential harm from these products. Therefore, member states may decide what degree of protection of health and life of humans is justified. But this is not an absolute discretion, because they must consider the Treaty requirements of the free movement of goods. The Community works through a Scientific Committee for Food whose members are either expert technologists, toxicologists or nutritionists. All are drawn from national specialist committees, to maintain effective contact with policies and action in the individual countries. Even if member states have to take into account the scientific research of international and EEC Committees, the Community suggestions are not mandatory, so that they must be incorporated in a regulation for each country under it own food law.

The use of any additive in manufactured products can be justified only if technologically necessary or because of meeting an economic or technical need. Community regulations about organoleptic and psychological aromas and colours allows a national authorization for selling foodstuffs containing dyes, taking care of public health and of the real need for colours in the manufactured products.

1.3. Some regulations

Directive 78725/EEC states that a dye is suitable for food or pharmaceutical use if it contains no more than 4% [10] of accessory colouring matter (isomers, homologues) and not more than 0.5% of intermediate synthetic products other than free aromatic amines. Owing to the admitted presence of these side-products, dyes are not denominated with the chemical term of the principal component, but often with

a casual name and some letters (e.g., CI, FD&C and E) and numbers. CI refers to the Color Index [13], which is a reference book that lists, divided into chemical classes, the colourants used in all kind of industries, each classification number describing a compound, its synthesis or natural origin, the official standard (if available) and relevant references. A second number in parentheses indicates the Index edition year in which the colourant is described.

The US Food and Drug Administration (FDA) nomenclature indicates the dyes with the letters F (food), D (drug) and C (cosmetics), followed by the colour of the dye and a number. A FD&C dye can be used for food, drug and cosmetics, whereas, for example, quinoline (D&C Yellow No. 10) is not admitted for colouring food. The EEC system has a list of purity specifications similar to the FDA and the nomenclature uses a number associated with the letter E. At least the WHO/FAO system (World Health Organization/United Nations Food and Agriculture Organization), mainly used in Australia, makes use of standard names.

The two main political areas in the world active in colourant regulations are the USA and Europe. The admitted colour lists are continuously updated, according the "Delaney clause" of Color Additives Amendment of 1960 to the FDA Act, which requires the banning from use in food of all additives that are carcinogenic at any level in animal tests. Because of suspected carcinogenicity in the 1970s, the dye amaranth (E123, FD&C Red No. 2), which was one of the most widely used, was delisted in the USA, being limited to only a few uses in other countries. In the 1980s FD&C Blue No. 2 (indigo carmine, E132) was also delisted, tartrazine (E102) was subjected to rigorous tests and recently (1990) the use of erythrosine (FD&C Red No. 3, E127 was discontinued). In the EEC, colours are regulated by Council Directives on colouring matters authorized for use in foodstuffs. Of relevant interest are the proposals in 1985 and 1992 in Foodstuff Directives for international harmonization of colourant regulations.

Table 1 lists some of the most widely used natural and synthetic dyes, taking into account

Table 1
Common natural and synthetic dyes

Colour	E No.	FD&C	Name	Comments
Yellow	E100		Curcumin	1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione
	E101		Riboflavine	7,8-Dimethyl-10-(D-ribo-2,3,4,5-tetrahydroxypentyl)isoalloxazine
	E102	FD&C Yellow No. 5	Tartrazine	4,5-Dihydro-5-oxo-1-(4-sulphophenyl)-4-[(4-sulphophenyl)azo]-1H-pyrazole-3-carboxylic acid trisodium salt
	E103		Crisoine S	2,4-Dihydroxy-azobenzene-4'-sulphonic acid
	E104	D&C Yellow No. 10	Quinoline Yellow	2-(2-Quinolyl)-1,3-indandione sulphanilic acid disodium salt
Orange	E110	FD&C Yellow No. 6	Sunset Yellow	1-(4'-Sulpho-1'-phenylazo)-2-naphthol-6-sulphonic acid disodium salt
Red	E120		Cochineal	Dried female insect *Coccus cacti* L. enclosing the young larvae
	E121		Orcein	Extract from lichens
	E123	FD&C Red No. 2	Amaranth	3-Hydroxy-4-[(4-sulpho-1-naphthalenyl)-azo]-2,7-naphthalenedisulphonic acid trisodium salt
	E124		Ponceau 4R	1-(4'-Sulpho-1'-naphthylazo)-2-naphthol-6,8-disulphonic acid trisodium salt
	E127	FD&C Red No. 3	Erythrosine	3',6'-Dihydroxy-2',4',5',7'-tetraiodospiro{isobenzofuran-1(3H)-[9H] xanthen}-3-one disodium salt
Blue	E130		Blue Anthraquinone	N,N'-Dihydro-1,2,1',2'-anthraquinonazine
	E132	FD&C Blue No. 2	Indigo Carmine	Indigotin-5,5-disulphonic acid disodium salt
	E133	FD&C Blue No. 1	Brilliant Blue FCF	N-Ethyl-N-{4-[(4-{ethyl[(3-sulpho-phenyl)methyl]amino}phenyl)(2-sulphophenyl)methylene]-2,5-cyclohexadien-1-ylidene}-3-sulphobenzenemethanaminium hydroxide inner salt, disodium salt
Green	E140		Chlorophyll	See text
Brown	E150		Caramel	
Black	E151		Brilliant Black BN	2-4'(4-Sulpho-1-phenylazo)-7'-sulpho-1-naphthylazo-1-hydroxy-8-acetylaminonaphthalene-3,5-disulphonic acid tetrasodium salt
Yellow, orange, red	E160		Carotenoids	See text
	E161		Xanthophylls	See text
	E162		Betalaines	Beetroot
	E163		Anthocyanines	See text

that in each country it is necessary to make reference to their own list. An important parameter, ADI (acceptable daily intake), was first formulated by the Joint WHO/FAO Expert Committee on Foodstuffs and is defined as the daily intake of a chemical which during the

lifetime appears to be without appreciable risk on the basis of all the known facts at the time.

2. Natural and synthetic colours

Food colours are usually divided into natural or nature-identical (synthetically prepared) and synthetic colours.

2.1. Natural colours

In the last few years, colourants of natural origin have been increasingly used, owing to consumer pressure. However, some of them are made synthetically and are termed nature-identical. World legislation [1] does not distinguish between naturally occurring colours and their chemical equivalents produced by a synthetic route, as long as the purity requirements are met. Anyway, when colours are prepared through chemical synthesis, the presence of toxic impurities must be considered, which can be present in the starting reagents or can be formed as by-products during processing or storage. Natural colours are generally characterized by a lower tinctorial strength with respect to the synthetic colours and, in addition, are generally more sensitive to light, temperature, pH and redox agents.

Natural colours are generally divided into the following classes: anthocyanins, carotenoids, betalaines, chlorophylls, and other colours such as caramel, carminic acid (an animal-source colour), oricel (a vegetable-source colour) and GSE (grape skin extract).

2.2. Synthetic colours

A study in the 1970s estimated that the annual average individual consumption of food in USA was 645 kg [7], 5.5 g of which were represented by synthetic colours and about 85% of this amount was represented by tartrazine, Sunset Yellow FCF and amaranth (which was subsequently banned). According an evaluation done in the UK (ref. 1, Ch. 3), the amount of consumed food which contains added colour is ca. 0.5 kg per head per day and represents nearly half of the total diet. The average colour content is less than 50 ppm in these foods and the average amount of synthetic colours consumed is less than 10 g per head per year. Yellow colours (tartrazine and Sunset Yellow FCF) and the red colours (carmoisine, Ponceau 4R and amaranth or Allura Red) were predominantly used.

The kind and the amounts of food and beverage with added colour (in amounts up to 120 mg per kg of product) are surprisingly high. Colour is added [1] to blackcurrant, raspberry, strawberry, plum, soft drinks, sugar and jelly confectionery, desserts, table jellies, canned fruits, jams, baked cakes, gravy mix, wafers, sausages, fish, pastes, broad beans, canned vegetables, drink powder, custard powder, breakfast cereals, haddock fillets, salad cream mayonnaise, piccalilli, meat pastes, etc. Different mixtures of two or more colours are also used for obtaining different shades and hues. Dyes can be used as water-soluble pigments or as the so-called lake form, prepared for precipitation of water-soluble colours with aluminium, calcium or magnesium salt on a substrate. The water-insoluble powder so obtained can be mixed to prepare new shades, can be dispersed in various carriers and assures greater homogeneity of colour, for example in frozen products. A new generation of non-toxic dye is the "polymeric dye", in which the colour is chemically bound to a polymer substrate. The resulting molecule, owing to its size, is able to pass through the gastrointestinal tract and to be excreted without being adsorbed or metabolized [1], provided that it is stable enough and it does not release absorbable and potentially toxic fragments.

Synthetic colours can be divided into classes as a function of their chemical structure: azo dyes, triarylmethane and phenylmethane dyes, xanthene dyes, quinoline dyes, anthraquinones, phenols.

2.3. Impurities

Owing to impurities contained in the reagents or due to formation in side-processes during the chemical synthesis of the dyes, aromatic amines (in particular naphthylamine, benzidine, 4-aminodiphenyl) and aromatic polycyclic hydro-

carbons can be present in many dyes and mainly in azo dyes. Carcinogenicity and toxicity must be checked, therefore, not only for dyes but also for impurities contained therein. Also, the toxicity of the colours used in food packaging must be considered, and also the possibility of their release into the contained food.

2.4. Analyses for dyes

As mentioned, many food dyes show possible carcinogenic effects and colours can be used in the adulteration of food. These considerations underline the importance of dye analysis in forensic science. Ever increasing numbers of forensic science laboratories will have to deal with the analysis of food dyes with respect to the adulteration of food.

Efficient analytical methods are therefore required in food quality control for evaluating toxicity and authenticity or adulteration, in order (a) to determine whether there are synthetic dyes present in foods and whether they are permitted, (b) to determine the levels, (c) to confirm the absence of added dyes in foods where they are not declared and (d) to check on the stability of dyes during processing and storage.

Difficulties are often encountered when a complex mixture has to be analysed, also because standard solutions are not easily available. Reviews and books [2,7,15–17] concerning food chemistry report HPLC methods published up to 1980 and the extraction procedures, which are often a very important and essential step in the analysis [1,2,18–20].

Recent HPLC methods for the identification and determination of natural and synthetic dyes and impurities contained in them are reviewed in this paper.

3. Natural colours

3.1. Anthocyanins

Anthocyanins are water-soluble bluish-red pigments that occur naturally in many plants as glycosides of anthocyanidins, which are characterized by a flavylium cation structure. They are naturally present in cranberry, roselle, hibiscus flowers, miracle fruit and grape or are formed as by-products in the wine and grape-juice industry and are receiving increasing attention after the delisting of amaranth. The most common anthocyanidins are pelargonidine, cyanidine, peonidine, delphinidine, petunidine and malvidine and the sugar moiety can be glucose, rhamnose, galactose, xylose or arabinose. Anthocyanin structures being correlated with genetic factors, their distribution has a taxonomic relevance and can be usefully employed in detecting adulteration.

Pigment extracts are commercially available, grape skin extract (GSE) being the most common. In the USA [21], only two pigments are allowed: GSE (grape skin extract) only for beverages and GCE (grape colour extract) for non-beverage foodstuffs, whereas in EEC countries the anthocyanin extracts from edible vegetables are generally allowed. Fruit juice and concentrates can be used without particular restrictions and represent important ingredients in the manufacture of foods and beverages.

3.1.1. HPLC determination

Many HPLC methods have been reported for the determination of anthocyanins and some of them are covered in a recent review [22]. The use of a reversed-phase C_{18} stationary phase and a water–methanol–formic acid mobile phase with linear gradient elution and photodiode-array detection was proposed by Mazza [23] for the separation of anthocyanins and related compounds in Saskatoon berry, which is native to the southern Yukon and Northwest Territories and has recently received interest in industrial cultivation. The extract is particularly complex because it contains, besides anthocyanin pigments, also gallic acid, cinnamic acid, quercetin, naringenin, rutin and caffeic acid. Fractions from HPLC analysis, re-analysed by paper chromatography and spectrophotometric analysis, permitted the identification of malvidine-3,5-diglucoside, malvidine chloride, cyanidine-3-galactoside, pelargonidine chloride, cyanidine-3-glucoside and cyanidine 3-xyloside. HPLC methods were employed by Hong and Wrolstad [21,24] in the identification of anthocyanin pigments (del-

phinidine, cyanidine, petunidine, pelargonidine, peonidine and malvidine) in blackcurrant, blackberry, black raspberry, elderberry, cherry, plum, grape, bilberry and red cabbage. The anthocyanin composition of many fruits being quite distinctive, the study has chemotaxonomic relevance and at the same time anthocyanin analysis is very useful for detecting adulteration and in product development and quality assurance fields. They also studied the colouring properties of the commercially available anthocyanin colourants. The same authors [25] used a polymer-based reversed phase as the stationary phase and water–acetonitrile–orthophosphoric acid as the mobile phase (pH < 2) for the determination of anthocyanins in their red flavylium cation form, while a reversed-phase C_{18} stationary phase and a water–acetonitrile–acetic acid mobile phase with isocratic elution were used for anthocyanidin determination. Spectrophotometric detection at 520 nm was employed. Derivatives of delphinidine, petunidine and malvidine could be distinguished from derivatives of cyanidine, peonidine and pelargonidine and it was shown that cranberry contains only cyanidine and peonidine, roselle contains delphinidine and cyanidine and strawberry contains pelargonidine as the major pigment and a smaller amount of cyanidine. By a combination of droplet counter-current chromatography and semi-preparative HPLC, Andersen [26] isolated antocyanin pigments from fruits of *Vaccinium uliginosum* L. (bog whortleberry). Fifteen pigments were identified, malvidine-3-glucoside being the major component (35.95% of the total pigment content). The pigments were identified as 3-monoglycosides in which the aglycones malvidine, delphinidine, cyanidine, and petunidine are combined with glucose, arabinose and galactose and peonidine with glucose. The method makes use of photodiode-array detection at 515 nm, a reversed-phase C_{18} column and a mobile phase of formic acid–water–methanol in different proportions with linear gradient elution.

In *Tradescantia pallida*, two major complex anthocyanins were identified with an HPLC method by Shi *et al.* [27]: one was cyanidine-3,7,3'-triglucoside with three molecules of ferulic acid and an extra terminal glucose and the other was similar but without the terminal glucose unit. These pigments have good properties as food colours because they are stable and highly coloured at pH > 4. A reversed-phase C_{18} 10-μm and a semi-preparative polymeric 10-μm stationary phase were used, with water–methanol–acetic acid–formic acid as the mobile phase. The increased demand for red raspberry (*Rubus ideaus* L.) and its relatively high cost make raspberry juice a likely target for adulteration. An HPLC method for anthocyanin composition analysis was developed by Spanos *et al.* [28] with the aim of creating a reference database for the authenticity of red raspberry juice. A reversed-phase C_{18} 10-μm stationary phase was used and the mobile phase was water–methanol–acetic acid with linear gradient elution. Cyanidine-3-sophoroside, cyanidine-3-glucoside, cyanidine-3-glucorutinoside, cyanidine-3-rutinoside and pelargonidine-3-sophoroside were identified, cyanidines being highly predominant. A comparison with the anthocyanin composition in other berries (blackcurrant, sour cherry, blackberry, strawberry) confirmed that the anthocyanin pigment composition depends greatly on variety but also on geographical origin, processing and storage. By HPLC Goiffon *et al.* [29] determined anthocyanins contained in bilberry, blackcurrant, strawberry, blackberry, black cherry, morello cherry, redcurrant, raspberry and elderberry. Identification was performed by semi-preparative chromatography, partial hydrolysis and analysis of fragments. Retention was shown to depend on two factors, one specific to the anthocyanidin and the other to the sugar. Glucose, galactose, arabinose, xylose and L-rhamnose were the sugars considered and cyanidine, pelargonidine, paenidine, petunidine and malvidine the aglycone moieties. The chromatographic profiles obtained for the mentioned fruits can be usefully employed in quality control analysis of commercial juices, sorbets, fruit wines, liquors and jams and can usefully assist in detecting adulteration. The chromatographic conditions are reversed-phase C_{18}-bonded silica (7 μm) as the stationary phase, different compo-

sitions of water–acetonitrile–formic acid (pH 1.9) as the mobile phase with isocratic and gradient elution and spectrophotometric detection at 546 nm.

Using HPLC, Williams et al. [30] separated twenty anthocyanin glucosides and diglucosides, making use of a reversed-phase C_{18} analytical column and different water–methanol–acetic acid (for the non-acetylated glucosides) or phosphoric acid (for the acetylated glucosides) mobile phases with isocratic and gradient elution with spectrophotometric detection at 520 nm. In particular, 3-glucosides, 3,5-diglucosides, 3-(6-o-coumaryl)glucosides and 3-(6-O-p-coumaryl-glucoside)-5-glucosides were separated in grape juice. The effects of temperature and mobile phase pH were also studied. Lunte [31] used a combination of spectrophotometric (photodiode-array detector) and amperometric (dual-electrode electrochemical detector, upstream electrode +1.00 V vs. Ag/AgCl, downstream electrode, 0.00 V vs. Ag/AgCl) detection to develop an HPLC method able to classify anthocyanidins and flavonoids in wine and grape juice in a single chromatographic run and without the need for component isolation. Gradient elutions with a mobile phase containing ammonium phosphate buffer (pH 2.5) and acetonitrile were performed. A reversed-phase C_{18} (5 μm) stationary phase thermostated at 35°C was used.

An HPLC method was employed by Velioglu and Mazza [32] for the separation of anthocyanins and other flavonoids in the petals of Rosa damascena, used to produce rose petal jam and attar of roses. The study proposed the characterization of both anthocyanins and colourless flavonoids (which contribute to the intensification and stabilization of the colour) in order to establish the experimental conditions for improving colour stability during the processing and storage of rose jam. About 25 components were detected and most of them identified. The total anthocyanin content was evaluated as 285 mg/kg of fresh petals. An ion-pair HPLC method was proposed by Drdàk et al. [33] for the determination of anthocyanins in red wines, with spectrophotometric detection. A silica-based C_{18} column was used with a mobile

phase containing water, methanol, perchloric acid and different alkylamines at pH 1.45, the best separation being obtained with butylamine as ion-pairing agent.

3.2. Carotenoids

Carotenoids can be of natural origin or synthesized. More than 500 naturally occurring carotenoids are known, which contribute to the yellow, orange and red colours in fruits, flowers, seeds and feathers. Carotenoid pigments are generally extracted from carrots, annatto, paprika, saffron, palm oil seed, citrus peel and maize. From reactions with proteins, blue, green and purple colours can also be obtained. Carotenoids are generally water insoluble and soluble in fats; food colours are prepared as dispersions in water. The most important carotenoids generally admitted as food colours are α-, β-, γ- and ζ-carotene, bissine, norbissine, capsantine, capsorubine, licopene, phytoene, phytofluene, β-apo-8'-carotenal (E160). β-Carotene is orange, ζ- is yellow and phytoene and phytofluene are almost colourless. Another group of xanthophylls (E161) includes flavoxanthine, luteine, cryptoxanthine, rubixanthine, violaxanthine, rodoxanthine and cantaxanthine. At concentrations between 1 and 10 ppm carotenoids are added to margarine, fruit juices, salad dressing, ice-cream, cheese, pasta, cakes, icings and soups.

In addition to their colouring properties, the importance of carotenoids is also correlated with their content in vitamin A and their properties as antioxidants. As carotenoids are one of the most abundant micronutrients in cancer-preventive food, methods for their identification and separation are particularly required in the development of epidemiological studies in the area of cancer prevention.

3.2.1. HPLC determination

Many HPLC methods have been developed for the determination of carotenoids, owing also to their antioxidant and radical scavenger properties. Problems associated with analysis have been discussed by Scott [34]. The analysis is

complicated because of the large number of naturally occurring carotenoids, the instability to light, heat, oxygen and acids and the difficulty of obtaining authentic reference standards. A review by Tee [35] was recently published concerning the determination of carotenoids in foods, underlining the importance for health and nutrition.

The ever more sophisticated adulteration methods require the development of ever more sophisticated analytical methods. Perfetti *et al.* [36] developed a method for the characterization of orange juice with the aim of detecting its adulteration by means of computer pattern recognition analysis. The experimental data were obtained with the use of a C_{18} stationary phase and acetonitrile–water–methanol–methylene chloride–acetic acid as the mobile phase, with spectrophotometric detection at 280 nm. The study is further complicated by the fact that fruit juice shows large natural variations induced by variety, growing location, season and processing methods. The presence of β-carotene, cryptoxanthine and zeaxanthine was identified. Principal components and K-nearest neighbour analysis were usefully employed to distinguish between authentic and adulterated samples for 99 orange juices.

Czinkotai *et al.* [37] studied the composition of paprika pigments (*Capsicum annuum*) whose dry powder and oleoresins are used as food colours. In addition to carotenoids, pigments also contain xanthines. A reversed-phase C_{18} stationary phase was used and different mobile phase compositions with isocratic and gradient elution were compared. The use of acetonitrile-2-propanol–water (39:57:4) permitted the separation of capsorubin, capsanthine, zeaxanthine and their esters and β-carotene, phytoene and phytofluene. The qualitative and quantitative variation in carotenoid composition of two paprika products was also followed during storage. The data of Czinkotai *et al.* were confirmed in a TLC study by Minguez-Mosquerra *et al.* [38].

Tee and Lim [39] developed an HPLC method for the separation of carotenoids (in particular α- and β-carotene and lycopene) and retinol using a C_{18} (10 μm) stationary phase, acetoni-

trile–methanol–ethyl acetate as the mobile phase and spectrophotometric detection at 313, 340 and 436 nm. The method was applied to the determination of β-carotene in 40 types of foods and was compared with the AOAC method. β-Carotene contents were determined in eggs (the highest level being in duck egg), fish and seafood (high concentrations were found in canned sardines and dried oysters), meat and meat products (the highest amount was in chicken liver), milk and milk products (butter had the highest content), oils (the highest content in margarine) and other processed foods such as canned baked beans and canned sardines, which also contain lycopene.

Taylor and McDowell [40] developed an HPLC method for the separation and classification of carotenoids and chlorophylls in the fresh leaf of tea (*Camellia sinensis* L.). From a Kenyan tea clone 28 pigments were identified and a correlation was proposed between levels of chlorophylls and/or carotenoids and the quality of black tea. The experimental conditions were a reversed-phase C_{18} (5 μm) column, acetonitrile–water–ethyl acetate as the mobile phase and photodiode-array detection at different wavelengths. Using HPLC, twelve carotenoids were identified in palm oil by Ng and Tan [41], the predominant ones being α- and β-carotene. A reversed-phase C_{18} column and acetonitrile–methanol–methylene chloride flowing as the mobile phase with isocratic elution were used, with UV–Vis diode-array detection.

Lesellier *et al.* [42] studied in dried carrots the separation between *trans* and *cis* isomers of α- and β-carotenes. The use of different reversed-phase silica- and polymer-based stationary phases and different mobile phase compositions was compared, and the effect of temperature was studied. It was shown that the industrial food treatment and cooking causes *trans–cis* isomerization in carotenoids, which in turn leads to a decrease in colour and to decreased provitamin A activity. Resolution of *cis*- and *trans*-α- and β-carotene isomers was achieved using a rapid HPLC method by Chandler and Schwartz [43] which employed a column packed with calcium hydroxide and acetone–hexane mixtures as the

mobile phase with spectrophotometric detection at 340 and 436 nm. In agreement with the results obtained by O'Neil and Schwartz [44], the highest percentages of isomers were found in processed foods. In fresh products, *cis* isomers were found in plums, nectarines and peaches and no isomer in sweet potatoes, carrots and tomatoes. In general, the processing of vegetables by cooking, freezing or canning does not usually cause significant decreases in total carotene content but a 15–35% decrease in vitamin A content.

Simon and Wolff [45] separated carotenes from typical and dark orange carrots (containing high concentrations of provitamin A) in raw and frozen samples; α-, β-, γ- and ζ-carotene and lycopene were identified, carotene accounting for 44–79% of the total while α-, β- and ζ-carotenes accounted for 94–97% of the total carotene. The method makes use of a C_{18} ODS-3 column together with a mobile phase of acetonitrile–methylene chloride–methanol. Phytofluene, β-carotene, phytoene, lycopene and, as minor components, α-carotene, lycoxanthine and *cis*-mutatoxanthine were identified by Tan [46] in tomato paste. An HPLC method was employed using a C_{18} (5 μm) stationary phase and different mixtures of acetonitrile–methylene chloride–methanol as the mobile phase with photodiode-array detection.

Razungles et al. [47] determined carotenoids in fruits of *Rosa canina* and *Rosa rugosa* and of chokoberry (*Aronia melanocarpa*). Nine carotenoids, three carotenes (lycopene, ζ-carotene and β-carotene) and six xanthophylls (neoxanthine, *trans*-violaxanthine, *cis*-violaxanthine, 5,6-epoxyluteine, luteine and β-cryptoxanthine) were identified. The fruits studied were characterized by large quantitative differences, *Rosa canina* containing the highest levels of β-carotene. The HPLC conditions were a C_{18} reversed-phase (5 μm) stationary phase, water–acetone as the mobile phase with gradient elution and spectrophotometric detection at 450 nm.

Using HPLC with a C_{18} stationary-phase, methanol–water–ethyl acetate as the mobile phase with convex gradient elution and spectro-

photometric detection, carotenoids were determined in different fruits by Philip and Chen [48]. Apricot, mango and cantaloupe contain β-carotene whereas cryptoxanthin predominates in papaya, persimmon and tangerine. Peach, red bell pepper, oranges and red grapefruits contain intermediate levels of provitamin A, whilst tomato, yellow bell pepper, pineapple and watermelon are poor sources. Normal-phase (with a mobile phase of hexane–ethyl acetate) and reversed-phase HPLC (with methanol–ethyl acetate as the mobile phase with gradient elution) were used by the same workers [49] for the determination of carotenoid esters in extracts of red bell pepper (*Capsicum annuum*), marigold flower petals (*Taget erecta*), navel orange peel (*Citrus sinensis*) and tangelo peel and also [50] in commercially processed Valencia and navel orange juice concentrates. An accurate measurement of cryptoxanthine was found to be very important in the evaluation of the provitamin A content in citrus juice and in detecting the adulteration of orange juices with mandarin juice.

Red bell peppers are good sources of carotenoids and paprika extracts are commercially used as colouring and flavoring agents for foods. Gregory et al. [51], in an HPLC study with methanol–ethyl acetate as the mobile phase with linear gradient elution and a reversed-phase C_{18} stationary phase, showed that in red bell peppers capsanthin accounts for 60% of the total carotenoids, with the simultaneous presence of β-carotene (around 11%) and capsorubin (about 20%). Khachik and Beecher [52] used an HPLC method with methanol–acetonitrile–methylene chloride as the mobile phase and a C_{18} (5 μm) spherical reversed-phase column for the determination of carotenoids in carrot, sweet potato, pumpkin and red palm oil and showed that the effect of cooking and processing leads to a loss of β-carotene of about 19%, of which about 82% is the *trans* isomer, in agreement with other workers [42–44] and with the results of Kimura et al. [53], who studied the effect induced by saponification. Chen and co-workers [54,55] identified and determined the major carotenoids contained in water convolvulus (*Ipomea aquatica*), a vege-

table grown in Taiwan and China, by using an HPLC method with a C_{18} (5 μm) stationary phase and a mobile phase of acetonitrile–methanol–ethyl acetate. With a mobile phase of acetonitrile–methanol–chloroform–hexane up to twelve pigments could be separated.

The determination of ten carotenoids (luteine, zeaxanthine, canthaxanthine, β-apo-8'carotenal, β-cryptoxanthine, echinenone, lycopene, γ-carotene, α-carotene, β- and 15-cis-β-carotene) was performed by Olmedilla et al. [56] with a C_{18} (5 μm) column and different mobile phases containing acetonitrile, dichloromethane and methanol in different ratios. In tomato extract, luteine, lycopene, γ-carotene and β-carotene were identified. Daood et al. [57] identified carotenoids in Diospyros kaki fruits, together with sugars and organic acids. A reversed-phase C_{18} column was used, with acetonitrile–2-propanol as the mobile phase and spectrophotometric detection at 438 nm. γ-Carotene and β-carotene were identified and some esters (mutatoxanthine, zeaxanthine, α-cryptoxanthine, β-cryptoxanthine, γ-carotene, β-carotene, α-cryptoxanthine ester, β-cryptoxanthine ester and zeaxanthine esters) were separated. In virgin olive oil seventeen pigments containing chlorophylls and carotenoids were separated by Minguez-Mosquera et al. [58]. The pigment content was shown to be very different for the different varieties so that the method allows routine control analysis. A reversed-phase ion-pair chromatographic method was employed, making use of a C_{18} (5 μm) column and a methanol–acetone mobile phase containing tetrabutylammonium and ammonium acetate, with spectrophotometric detection at 410 and 430 nm. Rouseff et al. [59] determined β-carotene and other carotenoids (lycopene, ζ-carotene, phytoene and phytofluene) in a red grapefruit cultivar. A C_{18} column was employed, together with an isocratic mobile phase of acetonitrile–methylene chloride–methanol.

The use of a polymeric C_{18} column and tetrahydrofuran as the mobile phase permitted the optimization of an isocratic separation of carotenoids by Craft et al. [60], who studied the influence of multiple solvent modifiers and tem-

perature on the separation of a complex carotenoid mixture. The major carotenoid fatty acids were determined by Philip and Chen [61] in persimmon (Diospyros kaki) and papaya (Carica papaya) by an HPLC method using a C_{18} stationary phase and methanol–ethyl acetate as the mobile phase with linear gradient elution, with spectrophotometric detection at 465 nm. Khachik et al. [62] performed the separation, identification and determination of the major carotenoids and carotenal fatty acid esters in extracts of apricot, peaches, cantaloupe and pink grapefruits using a C_{18} reversed-phase column and isocratic and gradient elution.

A procedure was developed by Philip et al. [63] for the detection of adulteration of California orange juice concentrates with externally added carotenoids. The method involves the treatment of the extracted carotenoids with methanolic HCl to convert carotenoids with 5,6-epoxide end-groups into 5,8-epoxides and HPLC measurement with a reversed-phase C_{18} stationary phase, methanol–ethyl acetate as the mobile phase with convex gradient elution and detection at 465 nm. Adulteration can be evidenced by the use of characteristic ratios that can be calculated between the concentrations of the esters of cryptoxanthine and luteine in orange concentrates. Nagy et al. [64] developed an HPLC method with a C_{18} stationary phase and water–acetonitrile–tetrahydrofuran as the mobile phase for the separation of browning pigments forming in white grapefruit juice when stored in glass and cans under non-refrigerated conditions. Up to 100 pigments that absorb in the range 382–400 nm are formed.

An HPLC method with a C_{18} column and acetonitrile–methanol–ethyl acetate as the mobile phase permitted Daood et al. [65] to identify carotenoids and chlorophylls in vegetables and to show that cooking vegetables with brine or acidic solutions leads to the formation of phephytins from chlorophylls. β-Carotene was determined in green peas, green pepper, cucumber, lettuce, spinach and celery. It is worth emphasizing the high content of β-carotene in celery leaves and the consequent high nutritive value of this vegetable.

3.3. Betalaines

Betalaines are pigments generally contained in beetroot and include two classes of pigments: betacyanines and betaxanthines. The chromophore group is the 1,7-diazaheptamethine. Betacyanines are red and betaxanthines are yellow. The most common is betanine, which accounts [2] for 75–95% of the betacyanine content of beetroot (*Beta vulgaris*) and whose extract is a natural colour listed in the EEC list as E162. The principal betaxanthines are vulgaxanthin I and vulgaxanthin II. Owing to their low stability, betalaines are generally employed to colour food having a relatively short life and not requiring thermal treatment, such as yoghurt, confectionery, ice-cream, jellies, dessert, soups, meat and meat-based products.

A betanine pigment was also extracted by Forni et al. [66,67] from the berries of the American pokeberry (*Phytolacca decandra*). This red pigment, known as phytolaccanine, is a betacyanine identical with the betanine of beetroot and can be used as food colour, after its extraction and purification from toxic saponins.

3.3.1. HPLC determination

Pourrat et al. [68] developed an HPLC method for the determination of betacyanine and betaxanthine pigments in fermented red beetroot extracts. A reversed-phase C_{18} (10 μm) stationary phase was used and the mobile phase was methanol–water containing phosphate buffer with gradient elution. Five betacyanines (betanine, isobetanine, betanidine, isobetanidine and prebetanidine) were identified at 538 nm and confirmed by enzymatic hydrolysis with β-glucosidase of a solution of *n*-betanine and isobetanine. Small amounts of betaxanthines (identified at 477 nm) and the probable presence of vulaxanthines were also evidenced. Forni et al. [69] obtained the separation of betalaines pigment in blood-red *Opuntia ficus indica* by an HPLC method with a reversed-phase RP-18 column and water-phosphate buffer (pH 5) containing different amounts of methanol as the mobile phase, with spectrophotometric detection. A yellow pigment (475 nm) was identified

as indicaxanthine and red-violet pigments (538 nm) were identified as betanine, isobetanine and a betalainic glucoside.

3.4. Porphyrin pigments (chlorophylls)

Porphyrins are macrocyclic compounds containing four methine-linked pyrrole rings. Chlorophylls are the most important examples. Chlorophylls (E140) and their copper complexes (E141) are generally admitted green pigments. Copper derivatives are more stable than the natural magnesium derivatives, but free ionizable copper must not exceed 200 ppm. Plant extracts normally contain a mixture of four different chlorophylls.

3.4.1. HPLC determination

A separation method for chlorophylls and pheophytins in fresh and frozen peas was developed by Forni et al. [70] with the aim of following the colour changes that take place during storage under frozen conditions. The HPLC method employs an end-capped RP-18 (5 μm) column and acetone–ethanol–water as the mobile phase, with fluorimetric detection (excitation at 413 nm, emission at 669 nm). During storage of peas, the colour can loose its bright greenness, owing to the conversion of chlorophylls into grey-brown pheophytins and the discolouration of frozen peas was therefore followed through chlorophyll composition changes. Garrido and Zapata [71] studied the chlorophyll pigments in algae by HPLC methods with C_{18} reversed-phase silica- and and polymer-based stationary phases. The mobile phases were methanol–ammonium acetate–acetone–acetonitrile mixtures with gradient elution. Minguez-Mosquera et al. [72] studied the degradation of chlorophylls in olives during fermentation and conservation processes and their transformation into pheophytins and pheophorbides. Eighteen different pigments, including chlorophylls, carotenoids and degradation products, were separated by reversed-phase ion-pair chromatography with a C_{18} (5 μm) reversed-phase column and a mobile phase of water–methanol–acetone–tetrabutylammoniumm acetate–ammonium acetate.

Photosynthetic pigments from chromophyte marine algae were analysed by Garrido and Zapata [73] by an HPLC method using different reversed-phase C_{18} columns and a mobile phase of methanol–acetonitrile–ammonium acetate–ethyl acetate in different ratios with linear gradient elution. In leaves of spinach (*Spinacia olearia*), Canjura and Schwartz [74] separated and identified chlorophylls, α- and β- chlorophyllides and degradation products by means of photodiode-array detection and the help of mass spectrometry.

3.5. Other natural pigments

Studies of natural non-toxic dyes led to the proposal of extraction of pigments from animal and vegetable sources, *e.g.*, cochineal or carminic acid (E120) extracted from *Coccus cacti*, oricel or orcein (E121) extracted from *Lichen roccelle* or curcumin or annatto extracted from a tree.

3.5.1. Lac colour

Lac colour is a natural colour that derives from a secretion of the insect *Coccus laccae* (*Laccifer lacca* J. Kerr) from India and Southeast Asia, which parasitizes especially on legumes. The colour constituents are laccaic acids (mono- and dicarboxylic acids) and are characterized by an anthraquinone moiety. The colour is water-soluble and is used in Japan to colour tomato ketchup, strawberry jam, candy and beverages. The colour was extracted, treated with diazomethane to produce two reddish-orange markers and then analysed by an HPLC method developed by Yamada *et al.* [75] using a reversed-phase C_{18} (5 μm) column and acetonitrile–water as the mobile phase with detection at 495 nm. The method was employed for the determination of lac colour in colour preparations and in jellies. Concentrations of *ca.* 1.92 μg/g of laccaic D1 acid and 8.53 μg/g of laccaic D2 acid were shown to be present in a jelly, in disagreement with the label declaring no added colour.

3.5.2. Annatto and turmeric

Rouseff [76] developed an HPLC method for determining annatto and turmeric pigments in food using a reversed-phase C_{18} column and water–tetrahydrofuran as the mobile phase with isocratic elution, with spectrophotometric and fluorescence detection. Annatto consists of pigments from the outer seed coat from the tree *Bixa orella*, while turmeric is an extract from the rhizomes of *Curcuma turmeric*. Both extracts are used in the food industry separately or in combination to impart yellow-orange-red hues to a wide variety of products. The major components were shown to be curcumin, dimethoxycurcumin and bismethoxycurcumin.

4. Synthetic colours

4.1. Azo dyes

The chromophoric system is an azo group in association with one or more aromatic systems. Some common example are Allura Red AC, amaranth, Ponceau 4R, Red 2G, Fast Yellow AB, Sunset Yellow 2G and Chocolate Brown HT. The colours can be red, orange, yellow, blue, violet, black and brown. Another azo class of dyes is the azopyrazolines such as tartrazine and Yellow 2G.

4.1.1. HPLC determination

Ion-pair chromatography was employed by Lawrence *et al.* [77] for the separation of twelve primary food colours and the analysis of grape beverages. The use of different mobile phases formed by methanol and water in different ratios and containing tetra-*n*-butylammonium phosphate was compared. Spectrophotometric detection at different wavelengths made possible the separation of Ponceau SX, Fast Red, Benzyl Violet 4B, erythrosine, Skyark (a subsidiary of Sunset Yellow FCF), indigotine, Fast Green FCF, Brilliant Blue FCF, amaranth, tartrazine, Allura Red AC and Benzyl Violet 4B. The method is interference free from natural colours contained in commercial grape soda drinks.

Puttemans *et al.* [78] developed an ion-pair chromatographic method with a C_{18} (10 μm) stationary phase and an aqueous methanol mobile phase with tri-*n*-octylamine added as an ion-pairing agent for the determination of syn-

thetic dyes in soft drinks in the presence of organic acids and saccharin. They analysed 45 commercial samples of soft drinks and 11 lemonade syrups for tartrazine, amaranth, Sunset Yellow and Cochineal Red. Of the fourteen samples of orange soft drink analysed, only two contained synthetic colours (a mixture of E102 and E110) and of the fourteen lemonades one sample contained the same mixture, the six cola samples contained no additive and of twelve soft drinks only a grenadine was coloured (with Cochineal Red).

An isotachophoretic method was proposed by Karovicova et al. [79] for the determination of the synthetic colours tartrazine, Sunset Yellow FCF, Cochineal Red, amaranth, azorubine, Patent Blue Brilliant and Black Indigotine. The method was applied to powdered beverages, powdered pudding, hard candies and beverages. A method for the determination of synthetic dyes in sugar-rich foods, such as boiled sweets, fruit gums, lemon curd, jelly, blancmange and soft drinks, was developed by Greenway et al. [80] and makes use of HPLC and on-line dialysis for sample preparation. The method was called ASTED (automated sample treatment through enrichment of dialysates). The mobile phase was water–methanol containing ammonium acetate buffer and the column was ODS-2 Spherisorb (5 μm). Spectrophotometric detection was performed at 475 nm. Detection limits obtained for amaranth, Brown FK, Ponceau 4R and Sunset Yellow were 0.64, 1.58, 0.50 and 0.50 μg/ml, respectively. The method was applied to the analysis of blancmange banana and vanilla dessert, lemon curd, yellow and red boiled sweets, jelly, fruit gums and drinks. Using an HPLC method with a spherical C_{18} stationary phase and a water–methanol–phosphate buffer (pH 3.6) mobile phase with gradient elution and spectrophotometric detection at 214 nm, Sunset Yellow was determined in the presence of saccharin, tartrazine, aspartame and benzoic acid [81] in fruit drinks, lemonade, vodka mixer, bitter lemon, strawberry drink, diet tonic, orange and passion fruit drink. Quantification was performed by a multi-level calibration method.

The colour additive Citrus Red No. 2 is

principally formed by 1(2,5-dimethoxyphenylazo)-2-naphthol and it is used to improve the colour of the skin of oranges. Its use is permitted up to 2 ppm (based on the mass of whole orange) in fruit that is not to be processed, whereas it is forbidden for orange peel intended for consumption (for flavouring herbal teas and for candies). An HPLC method with detection limits of 28 ppb and recoveries of >93% for dried orange peel and >87% for fresh oranges was developed by Hope and Connors [82]. A C_{18} (5 μm) column was used and the mobile phase was acetonitrile–water mixture, with spectrophotometric detection at 504 nm. No dye was found to be present in all the dried orange peels tested or in the tea blends, whereas among the fourteen fresh oranges randomly purchased in various supermarkets four contained Citrus Red above the limit.

4.2. Xanthene dyes

The chromophore group is xanthene (dibenzo-1,4-pyran with amino hydroxyl groups in the meta position), which gives brilliant red or fluorescent yellow colours.

4.2.1. HPLC determination

The determination of erythrosine and subsidiary dye intermediates and side-reaction products (such as fluoresceine and 2',4',5'- and 2',4',7'-triiodofluoresceine) was performed by Lancaster and Lawrence [83] using an ion-pair chromatographic method. The mobile phase was methanol–water in different ratios and containing tetrabutylammonium phosphate and the stationary phase was C_{18}, with spectrophotometric detection at 249 and 500 nm. The commercial samples of erythrosine analysed were found to contain no detectable amounts of resorcinol and allowable amounts of subsidiary dyes and intermediate or side-reaction products. Thermal decomposition of erythrosine and fluoresceine in sugar solutions and in candies was also investigated. An HPLC method was also developed by Van Liederkerke and De Leenheer [84] for the determination of xanthene dyes and derivatives, namely fluoresceine, 4',5'-dibromofluoresceine, eosine Y, ethyleosine, 2',7'-dichlorofluoresceine,

tetrachlorofluoresceine, 4',5'-diiodofluoresceine, erythrosine B and phloxine B. A polystyrene-divinylbenzene (10 μm) column was used and the mobile phase was water–acetonitrile mixtures containing tetramethylammonium hydroxide and phosphoric acid, with diode-array spectrophotometric detection. In analyses of the purity of different commercial dyes it was found that in many instances the label indications and the actual amounts of dyes present did not correspond. The method can be extended to the preparative scale for the purification of impure commercial dyes.

4.3. HPLC determination of antraquinone dyes

By means of centrifugal partition chromatography, a separation method was developed by Hermans–Lokkerbol et al. [85] for the separation of anthraquinones in an extract of Rubia tinctorum hairy root culture. Alizarin, alizarin 1-methyl ether, lucidine and nordamncanthal were separated. Okamura et al. [86] developed an HPLC method with reversed-phase C_{18} column and water–acetonitrile as the mobile phase with gradient elution for the determination of altersolanol pigments. These are rare but not unique examples of naturally occurring tetrahydroanthraquinones, which are produced by the fungus Alternaria solani, a pathogen of early blight disease of tomato and potato.

4.4. HPLC determination of phenol dyes

It was shown by Bailey et al. [87] that in black tea two main classes of phenolic pigments are formed during the manufacturing processes, i.e., theaflavins and thearubigins. Whereas theaflavins are compounds of known structure, thearubigins are represented by a non-homogeneous group of phenolic pigments of unknown structure, with different chemical functionalities, probably formed by oxidative degradation of theaflavins. Eight theaflavins could be separated by means of an HPLC method with the comparative use of reversed-phase C_{18} silica-based columns of different particle size and of a styrene–divinylbenzene copolymer. The mobile phase was water–

acetonitrile with addition of acetic and citric acid or EDTA disodium salt.

4.5. HPLC determination of toxic impurities contained in dyes

An HPLC method was developed by Richfield-Fratz et al. [88] for the determination of ppb levels of aniline, benzidine, 4-aminobiphenyl (4-ABP) and 4-aminoazobenzene (4-AAB) in the colour additive FD&C Yellow No. 6, a synthetic water-soluble, monoazo colour additive, principally composed of the disodium salt of 6-hydroxy-5-[(4-sulphophenyl)azo]-2-naphthalensulphonic acid. The dye is synthesized by coupling diazotized sulphanilic acid with Schaeffer salt and could be contaminated by impurities from the reagents or the intermediates. The mobile phase was ammonium acetate–acetonitrile–water and a reversed-phase C_{18} column was used, with diode-array detection. Aniline was found in all the 34 certified samples of FD&C Yellow No. 6 investigated and produced by different manufacturers, at average levels of 97.6 ppb, the highest concentration being 419 ppb. 4-ABP and 4-AAB were found in 30 and 23 samples, respectively, with maximum levels of 23 and 1098 ppb, while benzidine was not identified in any of the samples. The same authors [89] investigated the presence of 4-nitro-p-cresidine and p-cresidine (responsible for carcinogenic effects) in FD&C Red No. 40, performing separation and identification by HPLC and GC–MS techniques. In all the 28 certified samples of colour surveyed, both 4 nitro-p-cresidine (at average level 1035 ppb, concentration range 165–7526 ppb) and p-cresidine (average level 105 ppb, concentration range 4–920 ppb) were found. Aniline was found in thirteen samples (average level 26 ppb, concentration range 3–383 ppb).

An RP-HPLC method with a C_{18} column, ammonium acetate–acetonitrile as mobile phase with gradient elution and spectrophotometric detection was developed by Bailey [90] for the determination of unsulphonated aromatic amines (aniline, benzidine, 2-aminobiphenyl, 4-aminobiphenyl and 4-aminoazobenzene) in the reg-

ulated colour D&C Red No. 33. An RP-HPLC method developed by the same author [91] with ammonia–tetrahydrofuran as the mobile phase permitted the determination of the lower subsidiary colours 5-(phenylazo)-6-hydroxynaphthalene-2-sulphonic acid (ANSC) and 4-[(2-hydroxynaphthalene-1-yl)azo]benzenesulphonic acid (BNSC) in FD&C Yellow No. 6. The analysis of 31 commercial dyes from different manufacturers showed that the levels of BNSC ranged from 0.002 to 0.728% and those of ANSC from 0.002 to 0.521%.

Lancaster and Lawrence [92] developed an ion-pair HPLC method for the identification of impurities in some food colours that can be present as a result of incomplete reaction during commercial colour synthesis. In particular, the presence of the intermediates naphthionic acid and R salt (2-naphthol-3,6-disulphonic acid, sodium salt) were investigated in amaranth, of sulphanilic acid and Schaeffer salt (2-naphthol-6-sulphonic acid, sodium salt) in Sunset Yellow and of sulphanilic acid and 1-(4-sulphophenyl-3-carboxy-5-hydroxypyrazolone) in tartrazine. The stationary phase was C_{18} (10 μm) and the mobile phase was a methanol–water containing tetra-n-butylammonium phosphate, with spectrophotometric detection. The method was also employed by the same group [93], with spectrophotometric detection at 522 nm, in the determination of non-sulphonated aromatic amines (mainly 1- and 2-naphthylamine), which are known to be carcinogens. Most are diazotized and coupled during the colour manufacturing process but low levels of unreacted free amines can still be found in most food colours. After treatment with dithionite, dyes were extracted with chloroform and H_2SO_4, diazotized and coupled. Recoveries always greater than 80% were obtained, with detection levels of 8 ng/g. Analyses of nine commercial samples of amaranth from seven manufacturers and three countries gave levels up to 435 μg/g of total 1-naphthylamine and up to 214 μg/g of total 2-naphthylamine, most being bound to the coupling salt and less than 5% being in the free state in the dye.

The presence of various non-sulphonated aromatic amines was also studied by the same

workers [94] in other synthetic food colours such as tartrazine, Sunset Yellow and Allura Red, which are manufactured from reagents that contain aromatic amines as impurities. By using ion-pair HPLC recoveries always greater than 85% and detection limits always lower than 32 ng/g were obtained. The analysis of seven commercial samples of tartrazine indicated that benzidine was present in three of them at concentrations up to 326 ng/g, and all the samples contained total aniline in concentrations up to 83.2 μg/g. In some samples 1-naphthylamine, 2-aminobiphenyl and 4-aminobiphenyl were also present,. at concentrations lower than 0.1 μg/g. The analysis of nine samples of commercial Sunset Yellow FCF showed high aniline contents for all the samples (maximum concentration 519 μg/g), and one sample also contained 1-naphthylamine, 2-aminobiphenyl and 4-aminobiphenyl. All eight samples of Allura Red investigated contained aniline (maximum concentration 98.7 μg/g) and p-cresidine (maximum concentration 35.4 μg/g) and six samples contained 1-naphthylamine (maximum concentration 4.76 μg/g). Most of the aromatic amines are bound to the coupling agent and less than 7% remains as free amine in the dye. Lancaster and Lawrence [95] then applied the results of these studies to the determination of non-sulphonated aromatic amines in finished food products, such as soft drinks and hard candies. The effects of heat and of the presence of sugar and citric acid were studied. The recovery was not affected and the detection limits for aniline, 1- and 2-naphthylamine and 2- and 4-aminobiphenyl were always lower than 0.3 ng/ml. The analysis of commercial samples of soft drinks showed the presence of aniline (up to 12.6 ng/ml) in an orange beverage, of 1-naphthylamine (up to 8.25 ng/ml) and 2-naphthylamine (up to 1.12 ng/ml) in a grape product. It is noteworthy that notwithstanding, on the basis of the manufacturing process, the presence of naphthylamine could be expected in drinks containing amaranth and of aniline in beverages containing tartrazine and Sunset Yellow FCT, aniline was found in a grape drink coloured with amaranth and Brilliant Blue. For hard candies, aniline was unexpectedly de-

tected in a product containing amaranth (concentration 9.2 ng/g) together with 1-naphthylamine (10.6 ng/g).

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