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Journal:	*Food Additives and Contaminants*
Manuscript ID:	TFAC-2009-079.R1
Manuscript Type:	Review
Date Submitted by the Author:	31-Mar-2009
Complete List of Authors:	Scotter, Michael; DEFRA Central Science Laboratory, Food Safety and Quality
Methods/Techniques:	Analysis - NMR, Chromatography - GC/MS, Chromatography - HPLC, LC/MS
Additives/Contaminants:	Colours, Process contaminants, Volatiles
Food Types:	Ingredients, Processed foods

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Abstract

Annatto food colouring (E160b) has a long history of use in the food industry for the colouring of a wide range of food commodities. The principle colouring components of annatto is the oil-soluble diapo carotenoid bixin, which is the methyl ester of the dicarboxylic acid norbixin, which is soluble in aqueous alkali. Bixin and norbixin therefore exhibit not only physicochemical properties normally associated with carotenoids but also certain anomalous properties that have an impact on the stability, food colouring applications and importantly the analysis of annatto. This review summarizes the key aspects of the structural determination of bixin (and norbixin) with special attention to *cis-trans* isomerization and how this links with its chemical structure, spectroscopic properties and stability. The oxidative, thermal and photo stability of annatto and the subsequent implications for its use in the colouring of foods, food processing, and the analysis of foods and beverages are discussed along with important mechanistic, thermodynamic and kinetic aspects. The main analytical techniques used for the chemical characterization of annatto i.e. spectrophotometry, NMR, chromatography (particularly HPLC) and mass spectrometry are reviewed in detail and other methods discussed. This links in with a review of the methods available for the detection and measurement of annatto in colour formulations and foods and beverages, which highlights the importance of the need for a good understanding and knowledge of the chemistry of bixin and norbixin in order to meet new analytical challenges.

Keywords: annatto, bixin, norbixin, food additives, chemistry, stability,

analysis, E160b

Overview

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 and permitted as a food additive in the European Union and elsewhere, and have widespread use in the food industry for the colouring of many commodities including flour and sugar confectionery, dairy and savoury products, soft drinks and fish. The major colour principles of annatto are the carotenoids bixin and norbixin. Though chemically very similar, differences in their chemical properties present several challenges to the analytical chemist with respect to stereochemistry, solubility, chromatographic behaviour and stability. While current legislation on the extraction and use of annatto colours and their applications in food are addressed briefly, this review focuses on the chemistry, stability and analysis of annatto pertaining to its use as a permitted food colouring.

Annatto in foods

Legislative aspects

The use of food colours in the European Union is controlled by European Community Directive 94/36/EC (EC, 1994 as amended) which contains a list of permitted colours, a list of foodstuffs to which these colours may be added, and where appropriate, maximum limits on the level of addition. The permitted uses of annatto and the maximum levels of addition are given in Table 1. Annatto extracts are listed amongst those colours that may be used singly or in combination in certain foods up to the maximum levels specified (on a ready-for-consumption basis). Comprehensive on-line sources of information on permitted food colour regulations and specifications may be

found at the Nordic Food Additives Database (NNT, 2008) and the Food Law site of the Department of Food Biosciences, University of Reading (Jukes, 2008).

[Insert Table 1 about here]

In July 2006, the Commission published a set of four proposed Regulations that are intended to replace the current system and provide a common basis for controls on food additives, food flavourings and food enzymes. The proposals were published as separate Commission Documents on additives, flavourings, enzymes and a common authorization procedure (EC, 2006). The proposal brings together all of the existing food additive regulations and plans to introduce comitology for additive approvals in place of the cumbersome co-decision procedure.

The specifications for food colours are laid down in Commission Directive 95/45/EC (EC, 1995) in which separate definitions and purity criteria are prescribed for (i) solvent-extracted bixin and norbixin, (ii) alkali-extracted annatto and (iii) oil-extracted annatto. 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), and the limits for residual solvents and heavy metals.

Use of annatto in foods

Annatto was reported to be the most commonly consumed natural colour additive in the UK (MAFF, 1987 and 1993) where the *per capita* consumption was estimated to be 0.065 mg/kg bw/day based on pure colouring component, representing some 12.5% of the Acceptable Daily Intake (ADI). The chemistry and applications of oil- and water-soluble annatto colours in terms of their modes of applications to a wide range of food products and the usage levels required to obtain the desired colour shades has been reviewed (Collins, 1992; Levy and Rivadeneira, 2000). Crystalline bixin products of 80-97% purity may be obtained by extraction of annatto seed with certain permitted organic solvents and subsequent production of a solvent-free product, which is then processed to give a range of high purity oil- and water-soluble annatto formulations. Oil-soluble bixin is generally used in fatty food applications, whereas norbixin, because of its ability to bind strongly with protein, is especially suited for the colouring of high protein content foods. Annatto colours are often formulated with other additives such as emulsifiers to produce forms of water-soluble annatto that are stable to the effects of e.g. acids, metal ions and salts. The applications and stability of spray-dried annatto formulations in fruit and vegetable products have been studied (Satyanarayana *et al.*, 2006).

Annatto intake

Bixin is reported to be rapidly absorbed in the bloodstream, comparable to other dietary carotenoids, with complete plasma clearance after 8 hours and for norbixin after 24 hours (Levy, 1997). While annatto intake is an important issue within the regulatory context, intake estimates for annatto have in the past provided ambiguous results largely due to the lack of reliable data on the colour principals (bixin/norbixin)

content of annatto extracts (Levy and Rivadeneira, 2000). In response to a request by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for information relating to the toxicity, intake and specifications of annatto, the European annatto producers consulted with the food industry to determine usage levels of specific annatto extracts (JECFA, 2002). The data obtained were combined with the levels of bixin/norbixin in particular extracts to provide an estimate of their concentration in food. These data have been combined with food consumption data using various methods to estimate consumer intakes, which ranged from 1 to 163% of the ADI (Tennant and O'Callaghan, 2005). The actual levels of annatto in foodstuffs were well below maximum limits prescribed under EU regulations and Codex standards, which had been confirmed by an earlier analytical study (Scotter *et al*., 2002).

Annatto chemistry

Elucidation of the chemical constitution of bixin (and thereafter norbixin) was first put forward by Heiduschka and Panzer (1917) who suggested the correct molecular formula for bixin ($C_{25}H_{30}O_4$) as an unsymmetrical molecule. Herzig and Faltis (1923) recognised that bixin was the monomethyl ester of an unsaturated dicarboxylic acid. The results from their catalytic hydrogenation studies led them to conclude that bixin contains 9 carbon double bonds, which, evidenced by the intense red colour of the pigment, were conjugated. However, the unsymmetrical molecule hypothesis was abandoned when proposed the now accepted structure was proposed (Kuhn and Winterstein, 1932), later confirmed by Karrer *et al.* (1932). A new, higher melting form termed β-bixin was obtained during the course of pigment isolation (Herzig and Faltis, 1923), which was later proposed as the *trans*-isomer that the original form may be *cis*-isomer (Karrer *et al*., 1929). A stable form of bixin identical to the β-form by

treatment of the 'natural' (or 'labile') form with iodine was subsequently obtained (Kuhn and Winterstein, 1932). From the results of these investigations that it was concluded that bixin was the first known naturally occurring *cis*-polyene. The structural elucidation of bixin was confirmed from various oxidation and degradation experiments (Karrer and Jucker, 1950). During investigations to determine the stereochemical configuration of labile bixin, several stereoisomers were isolated (Zechmeister, 1960). The consequences of *cis-trans* isomerism on the chemistry, stability and analysis of annatto are significant and are discussed below.

The major colouring component of annatto is confirmed as the apo-carotenoid 9'-*cis*-bixin (methyl hydrogen 9'-*cis*-6,6'-diapocarotene-6,6'-dioate, $C_{25}H_{30}O_4$), the monomethyl ester of the dicarboxylic acid 9'-*cis*-norbixin, commonly referred to as *cis*-bixin (Figure 1). 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 ($C_{24}H_{28}O_4$) 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 (see below).

[Insert Figure 1 about here]

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*., 1997b). The preparation and use of ethylbixin has been discussed (Levy and Rivadeneira, 2000).

The presence of other minor carotenoids in annatto has also been postulated, which included β-carotene, cryptoxanthin, lutein, zeaxanthin and methyl bixin (Tirimanna, 1980). The presence of a range of lycopenoate analogues and other minor carotenoids in annatto has been reported in a series of papers by Mercadante and co-workers (1996, 1997a, 1997b and 1999) and has been reviewed by Mercadante (2001) and Satyanarayana, Prrabhakara Rao and Rao (2003). Bixin and three minor carotenoids have been chemically synthesized using the Wittig reaction of the (Z)-terminus followed by a Horner-Emmons reaction (Haberli and Pfander, 1999).

Molecular properties

It is the delocalisation of π-electrons along the polyene backbone that gives carotenoids their characteristic electronic spectra and is largely responsible for the photophysical and photochemical properties of these molecules, including *cis-trans* photoisomerization. Detailed explanations of *cis-trans* isomerization may be found in standard texts (Karrer and Jucker, 1950; Lunde and Zechmeister, 1954; Zechmeister,

1960; Kolher, 1995). Only the basic properties of linear conjugated molecules will be reviewed here along with a brief account of the simple concepts that apply to bixin and norbixin, in order to provide a background for discussion on the UV-VIS spectroscopy of these compounds and to show how UV-VIS spectra are affected by isomerization.

The sufficiently large barriers to rotation about the formal double bonds in polyenes or carotenoids allow double bond *cis*- and *trans*- isomers to be isolated as independent, distinct compounds. Since the differences in excitation energies for *cis*- and *trans*- isomers of a given molecule are small compared to the change in excitation energy that is associated with adding or subtracting a conjugated double bond, the basic electronic structure is *almost* independent of isomeric form. The four single bonds that surround a carbon-carbon double bond all lie in the same plane. In consequence, each of the disubstituted and trisubstituted acyclic double bonds that constitute the polyene chain of a carotenoid can exist in two forms i.e. geometric isomers. Nomenclature of the *cis*- or *trans*- isomers is designated in accordance with IUPAC rules (Weedon and Moss, 1995a, 1995b). In recent years however, these designations have been replaced largely by *Z* and *E* respectively.

Since each double bond in the polyene chain could, in principle, exist in one of two forms, a large number of geometric isomers are theoretically possible for any carotenoid. However, in practice few of these isomers are encountered. An explanation for this is provided by studying molecular models, which indicate that the introduction of a *cis*- double bond normally results in steric hindrance thus rendering the *cis*-isomer less stable than the *trans*- form. With both trisubstituted double bonds

and disubstituted double bonds in the 15,15'-position, the effect is relatively small, as it results from limited interference between two hydrogen atoms and hence these isomers may be formed quite readily. With other disubstituted double bonds the adoption of the *cis*- configuration results in major interference between a hydrogen atom and a methyl group. This renders such molecules less stable than the corresponding *trans*- form and hence less likely to be encountered (Karrer and Jucker, 1950; Zechmeister, 1960; Kolher, 1995; Weedon and Moss, 1995a).

Stereomutation studies, in which interconversion of geometrical isomers is deliberately promoted, lend support to this theory. Interconversion generally produces a 'set' of isomers that approximates to an equilibrium mixture of all possible geometric forms proportional to their relative thermodynamic stabilities. The all *trans*- form usually predominates, indicating that it is the most thermodynamically stable isomer. A number of mono- and di-*cis*-isomers are usually also present, however those isomers with more than two *cis*- double bonds and/or those that are sterically hindered usually occur only in trace amounts, if at all. It is not surprising that most naturally occurring carotenoids are predominantly in the all *trans*- form. However, bixin occurs predominantly as the *cis*-isomer, which has a *cis*- configuration about the 9'-trisubstituted double bond. Since asymmetric bixin has nine alkene bonds (n=9), theoretically 512 (i.e. $2^n = 2^9$) geometric isomers are possible, whereas symmetric norbixin has only 272 (i.e. $2^{(n-1/2)} \times (2^{(n-1/2)} + 1)$ possible isomers. However, the presence of stable *cis*-isomers at positions 7, 11, 12' and 8' are stearically hindered, hence the remaining 5 alkene bonds are capable of yielding 32 and 20 isomers for bixin and norbixin respectively (Figure 1).

Provided that an adequate sample of the pure isomer is available or the selected analytical technique is adequately sensitive, spectroscopic analysis will normally allow the unambiguous assignment of the geometrical configuration of any carotenoid isomer. All linear polyenes, the carotenoids included, possess similar low-lying excited singlet (S_1) states (Hudson and Kohler, 1974; Kohler, 1977; Hudson, Kohler and Schulten, 1982). This is critically important since virtually all photo processes in linear polyenes originate in the lowest-energy singlet excited state, the correct identification and characterization of which is therefore also important. As might be anticipated from the similarities in electronic structure, the electronic absorption spectrum of a given carotenoid closely resembles that of the unsubstituted polyene with the same number of conjugated double bonds. There are however well characterised principle differences due largely to the presence of methyl substituents along the carotenoid skeleton, which affect the basic polyene electronic structure. These are:

- a 10 to 30 nm shift of the lowest energy strong absorption band to longer wavelength
- decreased vibrational fine structure

Thus, *cis-* and *trans-* isomers may often be distinguished on the basis of their UV-VIS absorption spectra, but the most important differences observed between isomers are not related to excitation energies but to the relative intensities of high-energy absorption bands i.e. *'cis-'* peaks (Dale, 1954; Zechmeister, 1960). It is well established that the lowest excited state of linear polyenes (including carotenoids) is the 2^1A_g state and that the origin of the main absorption band is the strongly allowed $2^1A_g \rightarrow 1^1B_u$ transition. The shapes of electronic absorption (and fluorescence) bands

are derived from the vibrational levels that are associated with the initial and final electronic states. Thus, the typical three-peaked shape of the main absorption band of linear polyenes arises from transitions of the lowest vibrational level of the electronic ground state to the lowest vibrational levels of the electronic excited states. Broadening of these peaks is observed because of rotational levels and inhomogeneity leading to peak overlap. This is particularly relevant for many carotenoids measured as solutions at ambient temperatures (Kohler, 1995).

The positions of the absorption maxima and the shape or fine structure of the UV-VIS spectrum of carotenoids are therefore characteristic. But while the UV-VIS spectrum gives information about the chromophores of the molecule, it yields nothing about functional groups apart from conjugated carbonyl groups that form part of the molecule (Scott, 1964; Britton, 1995a and 1995b). In the case of carotenoids, the relevant transition is the $\pi \rightarrow \pi^*$ transition. For such a conjugated system, in which the π-electrons are highly delocalized, the excited state is of comparatively low energy. The energy required to bring about the transition is therefore relatively small and corresponds to light in the visible region. While the transition responsible for the main absorption band is strongly 'allowed', transitions from the ground state to higher electronic states are also possible, providing they obey the symmetry selection rules. These high energy transitions give rise to absorption bands in the UV region which are usually weak, but are observed particularly in the spectra of compounds with extended chromophores.

When the symmetry properties of a carotenoid change, absorption bands that are otherwise not detected may become a significant feature of the spectrum, as the

transitions that produce them become allowed (Britton, 1995a). For *trans*- isomers, the electronic structure has a centre of symmetry and the ground state is a *g* state, so transitions to a higher g state are forbidden. Transition to a higher excited *g* state only becomes allowed when at least one double bond becomes *cis*- and the original symmetry is lost. This gives rise to an absorption band in the UV region, known as the *cis*-band or *cis*-peak. The most important feature of the absorption spectrum of a carotenoid is the main absorption band in the visible region. Several important pieces of information can be obtained from the spectrum:

- The position of the main absorption band, specified by λ_{max}, provides structural information because it is determined by the chromophore of the molecule

- The intensity of the absorption at λ_{max}, (A) is related both to the structure and to the concentration of the carotenoid in the sample, and provides the basis for quantitative analysis

- The position or the intensity of the main absorption band of a carotenoid can be influenced by a number of factors such as a change in the molecular environment of the carotenoid e.g. solvent

Since the structure of the carotenoid chromophore is related to the overall shape or fine structure of the spectrum, the shape as well as the positions of the absorption maxima may therefore be used as a diagnostic tool, especially when comparing carotenoid spectra (Britton, 1995a). A numerical notation describing fine structure has

proved convenient and removes the requirement for presenting all spectra as diagrams (Kohler, 1995). In this notation, the baseline or zero value is taken as the minimum between the two peaks, the peak height of the longest wavelength absorption band is designated as III, that of the middle absorption band (usually λ_{max}) as II (Figure 2). Spectral fine structure is then expressed as the ratio of the peak heights III/II, as a percentage.

[Insert Figure 2 about here]

The annatto carotenoids bixin and norbixin are unusual in that they contain two carbonyl (i.e. carboxyl) groups, one at either end of the conjugated system and in conjugation with it, which formally extends the chromophore. This results in a spectral shift to longer wavelength, usually accompanied by loss of spectral fine structure but the degree of effect is solvent dependent. With dicarboxylic acids and their esters such as bixin (and norbixin), the further extension of the chromophore causes a large relative increase in λ_{max}. The spectral shift depends on the polarizability rather than the polarity of the solvent, and the frequency shifts to lower energy, i.e. a spectral shift to longer wavelength, as the refractive index of the solvent increases Britton, 1995a).

While water can have a major effect on the spectra of carotenoids in water-miscible solvents, both bixin and norbixin isomers are relatively polar carotenoid molecules, which are significantly soluble in solvents with a high aqueous content and as such similar solvent-related properties are not observed. *Cis*-norbixin is in fact highly soluble in 0.1M sodium hydroxide solution, and both compounds are fairly soluble in

mixtures of acetonitrile and dilute aqueous acetic acid, used as a mobile phase in HPLC analysis.

The differences that are observed consistently between the spectra of *trans*- and *cis*-isomers of carotenoids are therefore diagnostic for structural assignment. A small hypsochromic shift in λ_{max} of ca. 2 to 6 nm is usually observed for mono-*cis* isomers along with a significant hypochromic effect and a reduction in vibrational fine structure. A new absorption band appears at a characteristic position about 142 nm below the *longest* wavelength absorption maximum, often referred to as the *cis*-peak.

For the all-*trans* form of bixin, the main absorption band ($2^1A_g \rightarrow 1^1B_u$) is very intense. In the 9'-*cis* isomer), the intensity of this main band decreases as a weak *cis*-peak appears at 355 nm, corresponding to the transition to a higher energy level *g* state. From their studies on β-carotene isomers, it has been shown that the 15-*cis* isomer, in which the *cis* double bond is in the centre of the molecule, shows maximum bending of the chromophore and a well developed *cis*-band, with corresponding decrease in intensity of the main absorption band (Petterson and Jonsson, 1990).

The intensity of the *cis*-band is essentially greater as the *cis* double bond is nearer the centre of the molecule and is therefore empirically diagnostic. In a symmetrical di-*cis* carotenoid, the centre of symmetry may be restored so that the *cis*-band again becomes a weak feature. For di-*cis* and poly-*cis* carotenoids, a larger hypsochromic shift in the main absorption band may be seen e.g. 13 nm for di-*cis* norbixin (Figure 3) (Scotter *et al*., 1994).

[Insert Figure 3 about here]

A numerical notation similar to the % III/II notation to indicate spectral fine structure, has been adapted to designate the relative intensity of the *cis*-peak (Kohler, 1995). The intensity or absorbance of the *cis*-peak is expressed as a percentage of the absorbance of the middle main absorption band, which is usually the λ_{max}. Scotter *et al.* (1994) used this technique to study the spectra obtained by HPLC-photodiode array analysis of geometrical isomers of bixin and norbixin. The absorption intensity at λ_{max} (Figure 4, III) for each isomer was normalised and a comparison of relative intensity (REL (%)) made with the three other maxima at I, II and IV, to give REL(I), REL(II) and REL(IV) respectively. In all cases, λ_{max} (II) took the form of inflection rather than a peak, which made exact location of the wavelength maximum difficult. However, post-run analysis of the spectral data allowed first derivative spectra to be taken which facilitated location of λ_{max} (II), as shown in Figure 5.

[Insert Figure 4 about here]

[Insert Figure 5 about here]

Annatto stability

It is well known that the polyene chain in carotenoids is responsible for their instability i.e. their susceptibility to oxidation by various agents such as oxygen and peroxides, addition of electrophiles including H^+ and Lewis acids, and *cis-/trans-*isomerization due to various factors such as temperature and light as discussed above. Other undesirable reactions may also be promoted by higher temperatures and the light, and exposure to strong acids and alkalis should normally be avoided.

Oxidative stability

Annatto, especially norbixin, is susceptible to oxidation, particularly when applied in powdered form due to the large surface area and when incorporated into foodstuffs; although some foods can have a stabilizing effect (Berset and Marty, 1986; Collins, 1992; Levy and Rivadeneira, 2000). Spray-dried norbixin formulated with acaia gum or maltodextrin as carriers have been reported to be particularly susceptible to oxidation (Henry, 1992). The level of bixin prepared from annatto seeds and stored for ca. 1 year at 30°C in packages comprising materials with different oxygen transmission rates was reduced by ca. 10% during the first 2-3 weeks storage but stabilized thereafter except for polyethylene film, which exhibited a degradation rate of 0.04% per day, reflecting the permeability of the polyethylene (Carvalho *et al*., 1993). Several mechanisms have been put forward for the effect of water activity on the reduction of bixin oxidation in microcrystalline cellulose-based model systems to simulate dehydrated foods (Gloria *et al*., 1995). Bixin degradation followed first order kinetics and the observed half-lives showed greater stability in systems of intermediate and high water activity. It was postulated that this is because of the ability of water to exclude oxygen from liposoluble materials by surface adsorption, hydrogen bond with hydroperoxides, inactivate metal catalysts, reduce of free radicals and lower the stability of singlet oxygen. Annatto has been shown to inhibit hydroperoxide formation leading to triglyceride autoxidation by trapping peroxy radicals (Haila *et al*., 1996). Annatto was among a number of mediterranean spices whose antioxidant capacities were compared with permitted food antioxidants in lipid peroxidation (Martinez-Tomé *et al*., 2001). Annatto was reported to have a greater antioxidant capacity than either butylated hydroxylanisole (BHA) or butylated

hydroxytoluene (BHT) for preventing deoxyribose damage by hydroxyl radicals. In aqueous media, annatto exhibited a lower antioxidant activity than propyl gallate but was more effective at peroxide scavenging than BHA or BHT. Annatto oleoresin prepared by oil extraction of seeds was found to be more stable than a powdered formulation during storage over ca. 1 year. Samples were stored in glass bottles with a 3cm headspace of air. The greatest losses (60%) were observed for the powder at ambient temperature in daylight, compared to ambient temperature in the dark (54%) and at 5-8°C in the dark (23%). These results concur with the findings of Najar, Bobbio and Bobbio, (1988) that light is the main degradation factor. Moreover, photosensitized bixin is very reactive towards oxygen and thus may be considered as a an oxygen quencher; the reaction of bixin with singlet oxygen is a related issue and is discussed below.

Norbixin was the only carotenoid that inhibited the oxidative deterioration of lipids in both olive oil and oil-in-water emulsions stored at 60°C and displayed a similar activity to δ-tocopherol in stored oil (Kiokias and Gordon, 2003). In olive oil-in-water emulsions, norbixin reduced hydroperoxide formation and a synergistic effect between norbixin and ascorbic acid or ascorbyl palmitate was observed.

Bixin has been reported to be able to scavenge hydroxyl radicals generated by ferrous ions (Fe^{2+}) and hydrogen peroxide (H_2O_2) but no mechanism was suggested (Zhao *et al.*, 1998). Similarly, the behaviour of norbixin during in vivo plasmid DNA damage induced by reactive oxygen species Fe^{2+}, Sn^{2+} and H_2O_2 have been studied and it has proposed that since norbixin contains two free carboxyl moieties, its protective action

may rely on the formation of complexes (Kovary *et al*., 2001). Norbixin showed a stronger affinity for Sn^{2+} than for Fe^{2+} but was readily displaced by EDTA.

During the isolation and analysis of carotenoids, the exclusion of atmospheric air by inert gas or vacuum is strongly recommended in order to minimize the risk of destruction or undesired reactions (Scheidt and Liaaen-Jensen, 1995) and annatto is no different in this respect. The oxidation 'products' of bixin were identified tentatively after transformation of bixin in corn oil at 125°C using spectrophotometry and paper chromatography (McKeown and Mark, 1962). Evidence for the oxidative decomposition of *cis*-bixin on TLC plates has been reported, where both powdered colour formulations and chloroform solutions of *cis*-bixin exhibited decreased colour content when stored in the dark in air at ambient temperature (Reith and Gielen, 1971). This was concluded to be due to the presence of oxygen, supported by observing the relatively lower stability of cheese colour (norbixin in aqueous KOH) compared to butter colour (bixin in vegetable oil), due to the presence of tocopherols in the latter. The effects of light, air and pro-oxidants on the stability of annatto extracts in chloroform over a 12 day period were monitored by spectrophotometry. Air was much less effective at promoting loss of colour compared to light or to benzoyl peroxide, a free-radical promoter (Najar, Bobbio and Bobbio, 1988). The authors concluded that rapid loss of colour might occur whenever free radical formation is promoted.

Reaction with singlet oxygen

Model studies on the photosensitized isomerization of *cis*-bixin show that while bixin in the ground electronic state is stable to thermal isomerization, energy transfer via

photosensitization gives rise to the higher energy triplet state (^{3}BIX*) precursor, which readily isomerizes to the *trans*- isomer (Montenegro *et al*., 2004). The rate of isomerization is dependent on several factors which compete for deactivation of ^{3}BIX* e.g. ground state bixin and triplet oxygen ($^3O_2$). Primary reaction products are only degraded in the presence of air and under prolonged illumination, which is due to the formation of oxidation products by reaction with singlet oxygen ($^1O_2$). The associated reaction mechanisms are discussed very elegantly by the authors. In a similar study,), the ^{3}BIX* energy level was calculated used laser-induced photoacoustic calorimetry of bixin in methanol; acetonitrile solution (Rios *et al*., 2007). The results of the study showed that bixin is a very efficient quencher of $^1O_2$ in fluid solutions due to an efficient energy-transfer process, and confirmed that that the ^{3}BIX* energy level is lower than that of $^1O_2$ (18 ± 2 kcal/mol and 22.5 kcal/mol respectively).

Thermal stability

While bixin and norbixin have good heat stability during food processing compared to other carotenoids, 9'-*cis*-bixin undergoes a series of complex degradation reactions at commercial extraction temperatures to produce a range of products coloured pale yellow to orange (Iversen and Lam, 1953; Levy and Rivadeneira, 2000). Using paper chromatography, the pigments in commercial annatto preparations were separated into a series of bands that included a number of yellow bands comprising up to 40% of the total pigments and including bright yellow fluorescent (*sic*) band (McKeown, 1961). This band was thought to be the pale yellow breakdown product of bixin identified previously (Iversen and Lam, 1953). The main thermal degradation product of 9'-*cis*-bixin has since been isolated and identified using paper chromatography and UV/VIS

spectrophotometry as the yellow coloured 17-carbon polyene 4,8,dimethyl-tetradecahexaenedioc acid monomethyl ester "C_{17}" (McKeown and Mark, 1962; McKeown, 1963 and 1965; Preston and Rickard, 1980. The influence of heating time on the thermal degradation of bixin in alkaline extracts of annatto showed that pigment stability is related to the initial quantity of cis- and trans- bixin as well as to the method used to obtain the extracts (Prentice-Hernández, Rusig and Carvalho, 1993).

The C_{17} product has since been confirmed to be predominantly the *trans*- isomer and that *cis*-isomerisation of bixin was prerequisite to its formation (Scotter, 1995). However, this compound was shown to isomerise in solution to form small amounts of *cis*- isomers and to be susceptible to hydrolysis thus forming a range of compounds analogous to bixin and norbixin in terms of their chemical structures and chromatographic properties. In the light of the results obtained, the mechanism of C_{17} formation originally suggested (McKeown, 1963) was postulated as a concerted electrocyclic process followed by the elimination of *m*-xylene and, to a much lesser extent toluene, toluic acid and toluic acid methyl ester, and the formation of C_{17} which can degrade further by a similar mechanism.

The analytical HPLC-photodiode array (PDA) method developed by Scotter *et al.* (1994, 1995) provided superior qualitative and quantitative data compared with UV-VIS spectroscopic methods (McKeown and mark, 1962; Smith, Blake and Porter, 1983) for determining the colour content (as bixin and norbixin) in 21 commercial annatto formulations, particularly with respect to the coloured thermal degradation products (Scotter *et al.*, 1998). Moreover, the levels of the all-*trans* and di-*cis*-

siomers of norbixin determined from chromatographic profiles of two different norbixin formulations were found to be consistent with their known production history i.e. indicative of the degree of thermal treatment. The formulation obtained by direct aqueous alkaline extraction contained higher levels of these isomers compared to solvent pre-extracted bixin followed by alkaline hydrolysis obtained using lower temperatures. However, the authors pointed out that while the isomer profiles obtained by HPLC-PDA analysis support this, the different extraction procedures might also give rise to different isomer profiles due differential solubilities and stabilities in the extraction medium. The effects of light and oxygen may further complicate this during extraction and handling, and by the nature of the source material.

In a follow-up study, a method was developed which used ambient alkaline hydrolysis followed by solvent extraction and gas chromatography (GC), to analyse annatto colour formulations for the main aromatic hydrocarbon thermal degradation products m-xylene and toluene (Scotter *et al*., 2000). Of the 20 samples analysed, 15 contained <5 mg/kg toluene Four samples contained between 5 and 10 mg/kg and one sample contained 12 m/kg toluene but these levels were not indicative of significant toluene formation via thermal degradation of annatto. In contrast, 6 samples comprising both bixin and norbixin formulations contained m-xylene in the range 30 – 200 mg/kg with the highest level found in an oil-based bixin formulation. Moreover, the two norbixin formulations of known production history analysed in the previous study (Scotter *et al*., 1998) differed markedly in m-xylene content, which appeared to be consistent with the degree of thermal treatment.

For comparison with the alkaline hydrolysis-solvent extraction procedure, 7 of the annatto formulations were submitted for headspace (HS) GC analysis for toluene and m-xylene in order to monitor the effects of heating in a closed controlled environment (90°C for 20 minutes). An increase in m-xylene was observed, with the bixin in oil formulations showing the highest rise in m-xylene concentration on heating. The authors anticipated that HS-GC could be used to monitor the thermal degradation of annatto in food systems and thus conducted a number of experiments in combination with HPLC-PDA and GC-MS to study this (Scotter *et al*., 2001). Low levels (ca. 10 – 15 ug/kg) of m-xylene were detected in the headspace of annatto-coloured retail samples of custard powder, extruded snacks, margarine and breadcrumbs but not in control samples. Much higher levels of m-xylene were detected in annatto-coloured smoked herring (kippers) at ca. 150-200 ug/kg and m-xylene was observed in the headspace of heated Red Leicester cheese (not quantified). The C_{17} coloured annatto degradation product was also detected, indicating that thermal degradation of the principal annatto colouring agent 9'-*cis*-bixin in model systems and foods is facile. However, the degradation is complicated many competing isomerisation reactions which proceed at different rates towards equilibrium. This is further complicated by the simultaneous and irreversible formation of C_{17} associated with the production of m-xylene and to a lesser extent, toluene. While norbixin was reported to degrade similarly but more slowly, the levels of m-xylene formation were nonetheless consistent with bixin / norbixin concentration in the food and occurred more rapidly at higher temperatures.

In order to better understand the kinetics and yields for the formation of both the coloured and aromatic hydrocarbon thermal degradation products of annatto, the

authors carried out a number of experiments in model systems (*ibid.*). The thermal stability of bixin at the boiling point of three homologous alcohol solvents was evaluated using HPLC-PDA to monitor the rate of loss of 9'-*cis*-bixin as well as the appearance of a di-*cis*- and *trans*- isomer, and the C_{17} degradation product. Loss of linearity was observed at each temperature beyond 2 hours, suggesting that two or more competing reactions were taking place at different rates. From the rate constants calculated for the initial phase of the reaction, the Arrhenius activation energy for the *loss* of 9'-*cis*-bixin in refluxing alcohol solvent was 35.7 $kJ.mol^{-1}$. Since the rate of loss of 9'-*cis*-bixin was measured as a function of time regardless of reaction pathway i.e. isomerisation vs. degradation), the authors concluded that the rate data represented only the total (summed) values. Thus, several concurrent reaction pathways are available hence deviation from first order kinetics at long observation times was not unexpected as suggested in Figure 6.

[Insert Figure 6 about here]

Berset and Marty (1986) had reported previously an activation energy of 125 $kJ.mol^{-1}$ for the thermal degradation of annatto pigments in petroleum jelly using a simple first-order kinetic model for the complete decay. This disparity in values therefore suggests a controversy in the kinetic analysis or a misinterpretation of the experimental data. Interestingly, bixin was reported to be easily transformed to the all-*trans*- isomer at ambient temperature in the presence of a photosensitizer and light, where the activation energy for the excitation of bixin to an excited triplet state was ca. 25 $kJ.mol^{-1}$ as discussed above (Montenegro *et al.*, 2004), which suggests strongly

that a greater energy barrier may be anticipated for the thermal isomerisation of 9'-*cis*-bixin to *trans*-bixin.

A more detailed kinetic study on the thermal degradation of bixin in an aqueous model system comprising water: ethanol (8:2) as a function of temperature has been desribed, where HPLC was used to monitor the decay of 9'-*cis*-bixin and the formation of the di-*cis*- and *trans*- isomers, as well as C_{17} (Rios *et al*., 2005). The reactions were found not to follow first order rate characteristics but rather fitted well to a biexponential model. The rate constants for the *formation* of the primary products of bixin and the energy barriers for each step were calculated. Di-*cis*- isomers were formed immediately (energy barrier ca. 63 $kJ.mol^{-1}$) followed by a slow consumption (with the associated decay of 9'-*cis*-bixin), indicating their role as reaction intermediates. The di-*cis*- isomers can either revert readily to 9'-*cis*-bixin (ca. 13 $kJ.mol^{-1}$) or yield the primary C_{17} degradation product with a higher energy requirement of ca. 27 $kJ.mol^{-1}$). However, the isomerisation of 9'-*cis*-bixin to *trans*-bixin requires ca. 100 $kJ.mol^{-1}$, thereby explaining its relatively slow formation. The Arrhenius plot obtained from the initial decay component for 9'-*cis*-bixin yielded an activation energy of ca. 33 $kJ.mol^{-1}$, which concurs with earlier data (Scotter *et al*., 2001). In conclusion, while the activation energy obtained for the 9'-*cis*- → *trans*-isomerisation of bixin is very similar to that reported for β-carotene, the value of ca. 155 $kJ.mol^{-1}$ for the summed isomerisation steps of bixin is much higher than those reported for the thermal isomerisation of C_{40} carotenoids (ca. 105 $kJ.mol^{-1}$). Thus the reaction scheme suggested by Scotter *et al*. (2001) and the greater relative stability of bixin, especially during its isolation and manipulation were confirmed (Figure 7).

[Insert Figure 7 about here]

Thermogravimetric analysis has been used to investigate the thermal degradation of bixin derived from annatto seeds at different heating rates over the 25 – 900°C temperature range (Silva *et al*., 2005). The results indicated that the decomposition of solid 9'-*cis*-bixin occurs in the liquid phase and that four decomposition stages are evident over the temperature range 205-545°C, with isomerisation to the *trans*- isomer occurring between 200 and 240°C. The calculated activation energy was dependent upon heating rate (i.e. 5, 10 or 15 $K.min^{-1}$) at ca. 108, 147 and 128 $kJ.mol^{-1}$ respectively compared to the value of ca. 100 $kJ.mol^{-1}$ reported by Rios *et al*., (2005) obtained in solution. In a similar follow up study, *cis*-norbixin was heated at rates of 5, 10 and 20°$C.min^{-1}$ over the temperature range 25 – 900°C, where the thermal decomposition reactions occurred in the solid phase (Silva *et al*., 2007). Using the Coats-Redfern model, the calculated activation energy was dependent upon heating rate at ca. 154, 131 and 99 $kJ.mol^{-1}$ at 5, 10 and 20°$C.min^{-1}$ respectively for the first-order process.

Heating solid non-purified extracts of annatto seeds as a thin film deposited on a silicon wafer *in vacuo* and monitored using time of flight (ToF) secondary ion mass spectrometry (SIMS), does not give the same results as heating in solution (Bittencourt *et al*., 2005. Principal component analysis revealed that the thermal degradation of the annatto extracts under these conditions occurs in three distinct temperature ranges; below 70°C, the extracts remain thermally stable but above this temperature dimerization reactions occur and the signals attributed to bixin decrease. Near to 100°C, the bixin molecules begin to degrade, leading to fragmentation with

extensive degradation of bixin above 120°C. However, the nature of the degradation mechanism described is not fully understood since there was no evidence for the formation of C_{17} or related fragments from solid bixin.

Light stability

The effect of light at 900 lux intensity on the 30-day stability of a microencapsulated water-miscible extract of bixin compared to that of a purified bixin extract have been studied by measuring the loss of spectrophotometric absorbance at 470nm with time (Prentice-Hernández and Rusig, 1999). The degradation rate of bixin in the microencapsulated extract was ca. 0.05% compared to 0.11% per day for the purified extract.

Ferreira *et al*., (1999) submitted commercial water-soluble annatto (norbixin) solutions to different time and temperature treatments to investigate colour stability. The colour change was measured by spectrophotometry using the Hunter Lab System and the results presented in terms of changes in the norbixin concentration and L, a, b colour parameters. Data were analysed for reaction order and the temperature dependence was explained by the Arrhenius model, with activation energy values between 46 and 105 $kJ.mol^{-1}$ The changes in colour showed an increase in lightness and yellow colour and a decrease in red colour. Norbixin degradation reaction followed second order kinetics whereas for other colour parameters, first order kinetics was followed.

The light stability of spray-dried bixin encapsulated with gum Arabic or maltodextrin plus Tween 80 surfactant has been reported, where the kinetic behaviour of bixin

photo degradation in all systems was characterized by two first-order decays due to the presence of bixin both inside and outside the microcapsules (Barbosa *et al*., 2005). Unsurprisingly, approximately two orders of magnitude greater stability was observed for bixin in the dark compared to illuminated conditions and in the absence of light, and bixin stability in encapsulated solutions was ca. ten times greater than in non-encapsulated systems. The effect of processing conditions used for the preparation of traditional Indian foods on bixin stability including baking, frying, microwave cooking and pressure cooking have been monitored by following losses using spectrophotometry (Rao *et al*., 2005). The losses of bixin under model processing conditions was compared to the preparation of cakes, chegodis, biscuits and fried rice. The greatest losses of bixin were observed in direct exposure to oven baking (54% loss) and deep fat frying (47%), whereas microwave cooking did not affect the colour during direct exposure or in food products. The maximum loss of bixin (65%) was observed for deep fried snack due largely to leaching of the dye into the oil. Pressure cooking produced losses of between 25% and 33%. In cakes, the loss was 30% but negligible losses were found for biscuits (1.5%).

Similarly, the combined effects of light and temperature on annatto extract under different storage conditions were evaluated spectrophotometrically at 470nm in chloroform over a period of 360 days (Balaswamy, Prabhakara Rao, Satyanarayana and Rao, 2006). Annatto oleoresin was generally more stable during storage with respect to bixin content than annatto powder obtained by solvent extraction of annatto seeds. The bixin lost in oleoresin stored under cold (5-8°C), dark conditions was minor (11%) throughout the study, whereas considerable losses were observed for the powdered dye (23%). Likewise, the bixin lost after storage at ambient temperature in

the dark were 8% and 54% for oleoresin and powder respectively. Under diffused daylight ambient and temperature the losses were 14% and 60% respectively, whereas bixin seed stored in jute sacks showed a loss of only 15%. As expected, the rate constants for bixin degradation were much higher in powder compared to oleoresin and were reported to follow second order kinetics. It was proposed that the colour is protected from exposure to oxygen and light by the oleoresin compared to the dry powder, which has a large surface area.

Bixin complexed with α-cyclodextrin is also reported to be more resistant to the damage caused by light and air (Lyng, Passos and Fontana, 2005).

Analytical methods for annatto

Spectrophotometry

Historically, chloroform has been used as solvent for the spectrophotometric analysis of bixin and dilute sodium hydroxide (ca. 0.1M) for norbixin. Absorbance measurements at the two most intense spectral peaks (III and IV in Figure 4) are used for quantitative analysis, where peak IV is preferred because it is less prone to interference from yellow decomposition products. This interference was corrected by using a factor related to the absorbances at λ_{max} and at 404nm in determining the total pigment content of annatto formulations (McKeown and Mark, 1962). 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 ($E^{1\%}_{1cm}$) 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 $E^{1\%}_{1cm}$ = 3208 reported for pure norbixin, this equates to an extinction coefficient for bixin of $E^{1\%}_{1cm}$ = 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 (EC, 1995) or the FAO/WHO (1996), 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). Moreover, the same reservations were expressed over the value of 3473 at 453nm reported by the FAO/WHO specification (FAO/WHO, 1981).

An interesting and important aspect of the spectrophotometric analysis of bixin in chloroform is its rapid rate of degradation when contained in a quartz cuvette, which

unlike glass cuvettes allows the transmission of ultraviolet light (i.e. < 300nm) (Levy and Rivadeneira, 2000).

Planar chromatography

Prior to 1961, there were few references in the literature to paper and adsorption chromatography, which dealt mainly with the gross separation of different carotenoids, and from chlorophylls. The first paper chromatographic method for the direct separation of annatto colouring components used Whatman 3MM paper impregnated with 50% N,N-dimethylformamide (DMF) in acetone and which developed with cyclohexane: chloroform: DMF and acetic acid (85:10:3:2) (McKeown, 1961). This method was used in a number of subsequent studies on annatto and its main thermal degradation product, C_{17} (McKeown and Mark, 1962; McKeown, 1963). The first thin layer method for separation of annatto colour and other fat-soluble dyes shortly thereafter, employed silica gel G, plaster of Paris and silicic acid media with amyl acetate mobile phase (Ramamurthy and Bhalerao, 1964). However, of the 30 solvent systems studied, bixin was reported to migrate from the base line only when acetic acid was present (Francis, 1965). The findings suggested that the amyl acetate solvent used by Ramamurthy and Bhalerao (1964) must have contained acetic acid as an impurity, which was proven by subsequent experimentation. Later methods used silica gel with various solvent systems containing acetic acid for the separation of bixin and norbixin in colour formulations (Dendy, 1966) and cheese colour i.e. norbixin (Reith and Gielen, 1971), who also employed cellulose media for the analysis of butter colour i.e. bixin. Other methods include those developed by Preston and Rickard (1980) and Corradi and Micheli (1981). Chao *et al*. (1991) used reverse phase (C_{18}) plates with methanol:water mobile

phase to separate annatto pigments from supercritical CO_2 extractions of annatto seeds. More recently, TLC has been used for the detection of bixin and other food colour carotenoids derived from red pepper (Mínguez-Mosquera, Hornero-Méndez and Garrido-Fernández, 1995) and for the isolation and identification of new (trace) apocarotenoids from annatto seeds (Mercadante, Steck and Pfander, 1997b) and in the bioautographic detection of antimicrobial compounds in water-soluble annatto extracts (Galindo-Cuspinera and Rankin, 2005). The various methods are summarized in Table 2.

[Insert Table 2 about here]

HPLC

As discussed above, developments in HPLC techniques have enabled more detailed studies of other bixin and norbixin isomers as well as their degradation products compared to TLC methods and have been utilized to gain a greater understanding of the stability of annatto and which in turn have been applied to the detection and measurement of annatto colour in foodstuffs (below).

Literature references on the application of HPLC to the separation of annatto colouring components are sparse. Early methods include the HPLC analysis of annatto extract (Nishizawa *et al*., 1983) and Smith *et al.* (1983), who reported the use of an isocratic reverse-phase system employing an ODS column and methanol/aqueous acetic acid mobile phase. Using this system the *cis*- and *trans*-isomers of both bixin and norbixin were separated within 10 minutes. However, the *cis*- and *trans*-bixin peaks were not fully resolved and the peak shapes were generally very poor. A method for the reverse-

phase separation of bixin, norbixin and three curcuminoids using both isocratic and gradient elution systems, comprising a Zorbax ODS column and water/THF mobile phase was later developed that gave improved chromatographic separation (Rouseff, 1988) developed. However, only separation of the 'main' annatto colouring components were reported and no reference to stereoisomer separation was given. Other approaches have been reported for the analysis of cheese extracts (Luf and Brandl, 1988) and of foods after protease digestion (Chatani and Adachi, 1988). A procedure similar to that reported by Smith *et al.*, (1983) has been developed and appled to the determination of annatto in selected foodstuffs with reasonable success (Lancaster and Lawrence, 1995).

The method developed by Scotter *et al*. (1994) has played a key role in the advancement of HPLC capabilities for the separation and characterization of norbixin and bixin isomers, and has been refined and adapted for the study of annatto stability and for the determination of annatto colouring components in colour formulations, foodstuffs and human plasma. These are summarized along with other published methods in Table 3.

[Insert Table 3 about here]

While the development of column stationary phases been vital in allowing separation of geometrical isomers of bixin and norbixin, C_{17} analogues and other food components, it is the power of the detection systems that have enabled the development of highly useful qualitative and quantitative analyses. Many developed methods utilise detection with fixed wavelength UV-visible (UV-VIS) detectors at

wavelengths specific to bixin/norbixin isomer absorption maxima quite successfully. However, photodiode-array (PDA) technology offers combined sensitivity and specificity coupled to real-time qualitative (spectral) confirmatory analysis, thereby enabling powerful isomer identification and measurement. PDA allows isomer peaks with different λ_{max} wavelengths to be monitored using a spectral bandwidth that encompasses them. A reference wavelength can also be used to subtract background absorbances and to allow for baseline drift, which is usually set outside of the absorbance range of the main analyte and interfering peaks e.g. at 600nm x 4nm bandwidth. The lack of availability of authenticated reference standards is the main limiting factor in the HPLC analysis of annatto colouring components but methods are available for the isolation, purification and characterization of the main bixin and norbixin isomers (Scotter *et al*., 1994) and for C_{17} analogues (Scotter, 1995). Other workers have exploited the use of PDA detection for the identification of trace levels of other apocarotenoids in annatto seeds very successfully (Mercadante, Steck and Pfander, 1997b). Figure 8 shows the HPLC separation of bixin and norbixin isomers (Scotter *et al*., 1994).

[Insert Figure 8 about here]

Mass spectrometry(MS)

A comprehensive review on the use of mass spectrometry in the study of carotenoids in general may be found elsewhere (Enzell and Back, 1990). This work cites earlier reviews and studies that consolidate the importance of the technique not only for elucidation of structure but also for analytical research, not least those carried out by Vetter *et al*., (1971), Budzikiewicz, (1974) and Enzell and Wahlberg, (1980). The

1990 review covers in detail ionization techniques, tandem MS, combined chromatographic-MS techniques and elimination reactions of in-chain units and terminal groups. The first method for electrospray liquid chromatography-mass spectrometry (LC-ES-MS) of carotenoids employed gradient reversed-phase HPLC with PDA and MS detection in tandem (van Breemen, 1995). Molecular ions, $M^{(.+)}$, without evidence of any fragmentation, were observed in the ES mass spectra of both xanthophylls and carotenes but neither bixin nor norbixin were studied.

In common with other carotenoids, the MS spectra of bixin and norbixin are characterized by fragmentation leading to losses of toluene and xylene from the polyene chain and the structural significance of the intensity ratio of the $[M-92]^{+\cdot}$ and $[M-106]^{+\cdot}$ ions (and to a lesser extent the $[M-158]^{+\cdot}$ ion), which is related to the number of conjugated double bonds. It is the apo-configuration that gives rise to anomalous MS properties of bixin and norbixin that have diagnostic value i.e. the -CH_2-CH=CH-CH_2-COOH end group gives characteristic fragments at $[M-44]^{+\cdot}$ and $[M-99]^{+\cdot}$, whereas the -CH_2-CH=CH-CH_2-$COOCH_3$ end group gives characteristic fragments at $[M-31]^{+\cdot}$, $[M-59]^{+\cdot}$ and $[M-113]^{+\cdot}$. Solid probe electron ionization (EI+) was used to confirm the structures of isolated and purified bixin and norbixin isomers (Scotter *et al*., 1994). Both the 9´-*cis*- and *trans*- isomers gave a molecular ion at m/z 394 (bixin) and m/z 380 (norbixin), with major fragment ions at m/z [M-106], 106 (xylene), 105 (methyl tropylium) and 91. Using thermospray analysis, $[M+H]^+$ was identified as the base peak along with the presence of sodium and (possibly) water adducts, and fragment ions corresponding to $[M-H_2O]^+$ and $[M-CH_3OH]^+$. In a later study, similar analytical conditions were used to characterize the 17-carbon major thermal degradation product of annatto (Scotter, 1995). Solid probe EI revealed the

molecular ion at m/z 288 along with fragment ions at m/z [M-106], 106, 105 and 91, and thermospray analysis identified the base peak as $[M+H]^+$ as well as sodium adducts at m/z 311 ($[M+Na]^+$) and 333 $[M-H+2Na]^+$.

Complementary to other analytical techniques, EI+ and fast atom bombardment (FAB) MS was used to determine the structure of the bixin family of apocarotenoids (Kelly *et al*., 1996). Both *cis*- and *trans*- bixin isomers gave EI+ molecular ion abundancies equivalent to ca. 30% of the base peaks at m/z 59 or 91, and the [M+1] and [M+2] ion intensities were consistent with predictions based upon calculated ^{13}C isotope patterns. As expected, loss of xylene as a neutral group was most pronounced for *cis*-bixin but no loss of neutral toluene was observed although the m/z 91 peak was prominent. Fast-atom bombardment (FAB+) spectra of *cis*- and *trans*- bixin gave the molecular ion as the base peak but the abundance of the [M+1] peak exceeded the calculated isotopic abundance by 55-75%, indicating a small contribution from [M+H]. Small amounts of sodium adducts were observed but ions due to elimination of toluene were not. However, significant amounts of m/z 105, 115 and 165 were observed. These observations were consistent with other FAB spectra of carotenoids where odd electron molecular ions are frequently observed due presumably to their lower ionization potentials (Vetter and Meister, 1985).

Bixin was among the polyenes studied using electrospray ionization (EI) and high resolution (HR) matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (Guaratini *et al*., 2004). In this study, the ability of neutral organic molecules to give up an electron for oxidation was exploited, which is governed by the energy of their highest occupied molecular orbital (HOMO) and can

be estimated by measurement of the half-wave potential for solution oxidation. Strong evidence was reported for an ionization process that produces the molecular ion $M^{\cdot+}$ in ESI and HR-MALDI MS of polyenes, and the correlation of the observed ions to the oxidation potential. The formation of $M^{\cdot+}$ and $[M+H]^+$ species was shown to be dependent upon energetic variations and the presence of water or another protic solvent. Niether the $[M+H]^+$ nor the $[M+H-H2O]^+$ were detected as the major ions from ESI analysis of bixin, whereas $M^{\cdot+}$ was detected but only in the specific capillary voltage range of 0.1 to 0.7 kV . The accurate mass measurement afforded by the HR-MALDI-TOF analysis showed $M^{\cdot+}$ for bixin at an observed mass of 394.2147 with 40% ion intensity, but $[M+H]^+$ was not observed.

The major carotenoid composition of *Bixa orellana* seeds has been ascertained using TOF-MS with X-ray photoelectron spectroscopy (Felicissimo *et al*., 2004). The presence of bixin was revealed in the seed aril without any sample pretreatment from the detection of ions attributable to [M+2H] at m/z 396 with associated ^{13}C isotope analogues at m/z 397 and 398. The presence of characteristic fragments at m/z 337 was attributed to $C_{23}H_{29}O_2^+$ obtained from the previous molecular ion with loss of a $COOCH_3$ ester group, and at m/z 281, a fragment compatible with loss of a $C_6O_2H_8$ end group plus a hydrogen atom i.e. $C_{19}H_{21}O_2^+$. The characteristic presence of xylene was confirmed via the detection of the $C_8H_9^+$ ion at m/z 105. Analysis of the coloured interior of the seeds following cutting did not show any fragments consistent with bixin. A methanol:chloroform extract of the seeds was analysed immediately after preparation by blow-drying under nitrogen onto a silver subtstrate, and then after exposure to ambient light for 3 months. TOF-MS analysis of the fresh extract was dominated by the molecular peak at m/z 396 along with all other characteristic

fragments. As expected after 3 months exposure to light, the colour of the extract had lightened to a more yellow shade with an associated 5-fold decrease in the intensity of the $[M+2]^+$ ion and with a concomitant 2-fold increase in the intensity of the $C_8H_9^+$ ion, indicating the formation of xylene via degradation. In a related study, Bittencourt *et al*. (2005) analysed extracts of *Bixa orellana* using TOF-MS as a means of characterising thermal effects. The spectrum was characterised by a large number of peaks generated by the principal ions and their multiple fragmentation patterns but also, more notably, by the presence of ions at m/z 790 ($[C_{50}H_{62}O_8]^+$ = 2M+2H), 804 ($[C_{51}H_{64}O_8]^+$ = 2M+2H+CH_2) and 818 ($[C_{52}H_{66}O_8]^+$ = 2M+2H+2CH_2) attributed to the presence of dimers.

The confirmation of twelve different carotenoids used as food colorants was achieved utsing positive atmospheric pressure chemical ionization (APcI)+ LC-MS (Breithaupt, (2004). The [M+H]+ ions were monitored for norbixin and bixin at m/z 381 and 395 respectively. Based on the presence of at least one carboxyl group, APcI measurements in the negative mode were also carried out on bixin and norbixin but no significant enhancement in sensitivity was observed. A similar approach has been used for the analysis of water-soluble annatto extracts in both positive and negative electrospray detection modes (Galindo-Cuspinera and Rankin, 2005). ES- detection mode showed a major peak at m/z 379 corresponding to $[M-H]^-$ for norbixin, whereas the major peak at m/z 381 was found using ES+ mode. An ion at m/z 117 was identified in in the ES- spectrum of 9´-*cis*-norbixin but not in the spectrum of the *trans*- isomer. Conversely, the *trans*- isomer showed an ion at m/z 111.1 in ES+ mode that was not present on the spectrum of 9´-*cis*-isomer. This was thought to be due to differences in fragmentation patterns determined by stereochemical configuration.

More recently, it has been shown that HPLC-PDA in combination with ion-trap elelctrospray mass spectrometric confirmatory analysis can be used to identify and measure norbixin and bixin in meat products using precursor ions at m/z 379 and 395 respectively and monitoring characteristic product ions at m/z 253,291,310 and 335 (norbixin) and m/z 317, 335, 345, 363 and 377 (bixin) (Noppe *et al*., 2009).

Nuclear magnetic resonance (NMR) spectroscopy

A comprehensive review on the use of NMR spectroscopy in the study of carotenoids in general is given by Englert (1995), in which a detailed treatise on the experimental aspects, chemical shifts of end groups, chemical shifts and spin couplings, stereoisomerization , and simple and multidimensional experiments are given for ^{1}H and ^{13}C nuclei.

The earliest published use of NMR in the study of bixin stereochemistry used low resolution (40 MHz) instrumentation to assign ^{1}H frequencies and deduce that the *cis*-bond of the methyl analogue of 'natural or α-' bixin was in the 9´- (equivalent) position (Barber *et al*., 1961). The high frequency shift of the proton assigned to H-8´ was attributed to deshielding by the 11´-12´ alkene bond when compared to the *trans*-(or β-) isomer, which was confirmed via synthesis and more detailed structural assignments (Pattenden, Way and Weedon, 1970). Fourier transform (FT) NMR was used later to assign the ^{13}C spectra of methyl *cis*- and *trans*-bixin using deuterated compounds, however no experimental details were given and assignments were partly derived from spectra of carotenoids with similar structural characteristics (Moss, 1976). The ^{1}H FT-NMR spectrum of cis-bixin and cis-methyl bixin at 250Mhz has been reported but is limited to assignment of the terminal acrylate moieties (Jondiko

and Pattenden (1989). Proton NMR at 250MHz was used to confirm the structures of purified *trans*- and 9´-*cis*- bixin, where the chemical shifts and coupling constants associated with the change in stereochemistry were consistent with those reported previously (Barber *et al*., 1961) but afforded much higher resolution (Scotter *et al*., 1994). A similar approach was used to confirm the structure of the principal thermal degradation product of bixin as *trans*-4,8-dimethyltetradeca -hexaenedioc acid monomethyl ester or C_{17} (Scotter, 1995). The structure of a minor apocarotenoid isolated from *Bixa orellana* was confirmed as methyl 9´*Z*-apo-6´-lycopenate using proton NMR at 500 MHz (Mercadante *et al*., 1996) and a similar approach used to identify apocarotenoids not previousy found in annatto (Mercadante, Steck and Pfander, 1997b; 1999). NMR (300 MHz ^{1}H) was used alongside TLC and HPLC in the bioautographic detection of antimicrobial compounds in water-soluble annatto extracts where peak assignments were reported to be consistent with previous reports (Galindo-Cuspinera and Rankin, 2005).

The most comprehensive study to date on the determination of the structure of the bixin family of apocarotenoids is by Kelly *et al*. (1996), who utilised a combination of 1D and 2D NMR techniques in conjunction with mass spectrometry and X-ray diffraction analysis. Chemical shift, coupling constants and ^{1}H correlation data were examined alongside the ion abundances and intensity ratios from standard electron impact (EI+) and fast atom bombardment (FAB+) MS spectra, and bond measurement, cell dimension and degree of hydrogen bonding from X-ray diffraction data to elucidate and compare the crystal structures of the *cis*- and *trans*- isomers of bixin and methyl bixin.

Other analytical techniques

Notwithstanding where specific techniques have been discussed elsewhere in this review, there are several less widely known techniques that have been used in the study of annatto either alone or in conjunction with complementary techniques. These include infra-red spectroscopy, where the characteristic strong absorption due to the C=O stretching frequency between 1740 and 1700 cm^{-1} and the complex bands in the 1300-1050 cm-1 region due to C-O single bond characteristic of esters and carboxylic acids has been used (Lunde and Zechmeister, 1954; Reith and Gielen, 1971; Chao *et al*., 1991; Bernard and Grosjean, 1995). Photoacoustic spectrometry in the UV, VIS and IR regions has been used for the qualitative and quantitative analysis of annatto in commercial seasoning products (Haas and Vinha, 1995) and more recently in the determination of the triplet state energy of bixin (Rios *et al*., 2007). X-ray photoelectron spectroscopy was used by Felicissimo *et al*. (2004) to ascertain the major carotenoid composition of *Bixa orellana* seeds and X-ray diffraction in conjunction with NMR and mass spectrometry has been used to determine of the structure of the bixin family of apocarotenoids (Kelly *et al*., 1996).

Analysis of foods

Prior to 1970, there were very few published methods for the extraction of annatto from foods. The qualitative and quantitative analytical aspects of annatto extraction methods published prior to 1976 have been reviewed briefly (Aparnathi and Sharma, 1991). These relatively simple methods generally involve extraction with solvent (e.g. chloroform, benzene, petroleum spirit or ether) with or without some form of sample pre-treatment such as protein precipitation, washing and adsorption onto an inert

substance. The foodstuffs analysed by these methods largely comprise dairy products, which reflects the relatively narrow scope of annatto usage at that time.

Annatto has been extracted from whey solids with dilute ammonium hydroxide where proteins were precipitated by the addition of ethanol and phosphate buffer (Hammond, Chang and Reinhold, 1973), and from meats (McNeal, 1976). Annatto may be analysed in milk and ice-cream by precipitation with boiling acetic acid and extraction of the whey with diethyl ether, and the colour extracted from macaroni and noodles with 80% ethanol followed by back-extraction into diethyl ether under alkaline conditions (AOAC, 1980).

Rapid methods for the extraction of annatto from foods have been described where drinks and syrups were dissolved in water, acidified with acetic acid and annatto was partitioned into diethyl ether (Corradi and Micheli, (1981). Products with a high fat content *e.g*. butter and margarine, were dissolved in petroleum spirit and annatto was partitioned into aqueous ammonaical ethanol. Three extractions were required for quantitative extraction of the colour. The aqueous extracts were acidified with acetic acid and back-extracted with diethyl ether. For foods containing fat and protein *e.g*. yoghurt, cheese and pastries, samples were ground with sand and aqueous ammonaical ethanol. The mixture was transferred to a centrifuge tube and the fat was removed by agitation with petroleum spirit, centrifugation and siphoning off the petroleum spirit phase. The aqueous ammonaical phase was retained, acidified with acetic acid and the annatto partitioned into diethyl ether.

Methods for the extraction and determination of annatto in margarine, cheese and boiled sweets have been investigated using techniques similar to those described previously, with modifications to enable measurement by spectrophotometry and HPLC (Smith *et al*., 1983). Margarine samples were saponified to separate fat and to convert any bixin to norbixin, thereby facilitating its extraction into aqueous media and subsequent purification. However the reported HPLC conditions gave poor peak shapes and insufficient resolution. A method for the determination of annatto in cheese in which a simple acetone extraction was used, followed by concentration by rotary evaporation has been described (Luf and Brandl, 1988). Spectrophotometric (derivative) and HPLC techniques were used to quantify annatto in the presence of other carotenoids, based on the procedure described for the analysis of certain baked goods. However, the *cis*- and *trans*-isomers of bixin and norbixin were not identified separately under the stated conditions.

More recently, other workers have developed refined methods for the extraction of annatto from high-fat foods, dairy products and candy utilising solvent pre-extraction of fat and extraction of annatto into ethanolic aqueous ammonia (Lancaster and Lawrence, 1995) and to separate mixtures of bixin and norbixin from carminic acid in fruit beverages, yoghurt and candies (Lancaster and Lawrence, 1996). HPLC was used to measure both the *cis*- and *trans*-isomers of bixin and norbixin but no significant improvements in peak resolution were demonstrated compared to those reported previously (Smith *et al*., 1983), and impure reference materials were used for calibration. Recovery of norbixin from spiked cheese samples was reported to average 93% over the range 1 to 110 mg/kg, and the recovery of bixin from spiked wafers also

averaged 93% over the range 0.1 to 445 mg/kg. The recovery of norbixin from laboratory-prepared hard candies averaged 88%.

TLC and HPLC were used to determine bixin and other carotenoid colours in products derived from red pepper (Mínguez-Mosquera, Hornero-Méndez and Garrido-Fernández, 1995). A simple acetone extraction was used followed by partition with ether and sodium chloride solution and alkaline saponification. Back extraction with ether following acidification of the saponifying medium was necessary to recover the annatto colour (as norbixin). While good chromatographic separation of the carotenoids was obtained, no distinction between norbixin isomers was made. However, the method demonstrated the capability of detecting of colours added fraudulently to intensify the natural colour of paprika paste.

Whilst remaining an uncommon analytical technique in food laboratories, photoacoustic spectrometry (PAS) has been used for the analysis of annatto products (Haas and Vinha, 1995). The method is limited to semi quantitative (± 1% 'annatto content') and qualitative analysis of commercial seasonings comprising mixtures of corn meal and powdered annatto seeds or annatto extract known as '*Colorifico du Urucum*'. The particle size of the samples has a strong influence on the amplitude of the PAS signal and therefore requires close control.

Based on the methods described previously (Scotter *et al*., 1994; Lancaster and Lawrence, 1995), HPLC and spectrophotometric methods have been developed for the simple and rapid determination of annatto in cheese and milk products (Bareth, Strohmar and Kitzelmann, 2002). Solid phase extraction (SPE) on amino phase was

used to separate annatto components from fat and β-carotene. The choice of end method was determined by the presence of other colouring materials i.e. curcumin or β-apo-8´-carotenal but other food colours and emulsifiers did not affect the analysis. The recovery of annatto colouring spiked into cheese, processed cheese, butter and ice-cream ranged between 80 and 100%. Nine samples of cheese were analysed in which norbixin was found in the range <0.15 to 11.89 $mg.kg^{-1}$, whereas no bixin was detected (>0.15 $mg.kg^{-1}$).

The methods described by Scotter *et al*. (1994 and 1998), Lancaster and Lawrence (1995) and Navaz Diaz and Peinado (1992) were further developed and consolidated to encompass a wide range of food commodities (Scotter *et al*., 2002). Specific solvent extraction regimes were developed for specific sample matrices, with HPLC-PDA used for spectral confirmation and measurement of the main isomers of bixin and norbixin. The different extraction regimes are summarized in Table 4.

[Insert Table 4 about here]

With the exception of regime 5, samples were extracted essentially using ethanol:water:ammonia solution with or without a hexane partition to remove excess lipid. After centrifugation in the presence of Celite filter aid, the annatto colour was partitioned into chloroform:acetic acid solution, centrifuged and the solvent removed using vacuum-assisted rotary evaporation. To minimise analyte losses via oxidation, a 0.1% solution of butylated hydroxyl toluene (BHT) was added. For regime 5 matrices, samples were mixed with Celite in the presence of dilute hydrochloric acid and extracted using a biphasic solvent system comprising hexane (to remove excess lipid)

and acetonitrile, which was then concentrated using vacuum-assisted rotary evaporation.

Using this method, comprehensive quantitative and qualitative data on 165 composite and 2 single food samples covering a wide range of foods at levels above the analytical reporting limit of 0.1 $mg.kg^{-1}$ were obtained. Quantitative results were given for those annatto colouring components for which reference standards were available (9´-*cis*-bixin, *trans*-bixin and 9´-*cis*-norbixin), whereas semi-quantitative results were given for other bixin and norbixin isomers. The method was single-laboratory validated by the repeat (n = 4 to 9) analysis of 12 different sample types of food commodity covering the permitted range of annatto content, spiked with annatto at levels of between 1.7 and 27.7 $mg.kg^{-1}$ and by the analysis of in-house reference matrices. Mean recoveries of between 61 and 96% were obtained from foods spiked with annatto.

Using response surface methodology to establish optimum conditions, a method for the determination of annatto colour in extruded corn snack products has been developed that exhibits improved accuracy and precision compared to the method described by Scotter *et al*. (2002) (Rios and Mercadante, 2004). However, pre-treatment of the samples with α-amylase was necessary to remove starch and a total of 8 solvent extractions with ethyl acetate were required for complete extraction of the annatto colour. Lipids were removed using alkaline saponification therefore all of the bixin present was hydrolysed to norbixin and determined as such by HPLC.

Accelerated solvent extraction has been compared with manual solvent extraction to determine several food colouring carotenoids including bixin and norbixin in processed foods (Breithaupt, 2004). Reverse-phase HPLC with a C_{30} column successfully separated bixin and norbixin from 7 other carotenoids but the *cis*- and *trans*- isomers were not distinguishable. Due to its ostensibly higher polarity, lower recoveries of norbixin were reported for accelerated extraction (67 ± 1.0 $mg.kg^{-1}$) compared to manual extraction (88.7 ± 6.2 $mg.kg^{-1}$). However, a similar difference in recoveries was reported for less polar bixin (91.0 ± 2.7 and 98.0 ± 1.7 $mg.kg^{-1}$ respectively) although bixin recovery was higher than norbixin with improved precision. The limit of quantitation for bixin and norbixin was in the range 0.53 – 0.79 $mg.kg^{-1}$ for pudding mix and cereals. More recently, a method for the determination of norbixin and bixin in meat products using HPLC-PDA and LC-MS^n that gives recoveries of between 99 and 102% and a limit of quantitation of 0.5 mg/kg. (Noppe *et al*., 2009).

Annatto as an illegal food dye

The illegal use of annatto to colour milk goes back as far as the early 20th century where it was reported by UK Public Analyst laboratories (Richards, 1923; Collingwood Williams, 1925). Amongst other specific food commodities, annatto is currently permitted in the EU for the colouring of certain margarines and cheeses but is not permitted for the colouring of milk cream or butter (EC 1994 as amended). Moreover, 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 publicationshave not been forthcoming to date. Nevertheless, it is generally considered amongst analytical chemists working in this area that LC-MS/MS is capable of detecting bixin at ca. 0.01 mg/kg in certain commodities, but this is heavily dependent upon the degree of signal suppression caused by matrix effects. 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

Future aspects

There is a clear requirement is the future for the development and validation of highly sensitive methods of analysis for annatto in food commodities and other food ingredients, driven by the need to ensure compliance with food quality regulations and especially in the light of the pursuit of suitable alternatives to synthetic food colours. An in-depth understanding of the chemistry and stability of annatto is therefore

requisite and brings clear benefits to the production of annatto, and to the formulation and application of food colouring to a wide range of food commodities. Greater understanding of the processes of degradation may also benefit studies in the areas of food safety, particularly in risk assessment, and biomarkers of exposure such as circulating (plasma) levels of norbixin. Here, complementary analytical techniques such as HPLC-PDA, LC-MS/MS and NMRwill play a vital role in the detection, confirmation and measurement of comparatively low levels of bixin and norbixin isomers.

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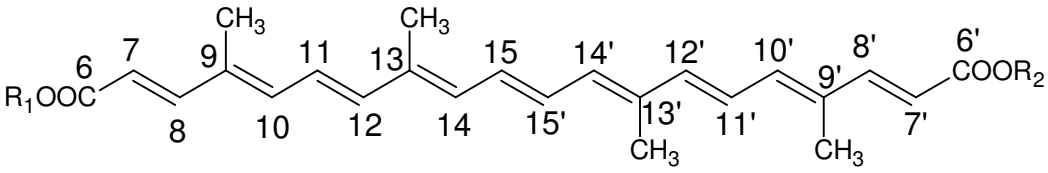

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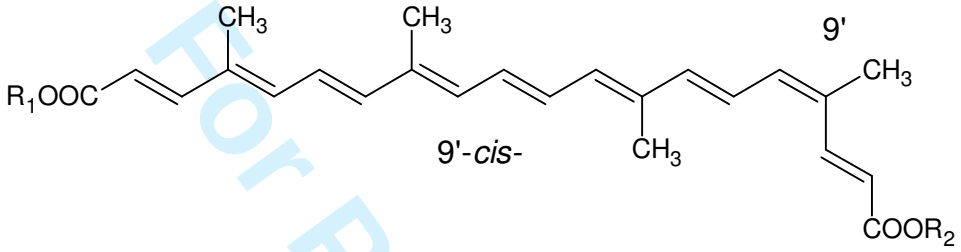

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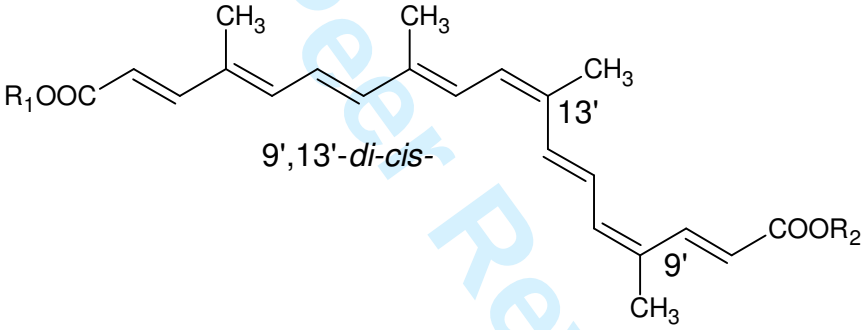

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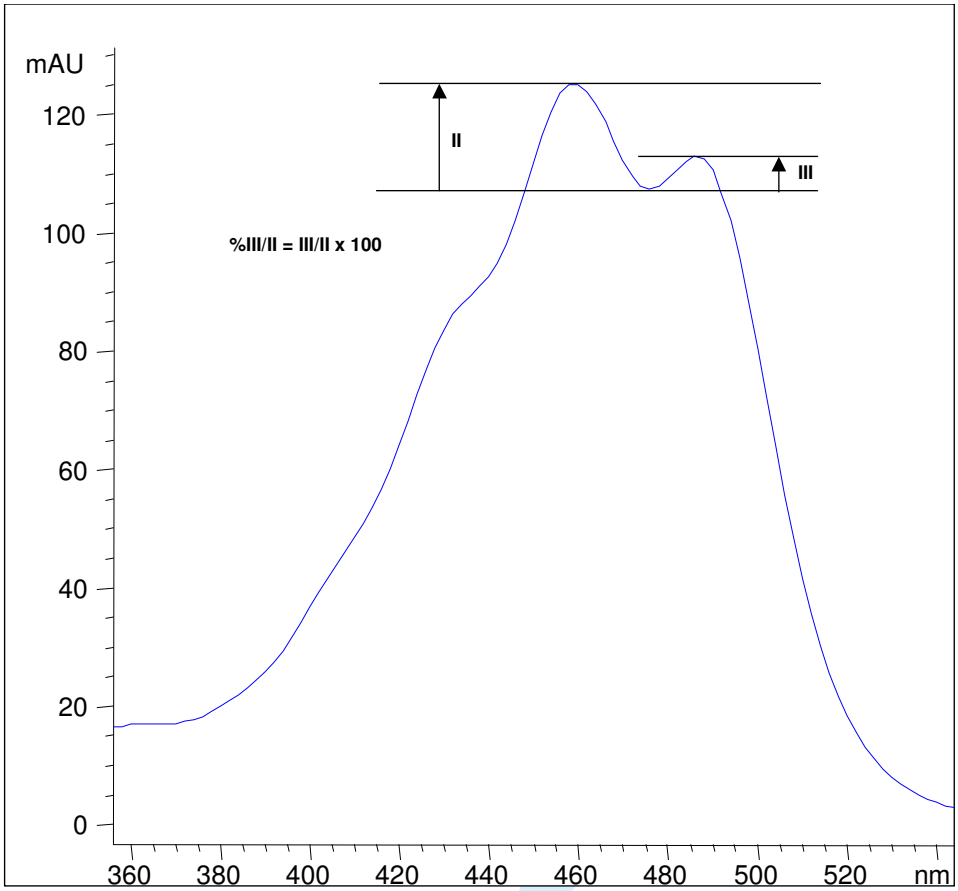

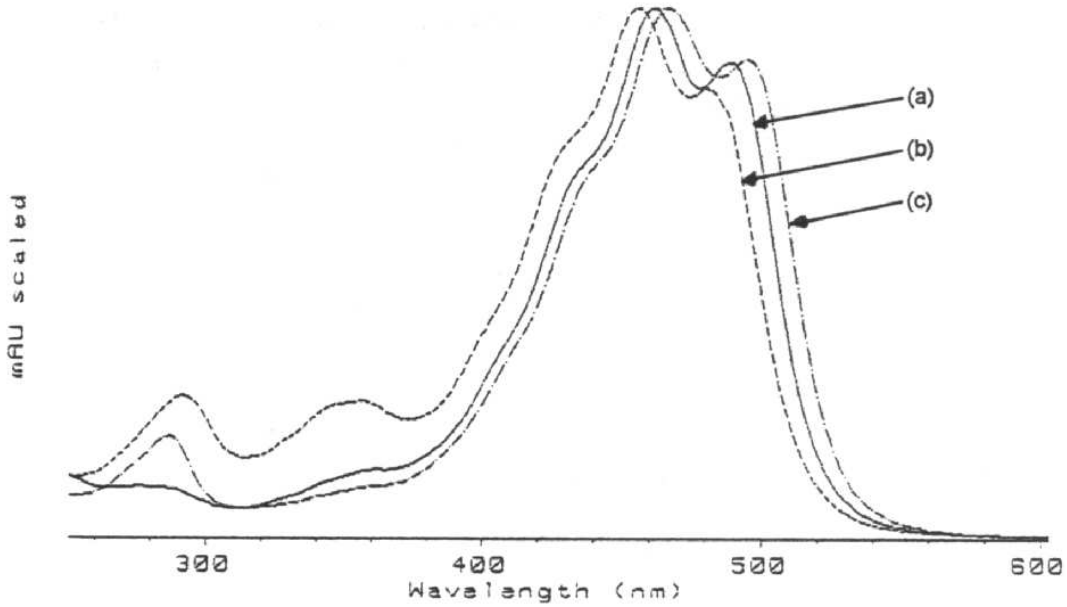

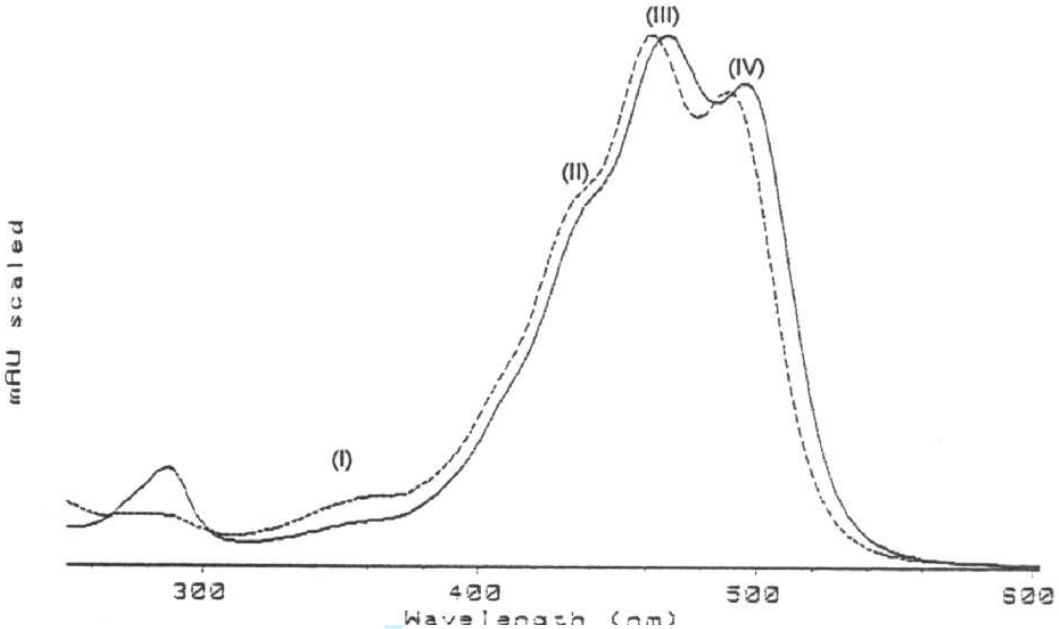

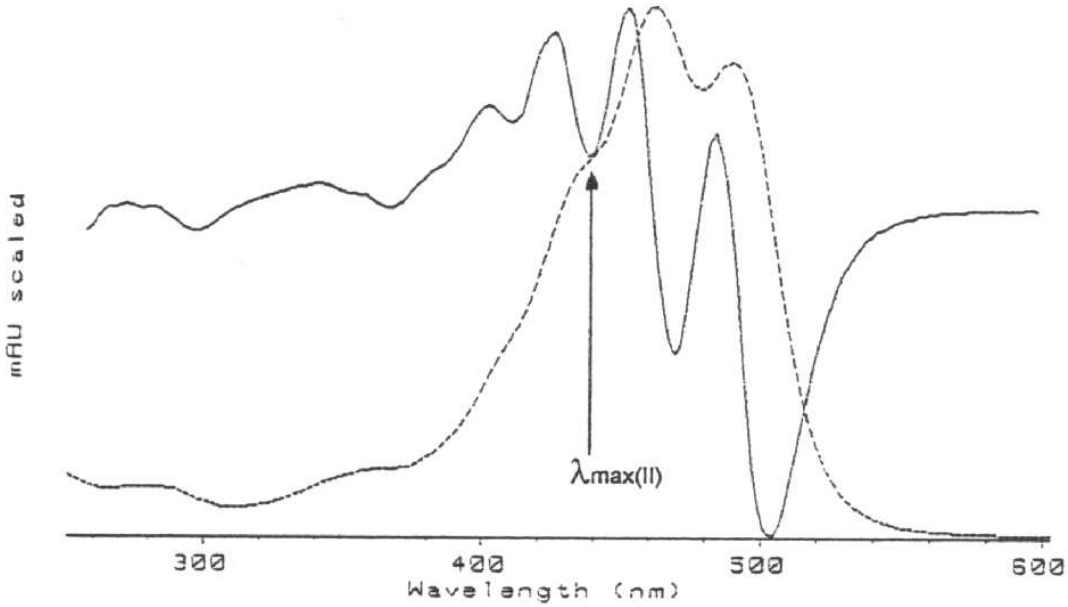

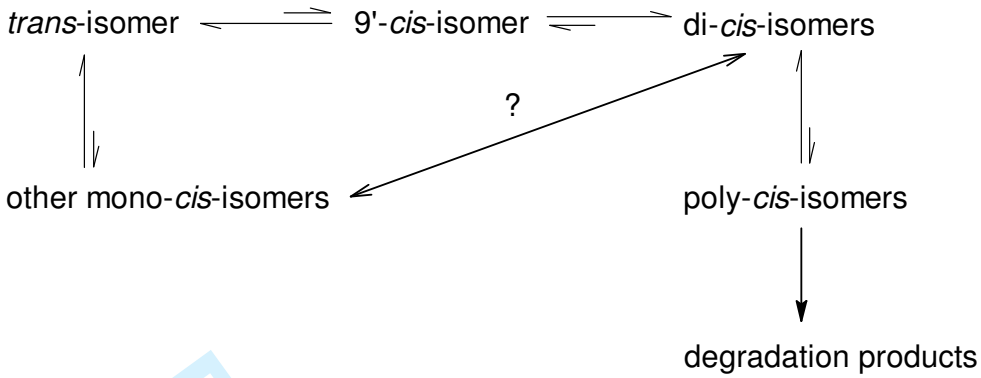

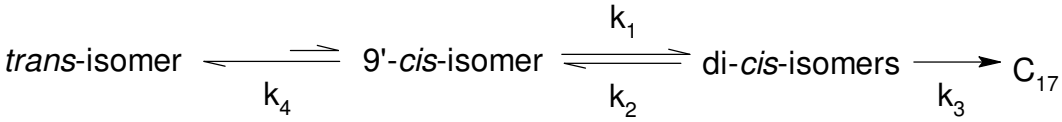

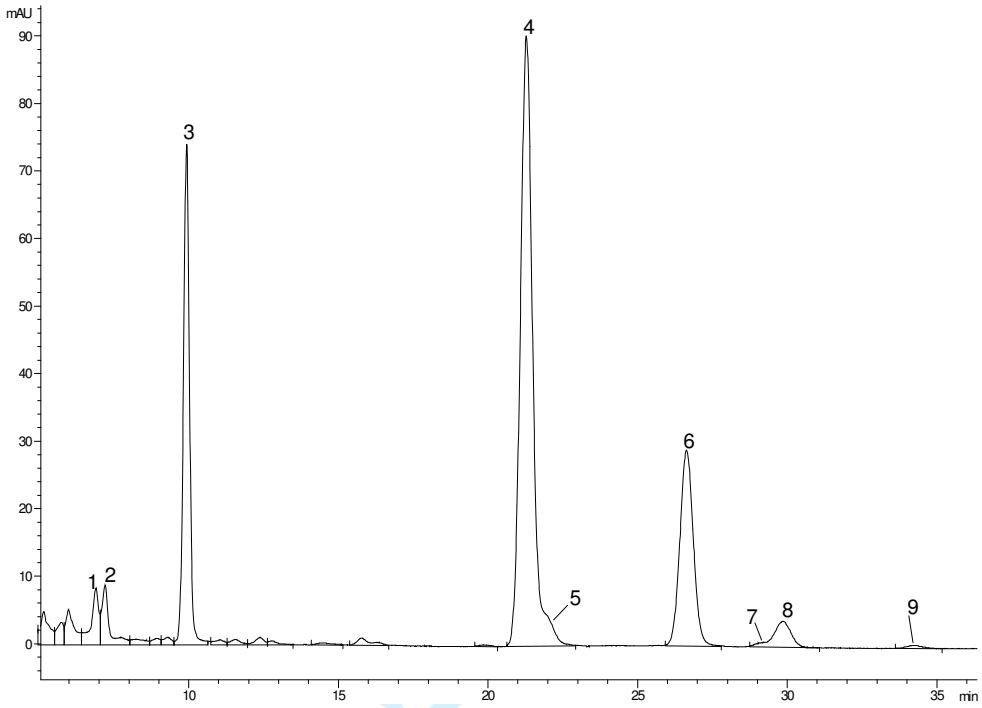

Legends for figures

Figure 1. Chemical structures of some bixin/norbixin isomers. R_1=H, R_2=H = norbixin; R_1=H, R_2=CH_3 = bixin.

Figure 2. Spectral fine structure. Calculation of %III/II for a carotenoid (9'-*cis*-norbixin).

Figure 3. UV-VIS spectra of norbixin isomers (by HPLC-photodiode array): (a) 9'-*cis*-norbixin, (b) di-*cis*-norbixin and (c) *trans*-norbixin (Scotter et al., 1994).

Figure 4. HPLC-photodiode array spectra of bixin isomers showing the locations of λ_{max} (I)-(IV) (Scotter et al., 1994).

Figure 5. HPLC-photodiode array spectrum of 9'-*cis*-bixin (broken line) and its first derivative spectrum (solid line) highlighting the inflection at λ_{max} (II) (Scotter et al., 1994).

Figure 6. Suggested reaction pathways for the thermal degradation of 9'-*cis*-bixin (Scotter et al., 2001).

Figure 7. Coupled reaction scheme proposed for the degradation of bixin and the formation of its primary products (Rios et al., 2005).

Figure 8. HPLC separation of bixin and norbixin isomers. Conditions: Column HRPB C_8/C_{18} 250 x 4.6mm, 5um; Mobile phase Acetonitrole: 0.4% acetic acid (65:35) isocratic elution at 1 $ml.min^{-1}$ 35°C; Detection photodiode array at 455 x 10nm. Assignment of peaks: 1. *Trans*-norbixin. 2. *Di-cis*-norbixin. 3. 9'-*cis*-norbixin. 4. *Trans*-bixin. 5 and 9. *Di-cis*-bixin isomers. 6. 9'-*cis*-bixin. 7. 15-*cis*-bixin*. 8. 13'-*cis*-bixin*. (*tentative).

Table 1. Permitted uses of annatto and maximum levels of addition (EC, 1994).

Food Commodity type	**Maximum permitted level (mg/kg)***
Margarine, minarine, other fat emulsions, and fats essentially free from water	10
Decorations and coatings	20
Fine bakery wares	10
Edible ices	20
Liqueurs, including fortified beverages with less than 15% alcohol by volume	10
Flavoured processed cheese	15
Ripened orange, yellow and broken white cheese; unflavoured processed cheese	15
Desserts	10
'Snacks': dry, savoury potato, cereal or starch-based products: Extruded or expanded savoury snack products	20
Other savoury snack products and savoury coated nuts	10
Smoked fish	10
Edible cheese rinds and edible casings	20
Red Leicester cheese	50
Mimolette cheese	35
Extruded, puffed and/or fruit-flavoured breakfast cereals	25

[* Refers to 100% bixin or norbixin]

Table 2. Summary of planar chromatographic methods for annatto colours.

Sample type	Adsorbent	Mobile phase	Reference(s)
Bixin, norbixin, C_{17}	Paper	CHX:$CHCl_3$:DMF:HOAc (85:10:3:2)	McKeown, 1961, 1963
Annatto and other fat soluble dyes	Silica gel G	Amyl acetate	Ramamurthy and Bhalerao, 1964
Annatto	Silica gel G	1% HOAc in amyl acetate	Francis, 1965
Bixin	Silica gel	$CHCl_3$:ACE:HOAc (50:50:1)	Dendy, 1966
(1) Bixin (2) Norbixin	(1) Cellulose (2) Silica gel	(1) CHX:$CHCl_3$:HOAc (65:5:1) (2) $CHCl_3$:EtOH:HOAc (68:2:1)	Reith and Gielen, 1971
Annatto and other pigments	Silica gel G (2-dimensional)	(1) $CHCl_3$:EtOAc (4:1) (2) Et_2O	Tirimanna, 1980
Bixin and norbixin commercial formulations	Silica gel GF	PE:Et_2O:HOAc (85:15:2.5)	Preston and Rickard, 1980
Ether extracts of foods	Silica gel	(1) $CHCl_3$: HOAc (9:1) (2) Et_2O: IPA (9:1)	Corradi and Micheli, 1981
Annatto seeds	KC18 reverse phase	MeOH:H2O (70:30)	Chao et al., 1991
Bixin and other carotenoids	Silica gel GF	(1) HEX:ACE (10:9) (2) DCM:Et2O (9:1) (3) PE: BZ (1:1) (4) PE	Mínguez-Mosquera, Hornero-Méndez and Garrido-Fernández (1995)
Annato seeds	(1) Silica gel (2) MgO/Kieselguhr	(1) HEX:t-BME (90:10) (2) HEX:ACE (85:15)	Mercadante, Steck and Pfander, 1997b
Annatto formulations	Silica gel GF	$CHCl_3$:HOAc:ACN:ACE (8:1:0.5:0.5)	Galindo-Cuspinera and Rankin, 2005

[Key: ACE = Acetone; ACN = Acetonitrile; BZ = Benzene; CHX = Cyclohexane, $CHCl_3$ = Chloroform; DMF = N,N-Dimethylformamide; EtOAc = Ethyl acetate; EtOH = Ethanol; Et_2O = Diethyl ether; HEX = Hexane; HOAc = Acetic acid; IPA = isopropyl alcohol; PE = Petroleum ether; t-BME = tertiary butylmethyl ether]

Table 3. Summary of HPLC methods used for analysis of annatto.

Sample matrix	Analyte(s)	HPLC conditions			Reference(s)
		Column	Mobile phase	Detector	
Annatto colour	Bixin and norbixin isomers	HRPB C_8/C_{18} 250 x 4.6mm, 5um	ACN: 2% HOAc (65:35) isocratic 1 $ml.min^{-1}$ 35°C	UV-VIS PDA 452, 460nm	Scotter et al., 1994
Annatto colour	Bixin and norbixin isomers and C_{17} isomers	HRPB C_8/C_{18} 250 x 4.6mm, 5um	ACN: 0.4% HOAc (65:35) isocratic 1 $ml.min^{-1}$ 35°C	UV-VIS PDA 435 x 60nm	Scotter, 1995; Scotter et al, 1998, 2001
Foods	*Cis/trans* bixin and norbixin	Supelco LC-18 250 x 4.6mm, 5um	MeOH: 2%HOAc (9:1) iscocratic 1 $ml.min^{-1}$	UV-VIS 500nm	Lancaster and Lawrence, 1995
Foods	Bixin, norbixin and carminic acid	Supelco LC-18 250 x 4.6mm, 5um	MeOH: 6%HOAc gradient 1 $ml.min^{-1}$	UV-VIS 493nm	Lancaster and Lawrence, 1996
Plasma	Bixin and norbixin isomers	S5ODS1	ACN: 2% HOAc isocratic 1.5 $ml.min^{-1}$	UV-VIS PDA 460nm	Levy et al., 1997
DNA	Bixin and norbixin	Supelco LC-8 250 x 4.6mm, 10um	ACN: 0.08% CF_3CO_2H (85:15) isocratic 1 $ml.min^{-1}$	UV-VIS 470nm	Kovary et al., 2001
Foods	Bixin and norbixin isomers	HRPB C_8/C_{18} 250 x 4.6mm, 5um	ACN: 0.4% HOAc (65:35) isocratic 1 $ml.min^{-1}$ 35°C	UV-VIS PDA 455 x 10nm	Scotter et al., 2002
Cheese	*Cis/trans* bixin and norbixin	ODS2 C_{18} 250 x 4mm, 5um	ACN: 2%HOAc (75:25) isocratic 1 $ml.min^{-1}$	UV-VIS 460nm	Bareth, Strohmar and Kitzelmann, 2002
Corn snacks	Norbixin	ODS2 C_{18} 150 x 4mm, 3um	ACN: 2%HOAc (65:35) isocratic 1 $ml.min^{-1}$ 29°C	UV-VIS PDA 450nm	O Rios and Mercadante, 2004
Food	Bixin, norbixin and other	YMC C30 250 x 4.6mm, 5um	A: MeOH: H_2O: TEA (90:10:0.1) B: MTBE: MeOH: H_2O:TEA	UV-VIS PDA 450 x 4nm	Breithaupt, 2004

	carotenoids		(90:6:4:0.1) gradient 1 ml.min^{-1} 35°C	+ LC-MS	
Bixin	Photodegradation products	(1) Vydac C18 250 x 4.6mm, 5um (2) ODS2 C18 150 x 4.6 3um	ACN: 2% HOAc: DCM (65:35:2) isocratic 1 ml.min^{-1} 25°C	UV-VIS PDA 450nm	Montenegro et al., 2004
Aqueous model system	Bixin thermal degradation products	ODS2 C_{18} 150 x 4mm, 3um	ACN: 2%HOAc (65:35) or ACN: 2% HOAc: DCM (65:35:2) isocratic 1 ml.min^{-1} 29°C	UV-VIS PDA 450nm	O Rios et al, 2005
Water soluble annatto	*Cis/trans* norbixin	Beckman C18 250 x 4.6mm, 5um	ACN: 0.4% HOAC + 5% ACN isocratic and gradient 1 ml.min^{-1}	UV-VIS PDA 250 - 600nm + LC-MS	Galindo-Cuspinera and Rankin, 2005

[Key: ACN = Acetonitrile; DCM = Dichloromethane; MeOH = Methanol; MTBE = Methyl tertiary butyl ether; TEA = Triethylamine]

Table 4. Summary of extraction regimes used for annatto in foods (Scotter et al., 2002).

Regime	Matrices
1	Cheese, cheese products and cheese-based compound foods
2	Custard powder and low-fat dessert dry mixes
3	Desserts, cake decorations, fine bakery wares, extruded snacks and breakfast cereals
4	Margarine, fat-based emulsions and spreads, butter and fat-based compound foods
5	Fish, ice cream and ice cream-based confectionery, yoghurt and other dairy desserts