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The chemistry and analysis of annatto food colouring: a review

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The chemistry and analysis of annatto food colouring: a review

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,

26 analysis, E160b

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27 **Overview**

28 Annatto is a natural colouring agent obtained from the outer coats of the seeds of the
29 tropical shrub *Bixa orellana*. Annatto and its extracts are designated collectively as
30 E160b and permitted as a food additive in the European Union and elsewhere, and
31 have widespread use in the food industry for the colouring of many commodities
32 including flour and sugar confectionery, dairy and savoury products, soft drinks and
33 fish. The major colour principles of annatto are the carotenoids bixin and norbixin.
34 Though chemically very similar, differences in their chemical properties present
35 several challenges to the analytical chemist with respect to stereochemistry, solubility,
36 chromatographic behaviour and stability. While current legislation on the extraction
37 and use of annatto colours and their applications in food are addressed briefly, this
38 review focuses on the chemistry, stability and analysis of annatto pertaining to its use
39 as a permitted food colouring.

41 **Annatto in foods**

42 *Legislative aspects*

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

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3 51 found at the Nordic Food Additives Database (NNT, 2008) and the Food Law site of
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5 52 the Department of Food Biosciences, University of Reading (Jukes, 2008).
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15 54 [Insert Table 1 about here]
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25 56 In July 2006, the Commission published a set of four proposed Regulations that are
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27 57 intended to replace the current system and provide a common basis for controls on
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29 58 food additives, food flavourings and food enzymes. The proposals were published as
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31 59 separate Commission Documents on additives, flavourings, enzymes and a common
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33 60 authorization procedure (EC, 2006). The proposal brings together all of the existing
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35 61 food additive regulations and plans to introduce comitology for additive approvals in
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37 62 place of the cumbersome co-decision procedure.
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64 The specifications for food colours are laid down in Commission Directive 95/45/EC
65 (EC, 1995) in which separate definitions and purity criteria are prescribed for (i)
66 solvent-extracted bixin and norbixin, (ii) alkali-extracted annatto and (iii) oil-
67 extracted annatto. Solvent-extracted bixin and norbixin formulations are often referred
68 to as indirectly-extracted annatto formulations, whereas alkali- and oil-extracted
69 annatto are termed directly-extracted. The purity specifications include definition of
70 the source material(s) and the solvents permitted for extraction, the identification and
71 the minimum content of the colouring material (measured by spectrophotometry), and
72 the limits for residual solvents and heavy metals.
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74 *Use of annatto in foods*

75 Annatto was reported to be the most commonly consumed natural colour additive in
76 the UK (MAFF, 1987 and 1993) where the *per capita* consumption was estimated to
77 be 0.065 mg/kg bw/day based on pure colouring component, representing some
78 12.5% of the Acceptable Daily Intake (ADI). The chemistry and applications of oil-
79 and water-soluble annatto colours in terms of their modes of applications to a wide
80 range of food products and the usage levels required to obtain the desired colour
81 shades has been reviewed (Collins, 1992; Levy and Rivadeneira, 2000). Crystalline
82 bixin products of 80-97% purity may be obtained by extraction of annatto seed with
83 certain permitted organic solvents and subsequent production of a solvent-free
84 product, which is then processed to give a range of high purity oil- and water-soluble
85 annatto formulations. Oil-soluble bixin is generally used in fatty food applications,
86 whereas norbixin, because of its ability to bind strongly with protein, is especially
87 suited for the colouring of high protein content foods. Annatto colours are often
88 formulated with other additives such as emulsifiers to produce forms of water-soluble
89 annatto that are stable to the effects of e.g. acids, metal ions and salts. The
90 applications and stability of spray-dried annatto formulations in fruit and vegetable
91 products have been studied (Satyanarayana *et al.*, 2006).

92
93 *Annatto intake*

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

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3 99 content of annatto extracts (Levy and Rivadeneira, 2000). In response to a request by
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6 100 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for information
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8 101 relating to the toxicity, intake and specifications of annatto, the European annatto
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10 102 producers consulted with the food industry to determine usage levels of specific
11
12 103 annatto extracts (JECFA, 2002). The data obtained were combined with the levels of
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14 104 bixin/norbixin in particular extracts to provide an estimate of their concentration in
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16 105 food. These data have been combined with food consumption data using various
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18 106 methods to estimate consumer intakes, which ranged from 1 to 163% of the ADI
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20 107 (Tennant and O'Callaghan, 2005). The actual levels of annatto in foodstuffs were well
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22 108 below maximum limits prescribed under EU regulations and Codex standards, which
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24 109 had been confirmed by an earlier analytical study (Scotter *et al.*, 2002).
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32 111 **Annatto chemistry**

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34 112 Elucidation of the chemical constitution of bixin (and thereafter norbixin) was first
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36 113 put forward by Heiduschka and Panzer (1917) who suggested the correct molecular
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38 114 formula for bixin ($C_{25}H_{30}O_4$) as an unsymmetrical molecule. Herzig and Faltis (1923)
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40 115 recognised that bixin was the monomethyl ester of an unsaturated dicarboxylic acid.
41
42 116 The results from their catalytic hydrogenation studies led them to conclude that bixin
43
44 117 contains 9 carbon double bonds, which, evidenced by the intense red colour of the
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46 118 pigment, were conjugated. However, the unsymmetrical molecule hypothesis was
47
48 119 abandoned when proposed the now accepted structure was proposed (Kuhn and
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50 120 Winterstein, 1932), later confirmed by Karrer *et al.* (1932). A new, higher melting
51
52 121 form termed β -bixin was obtained during the course of pigment isolation (Herzig and
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54 122 Faltis, 1923), which was later proposed as the *trans*-isomer that the original form may
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56 123 be *cis*-isomer (Karrer *et al.*, 1929). A stable form of bixin identical to the β -form by
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3 124 treatment of the 'natural' (or 'labile') form with iodine was subsequently obtained
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5 125 (Kuhn and Winterstein, 1932). From the results of these investigations that it was
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8 126 concluded that bixin was the first known naturally occurring *cis*-polyene. The
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10 127 structural elucidation of bixin was confirmed from various oxidation and degradation
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12 128 experiments (Karrer and Jucker, 1950). During investigations to determine the
13
14 129 stereochemical configuration of labile bixin, several stereoisomers were isolated
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16 130 (Zechmeister, 1960). The consequences of *cis-trans* isomerism on the chemistry,
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18 131 stability and analysis of annatto are significant and are discussed below.
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23 133 The major colouring component of annatto is confirmed as the apo-carotenoid 9'-*cis*-
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25 134 bixin (methyl hydrogen 9'-*cis*-6,6'-diapocarotene-6,6'-dioate, C₂₅H₃₀O₄), the
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27 135 monomethyl ester of the dicarboxylic acid 9'-*cis*-norbixin, commonly referred to as
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29 136 *cis*-bixin (Figure 1). 9'-*Cis*-bixin is soluble in most polar organic solvents to which it
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31 137 imparts an orange colour but is largely insoluble in vegetable oil. It may be readily
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33 138 converted to the all-*trans* isomer due to its instability in the isolated form in solution.
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35 139 *Trans*-bixin is the more stable isomer and has similar properties to the *cis*-isomer but
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37 140 exhibits a red colour in solution and is soluble in vegetable oil. Commercially,
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39 141 isomerisation is achieved by heating a suspension of the *cis*-isomer in oil to 130°C *in*
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41 142 *vacuo*. The water-soluble analogue 9'-*cis*-norbixin (C₂₄H₂₈O₄) can be isolated from
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43 143 annatto seeds by agitation in aqueous alkali at <70° C or formed by alkaline hydrolysis
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45 144 of *cis*-bixin to give either the sodium or potassium salt. The dicarboxylic acid is
46
47 145 soluble in polar solvents to which it imparts an orange colour. 9'-*Cis*-norbixin is only
48
49 146 sparingly soluble in chloroform and 0.1M sodium hydroxide (Preston and Rickard,
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51 147 1980). Under extraction conditions, 9'-*cis*-bixin undergoes isomerization to produce oil
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53 148 solutions containing approximately 0.2 - 0.5% of pigment comprising a mixture of all-

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3 149 *trans*- and 9'-*cis*-bixin in variable proportions and characteristic degradation products,
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5 150 dependent upon extraction temperature and time (see below).
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15 154 While it is reported that 80% of the carotenoids in the annatto seed coat comprise bixin
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17 155 (Preston and Rickard, 1980; Lauro, 1991), traces of bixin diesters may be found
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19 156 (Mercadante *et al.*, 1997b). The preparation and use of ethylbixin has been discussed
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21 157 (Levy and Rivadeneira, 2000).
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27 159 The presence of other minor carotenoids in annatto has also been postulated, which
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29 160 included β -carotene, cryptoxanthin, lutein, zeaxanthin and methyl bixin (Tirimanna,
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31 161 1980). The presence of a range of lycopenoate analogues and other minor carotenoids
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33 162 in annatto has been reported in a series of papers by Mercadante and co-workers (1996,
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35 163 1997a, 1997b and 1999) and has been reviewed by Mercadante (2001) and
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37 164 Satyanarayana, Prabhakara Rao and Rao (2003). Bixin and three minor carotenoids
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39 165 have been chemically synthesized using the Wittig reaction of the (Z)-terminus
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41 166 followed by a Horner-Emmons reaction (Haberli and Pfander, 1999).
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48 168 *Molecular properties*
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51 169 It is the delocalisation of π -electrons along the polyene backbone that gives
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53 170 carotenoids their characteristic electronic spectra and is largely responsible for the
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55 171 photophysical and photochemical properties of these molecules, including *cis-trans*
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57 172 photoisomerization. Detailed explanations of *cis-trans* isomerization may be found in
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59 173 standard texts (Karrer and Jucker, 1950; Lunde and Zechmeister, 1954; Zechmeister,
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3 174 1960; Kolher, 1995). Only the basic properties of linear conjugated molecules will be
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6 175 reviewed here along with a brief account of the simple concepts that apply to bixin
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8 176 and norbixin, in order to provide a background for discussion on the UV-VIS
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11 177 spectroscopy of these compounds and to show how UV-VIS spectra are affected by
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13 178 isomerization.

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17 180 The sufficiently large barriers to rotation about the formal double bonds in polyenes
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19 181 or carotenoids allow double bond *cis*- and *trans*- isomers to be isolated as
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22 182 independent, distinct compounds. Since the differences in excitation energies for *cis*-
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24 183 and *trans*- isomers of a given molecule are small compared to the change in excitation
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27 184 energy that is associated with adding or subtracting a conjugated double bond, the
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29 185 basic electronic structure is *almost* independent of isomeric form. The four single
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31 186 bonds that surround a carbon-carbon double bond all lie in the same plane. In
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34 187 consequence, each of the disubstituted and trisubstituted acyclic double bonds that
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36 188 constitute the polyene chain of a carotenoid can exist in two forms i.e. geometric
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38 189 isomers. Nomenclature of the *cis*- or *trans*- isomers is designated in accordance with
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41 190 IUPAC rules (Weedon and Moss, 1995a, 1995b). In recent years however, these
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43 191 designations have been replaced largely by *Z* and *E* respectively.

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48 193 Since each double bond in the polyene chain could, in principle, exist in one of two
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50 194 forms, a large number of geometric isomers are theoretically possible for any
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52 195 carotenoid. However, in practice few of these isomers are encountered. An
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54 196 explanation for this is provided by studying molecular models, which indicate that the
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56 197 introduction of a *cis*- double bond normally results in steric hindrance thus rendering
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58 198 the *cis*-isomer less stable than the *trans*- form. With both trisubstituted double bonds
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3 199 and disubstituted double bonds in the 15,15'-position, the effect is relatively small, as
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5 200 it results from limited interference between two hydrogen atoms and hence these
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7 201 isomers may be formed quite readily. With other disubstituted double bonds the
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9 202 adoption of the *cis*- configuration results in major interference between a hydrogen
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11 203 atom and a methyl group. This renders such molecules less stable than the
12
13 204 corresponding *trans*- form and hence less likely to be encountered (Karrer and Jucker,
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15 205 1950; Zechmeister, 1960; Kolher, 1995; Weedon and Moss, 1995a).
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22 207 Stereomutation studies, in which interconversion of geometrical isomers is
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24 208 deliberately promoted, lend support to this theory. Interconversion generally produces
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26 209 a 'set' of isomers that approximates to an equilibrium mixture of all possible geometric
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28 210 forms proportional to their relative thermodynamic stabilities. The all *trans*- form
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30 211 usually predominates, indicating that it is the most thermodynamically stable isomer.
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32 212 A number of mono- and di-*cis*-isomers are usually also present, however those
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34 213 isomers with more than two *cis*- double bonds and/or those that are sterically hindered
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36 214 usually occur only in trace amounts, if at all. It is not surprising that most naturally
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38 215 occurring carotenoids are predominantly in the all *trans*- form. However, bixin occurs
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40 216 predominantly as the *cis*-isomer, which has a *cis*- configuration about the
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42 217 9'-trisubstituted double bond. Since asymmetric bixin has nine alkene bonds ($n=9$),
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44 218 theoretically 512 (i.e. $2^n = 2^9$) geometric isomers are possible, whereas symmetric
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46 219 norbixin has only 272 (i.e. $2^{(n-1/2)} \times (2^{(n-1/2)} + 1)$) possible isomers. However, the
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48 220 presence of stable *cis*-isomers at positions 7, 11, 12' and 8' are sterically hindered,
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50 221 hence the remaining 5 alkene bonds are capable of yielding 32 and 20 isomers for
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52 222 bixin and norbixin respectively (Figure 1).
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3 224 Provided that an adequate sample of the pure isomer is available or the selected
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5 225 analytical technique is adequately sensitive, spectroscopic analysis will normally
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8 226 allow the unambiguous assignment of the geometrical configuration of any carotenoid
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10 227 isomer. All linear polyenes, the carotenoids included, possess similar low-lying
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12 228 excited singlet (S_1) states (Hudson and Kohler, 1974; Kohler, 1977; Hudson, Kohler
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14 229 and Schulten, 1982). This is critically important since virtually all photo processes in
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16 230 linear polyenes originate in the lowest-energy singlet excited state, the correct
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18 231 identification and characterization of which is therefore also important. As might be
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20 232 anticipated from the similarities in electronic structure, the electronic absorption
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22 233 spectrum of a given carotenoid closely resembles that of the unsubstituted polyene
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24 234 with the same number of conjugated double bonds. There are however well
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26 235 characterised principle differences due largely to the presence of methyl substituents
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28 236 along the carotenoid skeleton, which affect the basic polyene electronic structure.

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34 237 These are:

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36 238 • a 10 to 30 nm shift of the lowest energy strong absorption band to
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38 239 longer wavelength
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41 240 • decreased vibrational fine structure
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46 242 Thus, *cis*- and *trans*- isomers may often be distinguished on the basis of their UV-VIS
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48 243 absorption spectra, but the most important differences observed between isomers are
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50 244 not related to excitation energies but to the relative intensities of high-energy
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52 245 absorption bands i.e. '*cis*-' peaks (Dale, 1954; Zechmeister, 1960). It is well
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54 246 established that the lowest excited state of linear polyenes (including carotenoids) is
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56 247 the 2^1A_g state and that the origin of the main absorption band is the strongly allowed
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58 248 $2^1A_g \rightarrow 1^1B_u$ transition. The shapes of electronic absorption (and fluorescence) bands

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3 249 are derived from the vibrational levels that are associated with the initial and final
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5 250 electronic states. Thus, the typical three-peaked shape of the main absorption band of
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8 251 linear polyenes arises from transitions of the lowest vibrational level of the electronic
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10 252 ground state to the lowest vibrational levels of the electronic excited states.
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12 253 Broadening of these peaks is observed because of rotational levels and inhomogeneity
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14 254 leading to peak overlap. This is particularly relevant for many carotenoids measured
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16 255 as solutions at ambient temperatures (Kohler, 1995).
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22 257 The positions of the absorption maxima and the shape or fine structure of the UV-VIS
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24 258 spectrum of carotenoids are therefore characteristic. But while the UV-VIS spectrum
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26 259 gives information about the chromophores of the molecule, it yields nothing about
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28 260 functional groups apart from conjugated carbonyl groups that form part of the
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30 261 molecule (Scott, 1964; Britton, 1995a and 1995b). In the case of carotenoids, the
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32 262 relevant transition is the $\pi \rightarrow \pi^*$ transition. For such a conjugated system, in which the
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34 263 π -electrons are highly delocalized, the excited state is of comparatively low energy.
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36 264 The energy required to bring about the transition is therefore relatively small and
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38 265 corresponds to light in the visible region. While the transition responsible for the main
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40 266 absorption band is strongly 'allowed', transitions from the ground state to higher
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42 267 electronic states are also possible, providing they obey the symmetry selection rules.
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44 268 These high energy transitions give rise to absorption bands in the UV region which
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46 269 are usually weak, but are observed particularly in the spectra of compounds with
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48 270 extended chromophores.
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272 When the symmetry properties of a carotenoid change, absorption bands that are
273 otherwise not detected may become a significant feature of the spectrum, as the

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3 274 transitions that produce them become allowed (Britton, 1995a). For *trans*- isomers,
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5 275 the electronic structure has a centre of symmetry and the ground state is a *g* state, so
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8 276 transitions to a higher *g* state are forbidden. Transition to a higher excited *g* state only
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10 277 becomes allowed when at least one double bond becomes *cis*- and the original
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12 278 symmetry is lost. This gives rise to an absorption band in the UV region, known as the
13
14 279 *cis*-band or *cis*-peak. The most important feature of the absorption spectrum of a
15
16 280 carotenoid is the main absorption band in the visible region. Several important pieces
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19 281 of information can be obtained from the spectrum:
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24 283 • The position of the main absorption band, specified by λ_{\max} , provides
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26 284 structural information because it is determined by the chromophore of the
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28 285 molecule
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33 287 • The intensity of the absorption at λ_{\max} , (*A*) is related both to the structure and
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35 288 to the concentration of the carotenoid in the sample, and provides the basis for
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37 289 quantitative analysis
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42 291 • The position or the intensity of the main absorption band of a carotenoid can
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44 292 be influenced by a number of factors such as a change in the molecular
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46 293 environment of the carotenoid e.g. solvent
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53 295 Since the structure of the carotenoid chromophore is related to the overall shape or
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55 296 fine structure of the spectrum, the shape as well as the positions of the absorption
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57 297 maxima may therefore be used as a diagnostic tool, especially when comparing
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59 298 carotenoid spectra (Britton, 1995a). A numerical notation describing fine structure has
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4 299 proved convenient and removes the requirement for presenting all spectra as diagrams
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6 300 (Kohler, 1995). In this notation, the baseline or zero value is taken as the minimum
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8 301 between the two peaks, the peak height of the longest wavelength absorption band is
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10 302 designated as III, that of the middle absorption band (usually λ_{\max}) as II (Figure 2).
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12 303 Spectral fine structure is then expressed as the ratio of the peak heights III/II, as a
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14 304 percentage.
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20 306 [Insert Figure 2 about here]
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24
25 308 The annatto carotenoids bixin and norbixin are unusual in that they contain two
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27 309 carbonyl (i.e. carboxyl) groups, one at either end of the conjugated system and in
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29 310 conjugation with it, which formally extends the chromophore. This results in a
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31 311 spectral shift to longer wavelength, usually accompanied by loss of spectral fine
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33 312 structure but the degree of effect is solvent dependent. With dicarboxylic acids and
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35 313 their esters such as bixin (and norbixin), the further extension of the chromophore
36
37 314 causes a large relative increase in λ_{\max} . The spectral shift depends on the polarizability
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39 315 rather than the polarity of the solvent, and the frequency shifts to lower energy, i.e. a
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41 316 spectral shift to longer wavelength, as the refractive index of the solvent increases
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44 317 Britton, 1995a).
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51 319 While water can have a major effect on the spectra of carotenoids in water-miscible
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53 320 solvents, both bixin and norbixin isomers are relatively polar carotenoid molecules,
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55 321 which are significantly soluble in solvents with a high aqueous content and as such
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57 322 similar solvent-related properties are not observed. *Cis*-norbixin is in fact highly
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59 323 soluble in 0.1M sodium hydroxide solution, and both compounds are fairly soluble in
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3 324 mixtures of acetonitrile and dilute aqueous acetic acid, used as a mobile phase in
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5 325 HPLC analysis.
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10 327 The differences that are observed consistently between the spectra of *trans*- and *cis*-
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12 328 isomers of carotenoids are therefore diagnostic for structural assignment. A small
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15 329 hypsochromic shift in λ_{\max} of ca. 2 to 6 nm is usually observed for mono-*cis* isomers
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17 330 along with a significant hypochromic effect and a reduction in vibrational fine
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20 331 structure. A new absorption band appears at a characteristic position about 142 nm
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22 332 below the *longest* wavelength absorption maximum, often referred to as the *cis*-peak.
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27 334 For the all-*trans* form of bixin, the main absorption band ($2^1A_g \rightarrow 1^1B_u$) is very
28
29 335 intense. In the 9'-*cis* isomer), the intensity of this main band decreases as a weak *cis*-
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31 336 peak appears at 355 nm, corresponding to the transition to a higher energy level *g*
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33 337 state. From their studies on β -carotene isomers, it has been shown that the 15-*cis*
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35 338 isomer, in which the *cis* double bond is in the centre of the molecule, shows maximum
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37 339 bending of the chromophore and a well developed *cis*-band, with corresponding
38
39 340 decrease in intensity of the main absorption band (Pettersson and Jonsson, 1990).
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45 342 The intensity of the *cis*-band is essentially greater as the *cis* double bond is nearer the
46
47 343 centre of the molecule and is therefore empirically diagnostic. In a symmetrical di-*cis*
48
49 344 carotenoid, the centre of symmetry may be restored so that the *cis*-band again
50
51 345 becomes a weak feature. For di-*cis* and poly-*cis* carotenoids, a larger hypsochromic
52
53 346 shift in the main absorption band may be seen e.g. 13 nm for di-*cis* norbixin (Figure
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55 347 3) (Scotter *et al.*, 1994).
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3 349 [Insert Figure 3 about here]
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8 351 A numerical notation similar to the % III/II notation to indicate spectral fine structure,
9
10 352 has been adapted to designate the relative intensity of the *cis*-peak (Kohler, 1995).
11
12 353 The intensity or absorbance of the *cis*-peak is expressed as a percentage of the
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14 354 absorbance of the middle main absorption band, which is usually the λ_{\max} . Scotter *et*
15
16 355 *al.* (1994) used this technique to study the spectra obtained by HPLC-photodiode
17
18 356 array analysis of geometrical isomers of bixin and norbixin. The absorption intensity
19
20 357 at λ_{\max} (Figure 4, III) for each isomer was normalised and a comparison of relative
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22 358 intensity (REL (%)) made with the three other maxima at I, II and IV, to give REL(I),
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24 359 REL(II) and REL(IV) respectively. In all cases, λ_{\max} (II) took the form of inflection
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26 360 rather than a peak, which made exact location of the wavelength maximum difficult.
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28 361 However, post-run analysis of the spectral data allowed first derivative spectra to be
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30 362 taken which facilitated location of λ_{\max} (II), as shown in Figure 5.
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364 [Insert Figure 4 about here]

365 [Insert Figure 5 about here]

366

367 *Annatto stability*

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

374

375 *Oxidative stability*

376 Annatto, especially norbixin, is susceptible to oxidation, particularly when applied in

377 powdered form due to the large surface area and when incorporated into foodstuffs;

378 although some foods can have a stabilizing effect (Berset and Marty, 1986; Collins,

379 1992; Levy and Rivadeneira, 2000). Spray-dried norbixin formulated with acaia gum

380 or maltodextrin as carriers have been reported to be particularly susceptible to

381 oxidation (Henry, 1992). The level of bixin prepared from annatto seeds and stored

382 for ca. 1 year at 30°C in packages comprising materials with different oxygen

383 transmission rates was reduced by ca. 10% during the first 2-3 weeks storage but

384 stabilized thereafter except for polyethylene film, which exhibited a degradation rate

385 of 0.04% per day, reflecting the permeability of the polyethylene (Carvalho *et al.*,

386 1993). Several mechanisms have been put forward for the effect of water activity on

387 the reduction of bixin oxidation in microcrystalline cellulose-based model systems to

388 simulate dehydrated foods (Gloria *et al.*, 1995). Bixin degradation followed first order

389 kinetics and the observed half-lives showed greater stability in systems of

390 intermediate and high water activity. It was postulated that this is because of the

391 ability of water to exclude oxygen from liposoluble materials by surface adsorption,

392 hydrogen bond with hydroperoxides, inactivate metal catalysts, reduce of free radicals

393 and lower the stability of singlet oxygen. Annatto has been shown to inhibit

394 hydroperoxide formation leading to triglyceride autoxidation by trapping peroxy

395 radicals (Haila *et al.*, 1996). Annatto was among a number of mediterranean spices

396 whose antioxidant capacities were compared with permitted food antioxidants in lipid

397 peroxidation (Martinez-Tomé *et al.*, 2001). Annatto was reported to have a greater

398 antioxidant capacity than either butylated hydroxyanisole (BHA) or butylated

1
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3 399 hydroxytoluene (BHT) for preventing deoxyribose damage by hydroxyl radicals. In
4
5 400 aqueous media, annatto exhibited a lower antioxidant activity than propyl gallate but
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8 401 was more effective at peroxide scavenging than BHA or BHT. Annatto oleoresin
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10 402 prepared by oil extraction of seeds was found to be more stable than a powdered
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12 403 formulation during storage over ca. 1 year. Samples were stored in glass bottles with a
13
14 404 3cm headspace of air. The greatest losses (60%) were observed for the powder at
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16 405 ambient temperature in daylight, compared to ambient temperature in the dark (54%)
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18 406 and at 5-8°C in the dark (23%). These results concur with the findings of Najar,
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20 407 Bobbio and Bobbio, (1988) that light is the main degradation factor. Moreover,
21
22 408 photosensitized bixin is very reactive towards oxygen and thus may be considered as a
23
24 409 an oxygen quencher; the reaction of bixin with singlet oxygen is a related issue and is
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26 410 discussed below.

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31 412 Norbixin was the only carotenoid that inhibited the oxidative deterioration of lipids in
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33 413 both olive oil and oil-in-water emulsions stored at 60°C and displayed a similar
34
35 414 activity to δ -tocopherol in stored oil (Kiokias and Gordon, 2003). In olive oil-in-water
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37 415 emulsions, norbixin reduced hydroperoxide formation and a synergistic effect
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39 416 between norbixin and ascorbic acid or ascorbyl palmitate was observed.
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48 418 Bixin has been reported to be able to scavenge hydroxyl radicals generated by ferrous
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50 419 ions (Fe^{2+}) and hydrogen peroxide (H_2O_2) but no mechanism was suggested (Zhao *et*
51
52 420 *al.*, 1998). Similarly, the behaviour of norbixin during in vivo plasmid DNA damage
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54 421 induced by reactive oxygen species Fe^{2+} , Sn^{2+} and H_2O_2 have been studied and it has
55
56 422 proposed that since norbixin contains two free carboxyl moieties, its protective action
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3 423 may rely on the formation of complexes (Kovary *et al.*, 2001). Norbixin showed a
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5 424 stronger affinity for Sn^{2+} than for Fe^{2+} but was readily displaced by EDTA.
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10 426 During the isolation and analysis of carotenoids, the exclusion of atmospheric air by
11
12 427 inert gas or vacuum is strongly recommended in order to minimize the risk of
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14 428 destruction or undesired reactions (Scheidt and Liaaen-Jensen, 1995) and annatto is
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16 429 no different in this respect. The oxidation 'products' of bixin were identified
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18 430 tentatively after transformation of bixin in corn oil at 125°C using spectrophotometry
19
20 431 and paper chromatography (McKeown and Mark, 1962). Evidence for the oxidative
21
22 432 decomposition of *cis*-bixin on TLC plates has been reported, where both powdered
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24 433 colour formulations and chloroform solutions of *cis*-bixin exhibited decreased colour
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26 434 content when stored in the dark in air at ambient temperature (Reith and Gielen,
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28 435 1971). This was concluded to be due to the presence of oxygen, supported by
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30 436 observing the relatively lower stability of cheese colour (norbixin in aqueous KOH)
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32 437 compared to butter colour (bixin in vegetable oil), due to the presence of tocopherols
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34 438 in the latter. The effects of light, air and pro-oxidants on the stability of annatto
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36 439 extracts in chloroform over a 12 day period were monitored by spectrophotometry.
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38 440 Air was much less effective at promoting loss of colour compared to light or to
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40 441 benzoyl peroxide, a free-radical promoter (Najar, Bobbio and Bobbio, 1988). The
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42 442 authors concluded that rapid loss of colour might occur whenever free radical
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44 443 formation is promoted.
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54 445 *Reaction with singlet oxygen*

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56 446 Model studies on the photosensitized isomerization of *cis*-bixin show that while bixin
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58 447 in the ground electronic state is stable to thermal isomerization, energy transfer via
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3 448 photosensitization gives rise to the higher energy triplet state ($^3\text{BIX}^*$) precursor,
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5 449 which readily isomerizes to the *trans*- isomer (Montenegro *et al.*, 2004). The rate of
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7 450 isomerization is dependent on several factors which compete for deactivation of
8
9 451 $^3\text{BIX}^*$ e.g. ground state bixin and triplet oxygen ($^3\text{O}_2$). Primary reaction products are
10
11 452 only degraded in the presence of air and under prolonged illumination, which is due to
12
13 453 the formation of oxidation products by reaction with singlet oxygen ($^1\text{O}_2$). The
14
15 454 associated reaction mechanisms are discussed very elegantly by the authors. In a
16
17 455 similar study, the $^3\text{BIX}^*$ energy level was calculated used laser-induced
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19 456 photoacoustic calorimetry of bixin in methanol; acetonitrile solution (Rios *et al.*,
20
21 457 2007). The results of the study showed that bixin is a very efficient quencher of $^1\text{O}_2$ in
22
23 458 fluid solutions due to an efficient energy-transfer process, and confirmed that that the
24
25 459 $^3\text{BIX}^*$ energy level is lower than that of $^1\text{O}_2$ (18 ± 2 kcal/mol and 22.5 kcal/mol
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27 460 respectively).
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38 462 *Thermal stability*

39 463 While bixin and norbixin have good heat stability during food processing compared to
40
41 464 other carotenoids, 9'-*cis*-bixin undergoes a series of complex degradation reactions at
42
43 465 commercial extraction temperatures to produce a range of products coloured pale
44
45 466 yellow to orange (Iversen and Lam, 1953; Levy and Rivadeneira, 2000). Using paper
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47 467 chromatography, the pigments in commercial annatto preparations were separated into
48
49 468 a series of bands that included a number of yellow bands comprising up to 40% of the
50
51 469 total pigments and including bright yellow fluorescent (*sic*) band (McKeown, 1961).
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53 470 This band was thought to be the pale yellow breakdown product of bixin identified
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55 471 previously (Iversen and Lam, 1953). The main thermal degradation product of 9'-*cis*-
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57 472 bixin has since been isolated and identified using paper chromatography and UV/VIS
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3 473 spectrophotometry as the yellow coloured 17-carbon polyene 4,8,dimethyl-
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5 474 tetradecaheptaenedioic acid monomethyl ester “C₁₇” (McKeown and Mark, 1962;
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8 475 McKeown, 1963 and 1965; Preston and Rickard, 1980. The influence of heating time
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10 476 on the thermal degradation of bixin in alkaline extracts of annatto showed that
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12 477 pigment stability is related to the initial quantity of *cis*- and *trans*- bixin as well as to
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14 478 the method used to obtain the extracts (Prentice-Hernández, Rusig and Carvalho,
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16 479 1993).
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19 480
20
21 481 The C₁₇ product has since been confirmed to be predominantly the *trans*- isomer and
22
23 482 that *cis*-isomerisation of bixin was prerequisite to its formation (Scotter, 1995).
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25 483 However, this compound was shown to isomerise in solution to form small amounts
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27 484 of *cis*- isomers and to be susceptible to hydrolysis thus forming a range of compounds
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29 485 analogous to bixin and norbixin in terms of their chemical structures and
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31 486 chromatographic properties. In the light of the results obtained, the mechanism of C₁₇
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33 487 formation originally suggested (McKeown, 1963) was postulated as a concerted
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35 488 electrocyclic process followed by the elimination of *m*-xylene and, to a much lesser
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37 489 extent toluene, toluic acid and toluic acid methyl ester, and the formation of C₁₇ which
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39 490 can degrade further by a similar mechanism.
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43 492 The analytical HPLC-photodiode array (PDA) method developed by Scotter *et al.*
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45 493 (1994, 1995) provided superior qualitative and quantitative data compared with UV-
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47 494 VIS spectroscopic methods (McKeown and mark, 1962; Smith, Blake and Porter,
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49 495 1983) for determining the colour content (as bixin and norbixin) in 21 commercial
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51 496 annatto formulations, particularly with respect to the coloured thermal degradation
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53 497 products (Scotter *et al.*, 1998). Moreover, the levels of the all-*trans* and di-*cis*-

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3 498 isomers of norbixin determined from chromatographic profiles of two different
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5 499 norbixin formulations were found to be consistent with their known production
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8 500 history i.e. indicative of the degree of thermal treatment. The formulation obtained by
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10 501 direct aqueous alkaline extraction contained higher levels of these isomers compared
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12 502 to solvent pre-extracted bixin followed by alkaline hydrolysis obtained using lower
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15 503 temperatures. However, the authors pointed out that while the isomer profiles
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17 504 obtained by HPLC-PDA analysis support this, the different extraction procedures
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19 505 might also give rise to different isomer profiles due differential solubilities and
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21 506 stabilities in the extraction medium. The effects of light and oxygen may further
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23 507 complicate this during extraction and handling, and by the nature of the source
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26 508 material.

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31 510 In a follow-up study, a method was developed which used ambient alkaline hydrolysis
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33 511 followed by solvent extraction and gas chromatography (GC), to analyse annatto
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35 512 colour formulations for the main aromatic hydrocarbon thermal degradation products
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37 513 m-xylene and toluene (Scotter *et al.*, 2000). Of the 20 samples analysed, 15 contained
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39 514 <5 mg/kg toluene Four samples contained between 5 and 10 mg/kg and one sample
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41 515 contained 12 m/kg toluene but these levels were not indicative of significant toluene
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43 516 formation via thermal degradation of annatto. In contrast, 6 samples comprising both
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45 517 bixin and norbixin formulations contained m-xylene in the range 30 – 200 mg/kg with
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47 518 the highest level found in an oil-based bixin formulation. Moreover, the two norbixin
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49 519 formulations of known production history analysed in the previous study (Scotter *et*
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51 520 *al.*, 1998) differed markedly in m-xylene content, which appeared to be consistent
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53 521 with the degree of thermal treatment.
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3 523 For comparison with the alkaline hydrolysis-solvent extraction procedure, 7 of the
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5 524 annatto formulations were submitted for headspace (HS) GC analysis for toluene and
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8 525 m-xylene in order to monitor the effects of heating in a closed controlled environment
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10 526 (90°C for 20 minutes). An increase in m-xylene was observed, with the bixin in oil
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12 527 formulations showing the highest rise in m-xylene concentration on heating. The
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14
15 528 authors anticipated that HS-GC could be used to monitor the thermal degradation of
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17 529 annatto in food systems and thus conducted a number of experiments in combination
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19 530 with HPLC-PDA and GC-MS to study this (Scotter *et al.*, 2001). Low levels (ca. 10 –
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21 531 15 ug/kg) of m-xylene were detected in the headspace of annatto-coloured retail
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23 532 samples of custard powder, extruded snacks, margarine and breadcrumbs but not in
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25 533 control samples. Much higher levels of m-xylene were detected in annatto-coloured
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27 534 smoked herring (kippers) at ca. 150-200 ug/kg and m-xylene was observed in the
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29 535 headspace of heated Red Leicester cheese (not quantified). The C₁₇ coloured annatto
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31 536 degradation product was also detected, indicating that thermal degradation of the
32
33 537 principal annatto colouring agent 9'-*cis*-bixin in model systems and foods is facile.
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35 538 However, the degradation is complicated many competing isomerisation reactions
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37 539 which proceed at different rates towards equilibrium. This is further complicated by
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39 540 the simultaneous and irreversible formation of C₁₇ associated with the production of
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41 541 m-xylene and to a lesser extent, toluene. While norbixin was reported to degrade
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43 542 similarly but more slowly, the levels of m-xylene formation were nonetheless
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45 543 consistent with bixin / norbixin concentration in the food and occurred more rapidly at
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47 544 higher temperatures.
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51 546 In order to better understand the kinetics and yields for the formation of both the
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53 547 coloured and aromatic hydrocarbon thermal degradation products of annatto, the
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3 548 authors carried out a number of experiments in model systems (*ibid.*). The thermal
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5 549 stability of bixin at the boiling point of three homologous alcohol solvents was
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8 550 evaluated using HPLC-PDA to monitor the rate of loss of 9'-*cis*-bixin as well as the
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10 551 appearance of a di-*cis*- and *trans*- isomer, and the C₁₇ degradation product. Loss of
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12 552 linearity was observed at each temperature beyond 2 hours, suggesting that two or
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15 553 more competing reactions were taking place at different rates. From the rate constants
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17 554 calculated for the initial phase of the reaction, the Arrhenius activation energy for the
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19 555 loss of 9'-*cis*-bixin in refluxing alcohol solvent was 35.7 kJ.mol⁻¹. Since the rate of
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21 556 loss of 9'-*cis*-bixin was measured as a function of time regardless of reaction pathway
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23 557 i.e. isomerisation vs. degradation), the authors concluded that the rate data represented
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25 558 only the total (summed) values. Thus, several concurrent reaction pathways are
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27 559 available hence deviation from first order kinetics at long observation times was not
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29 560 unexpected as suggested in Figure 6.
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36 562 [Insert Figure 6 about here]
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41 564 Berset and Marty (1986) had reported previously an activation energy of 125 kJ.mol⁻¹
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43 565 for the thermal degradation of annatto pigments in petroleum jelly using a simple
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45 566 first-order kinetic model for the complete decay. This disparity in values therefore
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47 567 suggests a controversy in the kinetic analysis or a misinterpretation of the
48
49 568 experimental data. Interestingly, bixin was reported to be easily transformed to the all-
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51 569 *trans*- isomer at ambient temperature in the presence of a photosensitizer and light,
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53 570 where the activation energy for the excitation of bixin to an excited triplet state was
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55 571 ca. 25 kJ.mol⁻¹ as discussed above (Montenegro *et al.*, 2004), which suggests strongly
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3 572 that a greater energy barrier may be anticipated for the thermal isomerisation of 9'-*cis*-
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5 573 bixin to *trans*-bixin.

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10 575 A more detailed kinetic study on the thermal degradation of bixin in an aqueous
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12 576 model system comprising water: ethanol (8:2) as a function of temperature has been
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14 577 described, where HPLC was used to monitor the decay of 9'-*cis*-bixin and the
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16 578 formation of the di-*cis*- and *trans*- isomers, as well as C₁₇ (Rios *et al.*, 2005). The
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18 579 reactions were found not to follow first order rate characteristics but rather fitted well
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20 580 to a biexponential model. The rate constants for the *formation* of the primary products
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22 581 of bixin and the energy barriers for each step were calculated. Di-*cis*- isomers were
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24 582 formed immediately (energy barrier ca. 63 kJ.mol⁻¹) followed by a slow consumption
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26 583 (with the associated decay of 9'-*cis*-bixin), indicating their role as reaction
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28 584 intermediates. The di-*cis*- isomers can either revert readily to 9'-*cis*-bixin (ca. 13
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30 585 kJ.mol⁻¹) or yield the primary C₁₇ degradation product with a higher energy
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32 586 requirement of ca. 27 kJ.mol⁻¹). However, the isomerisation of 9'-*cis*-bixin to *trans*-
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34 587 bixin requires ca. 100 kJ.mol⁻¹, thereby explaining its relatively slow formation. The
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36 588 Arrhenius plot obtained from the initial decay component for 9'-*cis*-bixin yielded an
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38 589 activation energy of ca. 33 kJ.mol⁻¹, which concurs with earlier data (Scotter *et al.*,
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40 590 2001). In conclusion, while the activation energy obtained for the 9'-*cis*- → *trans*-
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42 591 isomerisation of bixin is very similar to that reported for β-carotene, the value of ca.
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44 592 155 kJ.mol⁻¹ for the summed isomerisation steps of bixin is much higher than those
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46 593 reported for the thermal isomerisation of C₄₀ carotenoids (ca. 105 kJ.mol⁻¹). Thus the
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48 594 reaction scheme suggested by Scotter *et al.* (2001) and the greater relative stability of
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50 595 bixin, especially during its isolation and manipulation were confirmed (Figure 7).
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3 597 [Insert Figure 7 about here]
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8 599 Thermogravimetric analysis has been used to investigate the thermal degradation of
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10 600 bixin derived from annatto seeds at different heating rates over the 25 – 900°C
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12 601 temperature range (Silva *et al.*, 2005). The results indicated that the decomposition of
13
14 602 solid 9'-*cis*-bixin occurs in the liquid phase and that four decomposition stages are
15
16 603 evident over the temperature range 205-545°C, with isomerisation to the *trans*- isomer
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18 604 occurring between 200 and 240°C. The calculated activation energy was dependent
19
20 605 upon heating rate (i.e. 5, 10 or 15 K.min⁻¹) at ca. 108, 147 and 128 kJ.mol⁻¹
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22 606 respectively compared to the value of ca. 100 kJ.mol⁻¹ reported by Rios *et al.*, (2005)
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24 607 obtained in solution. In a similar follow up study, *cis*-norbixin was heated at rates of
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26 608 5, 10 and 20°C.min⁻¹ over the temperature range 25 – 900°C, where the thermal
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28 609 decomposition reactions occurred in the solid phase (Silva *et al.*, 2007). Using the
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30 610 Coats-Redfern model, the calculated activation energy was dependent upon heating
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32 611 rate at ca. 154, 131 and 99 kJ.mol⁻¹ at 5, 10 and 20°C.min⁻¹ respectively for the first-
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34 612 order process.
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43 614 Heating solid non-purified extracts of annatto seeds as a thin film deposited on a
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45 615 silicon wafer *in vacuo* and monitored using time of flight (ToF) secondary ion mass
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47 616 spectrometry (SIMS), does not give the same results as heating in solution
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49 617 (Bittencourt *et al.*, 2005. Principal component analysis revealed that the thermal
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51 618 degradation of the annatto extracts under these conditions occurs in three distinct
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53 619 temperature ranges; below 70°C, the extracts remain thermally stable but above this
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55 620 temperature dimerization reactions occur and the signals attributed to bixin decrease.
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58 621 Near to 100°C, the bixin molecules begin to degrade, leading to fragmentation with
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3 622 extensive degradation of bixin above 120°C. However, the nature of the degradation
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5 623 mechanism described is not fully understood since there was no evidence for the
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8 624 formation of C₁₇ or related fragments from solid bixin.
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13 626 *Light stability*

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15 627 The effect of light at 900 lux intensity on the 30-day stability of a microencapsulated
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17 628 water-miscible extract of bixin compared to that of a purified bixin extract have been
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19 629 studied by measuring the loss of spectrophotometric absorbance at 470nm with time
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21
22 630 (Prentice-Hernández and Rusig, 1999). The degradation rate of bixin in the
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24 631 microencapsulated extract was ca. 0.05% compared to 0.11% per day for the purified
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26 632 extract.
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31 634 Ferreira *et al.*, (1999) submitted commercial water-soluble annatto (norbixin)
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33 635 solutions to different time and temperature treatments to investigate colour stability.
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35 636 The colour change was measured by spectrophotometry using the Hunter Lab System
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37 637 and the results presented in terms of changes in the norbixin concentration and L, a, b
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39 638 colour parameters. Data were analysed for reaction order and the temperature
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41 639 dependence was explained by the Arrhenius model, with activation energy values
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43 640 between 46 and 105 kJ.mol⁻¹. The changes in colour showed an increase in lightness
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45 641 and yellow colour and a decrease in red colour. Norbixin degradation reaction
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47 642 followed second order kinetics whereas for other colour parameters, first order
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49 643 kinetics was followed.
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53 645 The light stability of spray-dried bixin encapsulated with gum Arabic or maltodextrin
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55 646 plus Tween 80 surfactant has been reported, where the kinetic behaviour of bixin
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3 647 photo degradation in all systems was characterized by two first-order decays due to
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5 648 the presence of bixin both inside and outside the microcapsules (Barbosa *et al.*, 2005).
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7
8 649 Unsurprisingly, approximately two orders of magnitude greater stability was observed
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10 650 for bixin in the dark compared to illuminated conditions and in the absence of light,
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12 651 and bixin stability in encapsulated solutions was ca. ten times greater than in non-
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14 652 encapsulated systems. The effect of processing conditions used for the preparation of
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16 653 traditional Indian foods on bixin stability including baking, frying, microwave
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18 654 cooking and pressure cooking have been monitored by following losses using
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20 655 spectrophotometry (Rao *et al.*, 2005). The losses of bixin under model processing
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22 656 conditions was compared to the preparation of cakes, chegodis, biscuits and fried rice.
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24 657 The greatest losses of bixin were observed in direct exposure to oven baking (54%
25
26 658 loss) and deep fat frying (47%), whereas microwave cooking did not affect the colour
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28 659 during direct exposure or in food products. The maximum loss of bixin (65%) was
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30 660 observed for deep fried snack due largely to leaching of the dye into the oil. Pressure
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32 661 cooking produced losses of between 25% and 33%. In cakes, the loss was 30% but
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34 662 negligible losses were found for biscuits (1.5%).
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38 664 Similarly, the combined effects of light and temperature on annatto extract under
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40 665 different storage conditions were evaluated spectrophotometrically at 470nm in
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42 666 chloroform over a period of 360 days (Balaswamy, Prabhakara Rao, Satyanarayana
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44 667 and Rao, 2006). Annatto oleoresin was generally more stable during storage with
45
46 668 respect to bixin content than annatto powder obtained by solvent extraction of annatto
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48 669 seeds. The bixin lost in oleoresin stored under cold (5-8°C), dark conditions was
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50 670 minor (11%) throughout the study, whereas considerable losses were observed for the
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52 671 powdered dye (23%). Likewise, the bixin lost after storage at ambient temperature in
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3 672 the dark were 8% and 54% for oleoresin and powder respectively. Under diffused
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5 673 daylight ambient and temperature the losses were 14% and 60% respectively, whereas
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8 674 bixin seed stored in jute sacks showed a loss of only 15%. As expected, the rate
9
10 675 constants for bixin degradation were much higher in powder compared to oleoresin
11
12 676 and were reported to follow second order kinetics. It was proposed that the colour is
13
14 677 protected from exposure to oxygen and light by the oleoresin compared to the dry
15
16 678 powder, which has a large surface area.
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22 680 Bixin complexed with α -cyclodextrin is also reported to be more resistant to the
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24 681 damage caused by light and air (Lyng, Passos and Fontana, 2005).
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29 683 *Analytical methods for annatto*

30
31 684 *Spectrophotometry*

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33 685 Historically, chloroform has been used as solvent for the spectrophotometric analysis
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35 686 of bixin and dilute sodium hydroxide (ca. 0.1M) for norbixin. Absorbance
36
37 687 measurements at the two most intense spectral peaks (III and IV in Figure 4) are used
38
39 688 for quantitative analysis, where peak IV is preferred because it is less prone to
40
41 689 interference from yellow decomposition products. This interference was corrected by
42
43 690 using a factor related to the absorbances at λ_{\max} and at 404nm in determining the total
44
45 691 pigment content of annatto formulations (McKeown and Mark, 1962). In practice, the
46
47 692 spectrophotometric determination of annatto (as bixin or norbixin) is somewhat
48
49 693 confused by the use of conflicting extinction coefficients. This has been discussed in
50
51 694 detail and the published ($E_{1\text{cm}}^{1\%}$) extinction coefficients for norbixin and bixin
52
53 695 summarized and compared to highlight disparities (Levy and Rivadeneira, 2000).
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55 696 Depending upon the extinction coefficient used, large errors might be incurred and
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3 697 propose a practical conversion factor to correlate the relative absorbances at the two
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5 698 peak maxima. This is based upon the increase in absorbance observed upon hydrolysis
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7
8 699 of bixin to norbixin at constant concentration – thus proving that the extinction value
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10 700 for norbixin must be higher than that for bixin, which was also reported (Smith *et al.*,
11
12 701 1983). Furthermore, from data recorded by the authors from more than 1000
13
14 702 spectrophotometric measurements of different samples of bixin before and after
15
16 703 hydrolysis, the difference between the extinction values of bixin and norbixin was
17
18 704 reported to be of the order of 6%. When compared with a value of $E_{1\text{cm}}^{1\%} = 3208$
19
20 705 reported for pure norbixin, this equates to an extinction coefficient for bixin of $E_{1\text{cm}}^{1\%} =$
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22 706 3016, which concurs with the values reported for purified bixin in chloroform (Scotter
23
24 707 *et al.*, 1994).

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26 708
27
28 709 However, these extinction values do not agree with those adopted for colour purity
29
30 710 specifications by the European Union (EC, 1995) or the FAO/WHO (1996), largely
31
32 711 due to misassumptions made regarding solvent effects. The discrepancy in published
33
34 712 extinction values might be traced back to the ‘erroneous’ coefficient reported by Reith
35
36 713 and Gielen (1971) that has been used subsequently as a reference value by various
37
38 714 other workers. Serious doubt is expressed over the validity of the extinction values for
39
40 715 norbixin in aqueous alkaline solution at 453nm (2850) and 482nm (2550). Moreover,
41
42 716 the same reservations were expressed over the value of 3473 at 453nm reported by the
43
44 717 FAO/WHO specification (FAO/WHO, 1981).

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46 718
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48 719 An interesting and important aspect of the spectrophotometric analysis of bixin in
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50 720 chloroform is its rapid rate of degradation when contained in a quartz cuvette, which
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3 721 unlike glass cuvettes allows the transmission of ultraviolet light (i.e. < 300nm) (Levy
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5 722 and Rivadeneira, 2000).

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10 724 *Planar chromatography*

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12 725 Prior to 1961, there were few references in the literature to paper and adsorption

13
14 726 chromatography, which dealt mainly with the gross separation of different

15
16 727 carotenoids, and from chlorophylls. The first paper chromatographic method for the

17
18 728 direct separation of annatto colouring components used Whatman 3MM paper

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20 729 impregnated with 50% N,N-dimethylformamide (DMF) in acetone and which

21
22 730 developed with cyclohexane: chloroform: DMF and acetic acid (85:10:3:2)

23
24 731 (McKeown, 1961). This method was used in a number of subsequent studies on

25
26 732 annatto and its main thermal degradation product, C₁₇ (McKeown and Mark, 1962;

27
28 733 McKeown, 1963). The first thin layer method for separation of annatto colour and

29
30 734 other fat-soluble dyes shortly thereafter, employed silica gel G, plaster of Paris and

31
32 735 silicic acid media with amyl acetate mobile phase (Ramamurthy and Bhalerao, 1964).

33
34 736 However, of the 30 solvent systems studied, bixin was reported to migrate from the

35
36 737 base line only when acetic acid was present (Francis, 1965). The findings suggested

37
38 738 that the amyl acetate solvent used by Ramamurthy and Bhalerao (1964) must have

39
40 739 contained acetic acid as an impurity, which was proven by subsequent

41
42 740 experimentation. Later methods used silica gel with various solvent systems

43
44 741 containing acetic acid for the separation of bixin and norbixin in colour formulations

45
46 742 (Dendy, 1966) and cheese colour i.e. norbixin (Reith and Gielen, 1971), who also

47
48 743 employed cellulose media for the analysis of butter colour i.e. bixin. Other methods

49
50 744 include those developed by Preston and Rickard (1980) and Corradi and Micheli

51
52 745 (1981). Chao *et al.* (1991) used reverse phase (C₁₈) plates with methanol:water mobile

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2
3 746 phase to separate annatto pigments from supercritical CO₂ extractions of annatto
4
5
6 747 seeds. More recently, TLC has been used for the detection of bixin and other food
7
8 748 colour carotenoids derived from red pepper (Mínguez-Mosquera, Hornero-Méndez
9
10 749 and Garrido-Fernández, 1995) and for the isolation and identification of new (trace)
11
12 750 apocarotenoids from annatto seeds (Mercadante, Steck and Pfander, 1997b) and in the
13
14 751 bioautographic detection of antimicrobial compounds in water-soluble annatto
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16 752 extracts (Galindo-Cuspinera and Rankin, 2005). The various methods are summarized
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18 753 in Table 2.
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30 755 [Insert Table 2 about here]

31 756

32 757 *HPLC*

33 758 As discussed above, developments in HPLC techniques have enabled more detailed
34 759 studies of other bixin and norbixin isomers as well as their degradation products
35 760 compared to TLC methods and have been utilized to gain a greater understanding of
36 761 the stability of annatto and which in turn have been applied to the detection and
37 762 measurement of annatto colour in foodstuffs (below).
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47 764 Literature references on the application of HPLC to the separation of annatto colouring
48 765 components are sparse. Early methods include the HPLC analysis of annatto extract
49 766 (Nishizawa *et al.*, 1983) and Smith *et al.* (1983), who reported the use of an isocratic
50 767 reverse-phase system employing an ODS column and methanol/aqueous acetic acid
51 768 mobile phase. Using this system the *cis*- and *trans*-isomers of both bixin and norbixin
52 769 were separated within 10 minutes. However, the *cis*- and *trans*-bixin peaks were not
53 770 fully resolved and the peak shapes were generally very poor. A method for the reverse-

1
2
3 771 phase separation of bixin, norbixin and three curcuminoids using both isocratic and
4
5 772 gradient elution systems, comprising a Zorbax ODS column and water/THF mobile
6
7
8 773 phase was later developed that gave improved chromatographic separation (Rouseff,
9
10 774 1988) developed. However, only separation of the 'main' annatto colouring
11
12 775 components were reported and no reference to stereoisomer separation was given.
13
14 776 Other approaches have been reported for the analysis of cheese extracts (Luf and
15
16 777 Brandl, 1988) and of foods after protease digestion (Chatani and Adachi, 1988). A
17
18 778 procedure similar to that reported by Smith *et al.*, (1983) has been developed and
19
20 779 applied to the determination of annatto in selected foodstuffs with reasonable success
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22 780 (Lancaster and Lawrence, 1995).
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29 782 The method developed by Scotter *et al.* (1994) has played a key role in the
30
31 783 advancement of HPLC capabilities for the separation and characterization of norbixin
32
33 784 and bixin isomers, and has been refined and adapted for the study of annatto stability
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35 785 and for the determination of annatto colouring components in colour formulations,
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37 786 foodstuffs and human plasma. These are summarized along with other published
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39 787 methods in Table 3.
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45 789 [Insert Table 3 about here]
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51 791 While the development of column stationary phases been vital in allowing separation
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53 792 of geometrical isomers of bixin and norbixin, C₁₇ analogues and other food
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55 793 components, it is the power of the detection systems that have enabled the
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57 794 development of highly useful qualitative and quantitative analyses. Many developed
58
59 795 methods utilise detection with fixed wavelength UV-visible (UV-VIS) detectors at
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3 796 wavelengths specific to bixin/norbixin isomer absorption maxima quite successfully.
4
5 797 However, photodiode-array (PDA) technology offers combined sensitivity and
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8 798 specificity coupled to real-time qualitative (spectral) confirmatory analysis, thereby
9
10 799 enabling powerful isomer identification and measurement. PDA allows isomer peaks
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12 800 with different λ_{\max} wavelengths to be monitored using a spectral bandwidth that
13
14 801 encompasses them. A reference wavelength can also be used to subtract background
15
16 802 absorbances and to allow for baseline drift, which is usually set outside of the
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18 803 absorbance range of the main analyte and interfering peaks e.g. at 600nm x 4nm
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20
21 804 bandwidth. The lack of availability of authenticated reference standards is the main
22
23 805 limiting factor in the HPLC analysis of annatto colouring components but methods are
24
25 806 available for the isolation, purification and characterization of the main bixin and
26
27 807 norbixin isomers (Scotter *et al.*, 1994) and for C₁₇ analogues (Scotter, 1995). Other
28
29 808 workers have exploited the use of PDA detection for the identification of trace levels
30
31 809 of other apocarotenoids in annatto seeds very successfully (Mercadante, Steck and
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33 810 Pfander, 1997b). Figure 8 shows the HPLC separation of bixin and norbixin isomers
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35 811 (Scotter *et al.*, 1994).
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43 813 [Insert Figure 8 about here]
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815 *Mass spectrometry(MS)*

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51 816 A comprehensive review on the use of mass spectrometry in the study of carotenoids
52
53 817 in general may be found elsewhere (Enzell and Back, 1990). This work cites earlier
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55 818 reviews and studies that consolidate the importance of the technique not only for
56
57 819 elucidation of structure but also for analytical research, not least those carried out by
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59 820 Vetter *et al.*, (1971), Budzikiewicz, (1974) and Enzell and Wahlberg, (1980). The
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2
3 821 1990 review covers in detail ionization techniques, tandem MS, combined
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5 822 chromatographic-MS techniques and elimination reactions of in-chain units and
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7
8 823 terminal groups. The first method for electrospray liquid chromatography-mass
9
10 824 spectrometry (LC-ES-MS) of carotenoids employed gradient reversed-phase HPLC
11
12 825 with PDA and MS detection in tandem (van Breemen, 1995). Molecular ions, $M^{(+)}$,
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15 826 without evidence of any fragmentation, were observed in the ES mass spectra of both
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17 827 xanthophylls and carotenes but neither bixin nor norbixin were studied.
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22 829 In common with other carotenoids, the MS spectra of bixin and norbixin are
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24 830 characterized by fragmentation leading to losses of toluene and xylene from the
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26
27 831 polyene chain and the structural significance of the intensity ratio of the $[M-92]^+$ and
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29 832 $[M-106]^+$ ions (and to a lesser extent the $[M-158]^+$ ion), which is related to the
30
31 833 number of conjugated double bonds. It is the apo-configuration that gives rise to
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33 834 anomalous MS properties of bixin and norbixin that have diagnostic value i.e. the -
34
35 835 $CH_2-CH=CH-CH_2-COOH$ end group gives characteristic fragments at $[M-44]^+$ and
36
37 836 $[M-99]^+$, whereas the $-CH_2-CH=CH-CH_2-COOCH_3$ end group gives characteristic
38
39 837 fragments at $[M-31]^+$, $[M-59]^+$ and $[M-113]^+$. Solid probe electron ionization (EI+)
40
41 838 was used to confirm the structures of isolated and purified bixin and norbixin isomers
42
43 839 (Scotter *et al.*, 1994). Both the 9'-*cis*- and *trans*- isomers gave a molecular ion at m/z
44
45 840 394 (bixin) and m/z 380 (norbixin), with major fragment ions at m/z $[M-106]$, 106
46
47 841 (xylene), 105 (methyl tropylium) and 91. Using thermospray analysis, $[M+H]^+$ was
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49 842 identified as the base peak along with the presence of sodium and (possibly) water
50
51 843 adducts, and fragment ions corresponding to $[M-H_2O]^+$ and $[M-CH_3OH]^+$. In a later
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53 844 study, similar analytical conditions were used to characterize the 17-carbon major
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55 845 thermal degradation product of annatto (Scotter, 1995). Solid probe EI revealed the
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3 846 molecular ion at m/z 288 along with fragment ions at m/z [M-106], 106, 105 and 91,
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5 847 and thermospray analysis identified the base peak as [M+H]⁺ as well as sodium
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8 848 adducts at m/z 311 ([M+Na]⁺) and 333 [M-H+2Na]⁺.
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10 849
11
12 850 Complementary to other analytical techniques, EI⁺ and fast atom bombardment
13
14 851 (FAB) MS was used to determine the structure of the bixin family of apocarotenoids
15
16 852 (Kelly *et al.*, 1996). Both *cis*- and *trans*- bixin isomers gave EI⁺ molecular ion
17
18 853 abundancies equivalent to ca. 30% of the base peaks at m/z 59 or 91, and the [M+1]
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20 854 and [M+2] ion intensities were consistent with predictions based upon calculated ¹³C
21
22 855 isotope patterns. As expected, loss of xylene as a neutral group was most pronounced
23
24 856 for *cis*-bixin but no loss of neutral toluene was observed although the m/z 91 peak was
25
26 857 prominent. Fast-atom bombardment (FAB⁺) spectra of *cis*- and *trans*- bixin gave the
27
28 858 molecular ion as the base peak but the abundance of the [M+1] peak exceeded the
29
30 859 calculated isotopic abundance by 55-75%, indicating a small contribution from
31
32 860 [M+H]. Small amounts of sodium adducts were observed but ions due to elimination
33
34 861 of toluene were not. However, significant amounts of m/z 105, 115 and 165 were
35
36 862 observed. These observations were consistent with other FAB spectra of carotenoids
37
38 863 where odd electron molecular ions are frequently observed due presumably to their
39
40 864 lower ionization potentials (Vetter and Meister, 1985).
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42 865
43
44 866 Bixin was among the polyenes studied using electrospray ionization (EI) and high
45
46 867 resolution (HR) matrix-assisted laser desorption ionization (MALDI) time-of-flight
47
48 868 (TOF) mass spectrometry (Guaratini *et al.*, 2004). In this study, the ability of neutral
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50 869 organic molecules to give up an electron for oxidation was exploited, which is
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52 870 governed by the energy of their highest occupied molecular orbital (HOMO) and can
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3 871 be estimated by measurement of the half-wave potential for solution oxidation. Strong
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5 872 evidence was reported for an ionization process that produces the molecular ion M^+ in
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7
8 873 ESI and HR-MALDI MS of polyenes, and the correlation of the observed ions to the
9
10 874 oxidation potential. The formation of M^+ and $[M+H]^+$ species was shown to be
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12
13 875 dependent upon energetic variations and the presence of water or another protic
14
15 876 solvent. Neither the $[M+H]^+$ nor the $[M+H-H_2O]^+$ were detected as the major ions
16
17 877 from ESI analysis of bixin, whereas M^+ was detected but only in the specific capillary
18
19 878 voltage range of 0.1 to 0.7 kV. The accurate mass measurement afforded by the HR-
20
21 879 MALDI-TOF analysis showed M^+ for bixin at an observed mass of 394.2147 with
22
23 880 40% ion intensity, but $[M+H]^+$ was not observed.
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28
29 882 The major carotenoid composition of *Bixa orellana* seeds has been ascertained using
30
31 883 TOF-MS with X-ray photoelectron spectroscopy (Felicissimo *et al.*, 2004). The
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33 884 presence of bixin was revealed in the seed aril without any sample pretreatment from
34
35 885 the detection of ions attributable to $[M+2H]^+$ at m/z 396 with associated ^{13}C isotope
36
37 886 analogues at m/z 397 and 398. The presence of characteristic fragments at m/z 337
38
39 887 was attributed to $C_{23}H_{29}O_2^+$ obtained from the previous molecular ion with loss of a
40
41 888 $COOCH_3$ ester group, and at m/z 281, a fragment compatible with loss of a $C_6O_2H_8$
42
43 889 end group plus a hydrogen atom i.e. $C_{19}H_{21}O_2^+$. The characteristic presence of xylene
44
45 890 was confirmed via the detection of the $C_8H_9^+$ ion at m/z 105. Analysis of the coloured
46
47 891 interior of the seeds following cutting did not show any fragments consistent with
48
49 892 bixin. A methanol:chloroform extract of the seeds was analysed immediately after
50
51 893 preparation by blow-drying under nitrogen onto a silver substrate, and then after
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53 894 exposure to ambient light for 3 months. TOF-MS analysis of the fresh extract was
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55 895 dominated by the molecular peak at m/z 396 along with all other characteristic
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3 896 fragments. As expected after 3 months exposure to light, the colour of the extract had
4
5 897 lightened to a more yellow shade with an associated 5-fold decrease in the intensity of
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7
8 898 the $[M+2]^+$ ion and with a concomitant 2-fold increase in the intensity of the $C_8H_9^+$
9
10 899 ion, indicating the formation of xylene via degradation. In a related study, Bittencourt
11
12 900 *et al.* (2005) analysed extracts of *Bixa orellana* using TOF-MS as a means of
13
14 901 characterising thermal effects. The spectrum was characterised by a large number of
15
16 902 peaks generated by the principal ions and their multiple fragmentation patterns but
17
18 903 also, more notably, by the presence of ions at m/z 790 ($[C_{50}H_{62}O_8]^+ = 2M+2H$), 804
19
20 904 ($[C_{51}H_{64}O_8]^+ = 2M+2H+CH_2$) and 818 ($[C_{52}H_{66}O_8]^+ = 2M+2H+2CH_2$) attributed to
21
22 905 the presence of dimers.
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27 906

28
29 907 The confirmation of twelve different carotenoids used as food colorants was achieved
30
31 908 using positive atmospheric pressure chemical ionization (APCI)+ LC-MS (Breithaupt,
32
33 909 (2004). The $[M+H]^+$ ions were monitored for norbixin and bixin at m/z 381 and 395
34
35 910 respectively. Based on the presence of at least one carboxyl group, APCI
36
37 911 measurements in the negative mode were also carried out on bixin and norbixin but no
38
39 912 significant enhancement in sensitivity was observed. A similar approach has been
40
41 913 used for the analysis of water-soluble annatto extracts in both positive and negative
42
43 914 electrospray detection modes (Galindo-Cuspinera and Rankin, 2005). ES- detection
44
45 915 mode showed a major peak at m/z 379 corresponding to $[M-H]^-$ for norbixin, whereas
46
47 916 the major peak at m/z 381 was found using ES+ mode. An ion at m/z 117 was
48
49 917 identified in in the ES- spectrum of 9'-*cis*-norbixin but not in the spectrum of the
50
51 918 *trans*- isomer. Conversely, the *trans*- isomer showed an ion at m/z 111.1 in ES+ mode
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53 919 that was not present on the spectrum of 9'-*cis*-isomer. This was thought to be due to
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55 920 differences in fragmentation patterns determined by stereochemical configuration.
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2
3 921 More recently, it has been shown that HPLC-PDA in combination with ion-trap
4
5 922 electrospray mass spectrometric confirmatory analysis can be used to identify and
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7
8 923 measure norbixin and bixin in meat products using precursor ions at m/z 379 and 395
9
10 924 respectively and monitoring characteristic product ions at m/z 253,291,310 and 335
11
12 925 (norbixin) and m/z 317, 335, 345, 363 and 377 (bixin) (Noppe *et al.*, 2009).
13
14

926

927 *Nuclear magnetic resonance (NMR) spectroscopy*

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18 927
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20 928 A comprehensive review on the use of NMR spectroscopy in the study of carotenoids
21
22 929 in general is given by Englert (1995), in which a detailed treatise on the experimental
23
24 930 aspects, chemical shifts of end groups, chemical shifts and spin couplings,
25
26 931 stereoisomerization, and simple and multidimensional experiments are given for ^1H
27
28 932 and ^{13}C nuclei.
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33
34 934 The earliest published use of NMR in the study of bixin stereochemistry used low
35
36 935 resolution (40 MHz) instrumentation to assign ^1H frequencies and deduce that the *cis*-
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38 936 bond of the methyl analogue of 'natural or α -' bixin was in the 9'- (equivalent)
39
40 937 position (Barber *et al.*, 1961). The high frequency shift of the proton assigned to H-8'
41
42 938 was attributed to deshielding by the 11'-12' alkene bond when compared to the *trans*-
43
44 939 (or β -) isomer, which was confirmed via synthesis and more detailed structural
45
46 940 assignments (Pattenden, Way and Weedon, 1970). Fourier transform (FT) NMR was
47
48 941 used later to assign the ^{13}C spectra of methyl *cis*- and *trans*-bixin using deuterated
49
50 942 compounds, however no experimental details were given and assignments were partly
51
52 943 derived from spectra of carotenoids with similar structural characteristics (Moss,
53
54 944 1976). The ^1H FT-NMR spectrum of *cis*-bixin and *cis*-methyl bixin at 250Mhz has
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56 945 been reported but is limited to assignment of the terminal acrylate moieties (Jondiko
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3 946 and Pattenden (1989). Proton NMR at 250MHz was used to confirm the structures of
4
5 947 purified *trans*- and 9'-*cis*- bixin, where the chemical shifts and coupling constants
6
7 948 associated with the change in stereochemistry were consistent with those reported
8
9 949 previously (Barber *et al.*, 1961) but afforded much higher resolution (Scotter *et al.*,
10
11 950 1994). A similar approach was used to confirm the structure of the principal thermal
12
13 951 degradation product of bixin as *trans*-4,8-dimethyltetradeca-hexaenedioic acid
14
15 952 monomethyl ester or C₁₇ (Scotter, 1995). The structure of a minor apocarotenoid
16
17 953 isolated from *Bixa orellana* was confirmed as methyl 9'-Z-apo-6'-lycopenate using
18
19 954 proton NMR at 500 MHz (Mercadante *et al.*, 1996) and a similar approach used to
20
21 955 identify apocarotenoids not previously found in annatto (Mercadante, Steck and
22
23 956 Pfander, 1997b; 1999). NMR (300 MHz ¹H) was used alongside TLC and HPLC in
24
25 957 the bioautographic detection of antimicrobial compounds in water-soluble annatto
26
27 958 extracts where peak assignments were reported to be consistent with previous reports
28
29 959 (Galindo-Cuspinera and Rankin, 2005).
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31 960
32
33 961 The most comprehensive study to date on the determination of the structure of the
34
35 962 bixin family of apocarotenoids is by Kelly *et al.* (1996), who utilised a combination of
36
37 963 1D and 2D NMR techniques in conjunction with mass spectrometry and X-ray
38
39 964 diffraction analysis. Chemical shift, coupling constants and ¹H correlation data were
40
41 965 examined alongside the ion abundances and intensity ratios from standard electron
42
43 966 impact (EI+) and fast atom bombardment (FAB+) MS spectra, and bond
44
45 967 measurement, cell dimension and degree of hydrogen bonding from X-ray diffraction
46
47 968 data to elucidate and compare the crystal structures of the *cis*- and *trans*- isomers of
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49 969 bixin and methyl bixin.
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971 *Other analytical techniques*

972 Notwithstanding where specific techniques have been discussed elsewhere in this
973 review, there are several less widely known techniques that have been used in the
974 study of annatto either alone or in conjunction with complementary techniques. These
975 include infra-red spectroscopy, where the characteristic strong absorption due to the
976 C=O stretching frequency between 1740 and 1700 cm⁻¹ and the complex bands in the
977 1300-1050 cm⁻¹ region due to C-O single bond characteristic of esters and carboxylic
978 acids has been used (Lunde and Zechmeister, 1954; Reith and Gielen, 1971; Chao *et*
979 *al.*, 1991; Bernard and Grosjean, 1995). Photoacoustic spectrometry in the UV, VIS
980 and IR regions has been used for the qualitative and quantitative analysis of annatto in
981 commercial seasoning products (Haas and Vinha, 1995) and more recently in the
982 determination of the triplet state energy of bixin (Rios *et al.*, 2007). X-ray
983 photoelectron spectroscopy was used by Felicissimo *et al.* (2004) to ascertain the
984 major carotenoid composition of *Bixa orellana* seeds and X-ray diffraction in
985 conjunction with NMR and mass spectrometry has been used to determine of the
986 structure of the bixin family of apocarotenoids (Kelly *et al.*, 1996).

987

988 *Analysis of foods*

989 Prior to 1970, there were very few published methods for the extraction of annatto
990 from foods. The qualitative and quantitative analytical aspects of annatto extraction
991 methods published prior to 1976 have been reviewed briefly (Aparnathi and Sharma,
992 1991). These relatively simple methods generally involve extraction with solvent (e.g.
993 chloroform, benzene, petroleum spirit or ether) with or without some form of sample
994 pre-treatment such as protein precipitation, washing and adsorption onto an inert

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3 995 substance. The foodstuffs analysed by these methods largely comprise dairy products,
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5 996 which reflects the relatively narrow scope of annatto usage at that time.
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10 998 Annatto has been extracted from whey solids with dilute ammonium hydroxide where
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12 999 proteins were precipitated by the addition of ethanol and phosphate buffer
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15 1000 (Hammond, Chang and Reinhold, 1973), and from meats (McNeal, 1976). Annatto
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17 1001 may be analysed in milk and ice-cream by precipitation with boiling acetic acid and
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20 1002 extraction of the whey with diethyl ether, and the colour extracted from macaroni and
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22 1003 noodles with 80% ethanol followed by back-extraction into diethyl ether under
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24 1004 alkaline conditions (AOAC, 1980).
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29 1006 Rapid methods for the extraction of annatto from foods have been described where
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31 1007 drinks and syrups were dissolved in water, acidified with acetic acid and annatto was
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34 1008 partitioned into diethyl ether (Corradi and Micheli, (1981). Products with a high fat
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36 1009 content *e.g.* butter and margarine, were dissolved in petroleum spirit and annatto was
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38 1010 partitioned into aqueous ammoniacal ethanol. Three extractions were required for
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40 1011 quantitative extraction of the colour. The aqueous extracts were acidified with acetic
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42 1012 acid and back-extracted with diethyl ether. For foods containing fat and protein *e.g.*
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44 1013 yoghurt, cheese and pastries, samples were ground with sand and aqueous
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46 1014 ammoniacal ethanol. The mixture was transferred to a centrifuge tube and the fat was
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48 1015 removed by agitation with petroleum spirit, centrifugation and siphoning off the
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51 1016 petroleum spirit phase. The aqueous ammoniacal phase was retained, acidified with
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53 1017 acetic acid and the annatto partitioned into diethyl ether.
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3 1019 Methods for the extraction and determination of annatto in margarine, cheese and
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5 1020 boiled sweets have been investigated using techniques similar to those described
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8 1021 previously, with modifications to enable measurement by spectrophotometry and
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10 1022 HPLC (Smith *et al.*, 1983). Margarine samples were saponified to separate fat and to
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12 1023 convert any bixin to norbixin, thereby facilitating its extraction into aqueous media
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14 1024 and subsequent purification. However the reported HPLC conditions gave poor peak
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16 1025 shapes and insufficient resolution. A method for the determination of annatto in
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18 1026 cheese in which a simple acetone extraction was used, followed by concentration by
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20 1027 rotary evaporation has been described (Luf and Brandl, 1988). Spectrophotometric
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22 1028 (derivative) and HPLC techniques were used to quantify annatto in the presence of
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24 1029 other carotenoids, based on the procedure described for the analysis of certain baked
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26 1030 goods. However, the *cis*- and *trans*-isomers of bixin and norbixin were not identified
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28 1031 separately under the stated conditions.
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36 1033 More recently, other workers have developed refined methods for the extraction of
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38 1034 annatto from high-fat foods, dairy products and candy utilising solvent pre-extraction
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40 1035 of fat and extraction of annatto into ethanolic aqueous ammonia (Lancaster and
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42 1036 Lawrence, 1995) and to separate mixtures of bixin and norbixin from carminic acid in
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44 1037 fruit beverages, yoghurt and candies (Lancaster and Lawrence, 1996). HPLC was
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46 1038 used to measure both the *cis*- and *trans*-isomers of bixin and norbixin but no
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48 1039 significant improvements in peak resolution were demonstrated compared to those
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50 1040 reported previously (Smith *et al.*, 1983), and impure reference materials were used for
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52 1041 calibration. Recovery of norbixin from spiked cheese samples was reported to average
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54 1042 93% over the range 1 to 110 mg/kg, and the recovery of bixin from spiked wafers also
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3 1043 averaged 93% over the range 0.1 to 445 mg/kg. The recovery of norbixin from
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5 1044 laboratory-prepared hard candies averaged 88%.
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10 1046 TLC and HPLC were used to determine bixin and other carotenoid colours in products
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12 1047 derived from red pepper (Mínguez-Mosquera, Hornero-Méndez and Garrido-
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14 1048 Fernández, 1995). A simple acetone extraction was used followed by partition with
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16 1049 ether and sodium chloride solution and alkaline saponification. Back extraction with
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18 1050 ether following acidification of the saponifying medium was necessary to recover the
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20 1051 annatto colour (as norbixin). While good chromatographic separation of the
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22 1052 carotenoids was obtained, no distinction between norbixin isomers was made.
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24 1053 However, the method demonstrated the capability of detecting of colours added
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26 1054 fraudulently to intensify the natural colour of paprika paste.
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31 1056 Whilst remaining an uncommon analytical technique in food laboratories,
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33 1057 photoacoustic spectrometry (PAS) has been used for the analysis of annatto products
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35 1058 (Haas and Vinha, 1995). The method is limited to semi quantitative ($\pm 1\%$ 'annatto
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37 1059 content') and qualitative analysis of commercial seasonings comprising mixtures of
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39 1060 corn meal and powdered annatto seeds or annatto extract known as '*Colorifico du*
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41 1061 *Urucum*'. The particle size of the samples has a strong influence on the amplitude of
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43 1062 the PAS signal and therefore requires close control.
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48 1064 Based on the methods described previously (Scotter *et al.*, 1994; Lancaster and
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50 1065 Lawrence, 1995), HPLC and spectrophotometric methods have been developed for
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52 1066 the simple and rapid determination of annatto in cheese and milk products (Bareth,
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54 1067 Strohmar and Kitzelmann, 2002). Solid phase extraction (SPE) on amino phase was

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3 1068 used to separate annatto components from fat and β -carotene. The choice of end
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6 1069 method was determined by the presence of other colouring materials i.e. curcumin or
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8 1070 β -apo-8'-carotenal but other food colours and emulsifiers did not affect the analysis.
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11 1071 The recovery of annatto colouring spiked into cheese, processed cheese, butter and
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13 1072 ice-cream ranged between 80 and 100%. Nine samples of cheese were analysed in
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15 1073 which norbixin was found in the range <0.15 to 11.89 mg.kg^{-1} , whereas no bixin was
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17 1074 detected ($>0.15 \text{ mg.kg}^{-1}$).
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22 1076 The methods described by Scotter *et al.* (1994 and 1998), Lancaster and Lawrence
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24 1077 (1995) and Navaz Diaz and Peinado (1992) were further developed and consolidated
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26 1078 to encompass a wide range of food commodities (Scotter *et al.*, 2002). Specific
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28 1079 solvent extraction regimes were developed for specific sample matrices, with HPLC-
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30 1080 PDA used for spectral confirmation and measurement of the main isomers of bixin
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32 1081 and norbixin. The different extraction regimes are summarized in Table 4.
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36 1082
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39 1083 [Insert Table 4 about here]
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41 1084
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44 1085 With the exception of regime 5, samples were extracted essentially using
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46 1086 ethanol:water:ammonia solution with or without a hexane partition to remove excess
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48 1087 lipid. After centrifugation in the presence of Celite filter aid, the annatto colour was
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50 1088 partitioned into chloroform:acetic acid solution, centrifuged and the solvent removed
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52 1089 using vacuum-assisted rotary evaporation. To minimise analyte losses via oxidation, a
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54 1090 0.1% solution of butylated hydroxyl toluene (BHT) was added. For regime 5 matrices,
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56 1091 samples were mixed with Celite in the presence of dilute hydrochloric acid and
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58 1092 extracted using a biphasic solvent system comprising hexane (to remove excess lipid)
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3 1093 and acetonitrile, which was then concentrated using vacuum-assisted rotary
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5 1094 evaporation.
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10 1096 Using this method, comprehensive quantitative and qualitative data on 165 composite
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12 1097 and 2 single food samples covering a wide range of foods at levels above the
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14 1098 analytical reporting limit of 0.1 mg.kg⁻¹ were obtained. Quantitative results were
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16 1099 given for those annatto colouring components for which reference standards were
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18 1100 available (9'-*cis*-bixin, *trans*-bixin and 9'-*cis*-norbixin), whereas semi-quantitative
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20 1101 results were given for other bixin and norbixin isomers. The method was single-
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22 1102 laboratory validated by the repeat (n = 4 to 9) analysis of 12 different sample types of
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24 1103 food commodity covering the permitted range of annatto content, spiked with annatto
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26 1104 at levels of between 1.7 and 27.7 mg.kg⁻¹ and by the analysis of in-house reference
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28 1105 matrices. Mean recoveries of between 61 and 96% were obtained from foods spiked
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30 1106 with annatto.
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34 1108 Using response surface methodology to establish optimum conditions, a method for
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36 1109 the determination of annatto colour in extruded corn snack products has been
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38 1110 developed that exhibits improved accuracy and precision compared to the method
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40 1111 described by Scotter *et al.* (2002) (Rios and Mercadante, 2004). However, pre-
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42 1112 treatment of the samples with α -amylase was necessary to remove starch and a total
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44 1113 of 8 solvent extractions with ethyl acetate were required for complete extraction of the
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46 1114 annatto colour. Lipids were removed using alkaline saponification therefore all of the
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48 1115 bixin present was hydrolysed to norbixin and determined as such by HPLC.
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3 1117 Accelerated solvent extraction has been compared with manual solvent extraction to
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5 1118 determine several food colouring carotenoids including bixin and norbixin in
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8 1119 processed foods (Breithaupt, 2004). Reverse-phase HPLC with a C₃₀ column
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10 1120 successfully separated bixin and norbixin from 7 other carotenoids but the *cis*- and
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12 1121 *trans*- isomers were not distinguishable. Due to its ostensibly higher polarity, lower
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14 1122 recoveries of norbixin were reported for accelerated extraction ($67 \pm 1.0 \text{ mg.kg}^{-1}$)
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16 1123 compared to manual extraction ($88.7 \pm 6.2 \text{ mg.kg}^{-1}$). However, a similar difference in
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18 1124 recoveries was reported for less polar bixin (91.0 ± 2.7 and $98.0 \pm 1.7 \text{ mg.kg}^{-1}$
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21 1125 respectively) although bixin recovery was higher than norbixin with improved
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23 1126 precision. The limit of quantitation for bixin and norbixin was in the range 0.53 – 0.79
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25 1127 mg.kg^{-1} for pudding mix and cereals. More recently, a method for the determination
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27 1128 of norbixin and bixin in meat products using HPLC-PDA and LC-MSⁿ that gives
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29 1129 recoveries of between 99 and 102% and a limit of quantitation of 0.5 mg/kg. (Noppe
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31 1130 *et al.*, 2009).

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1132 *Annatto as an illegal food dye*

1133 The illegal use of annatto to colour milk goes back as far as the early 20th century
1134 where it was reported by UK Public Analyst laboratories (Richards, 1923;
1135 Collingwood Williams, 1925). Amongst other specific food commodities, annatto is
1136 currently permitted in the EU for the colouring of certain margarines and cheeses but
1137 is not permitted for the colouring of milk cream or butter (EC 1994 as amended).
1138 Moreover, while annatto is permitted for use in food commodities such as savoury
1139 snack products, coated nuts, extruded products and flavoured breakfast cereals, it is
1140 not permitted for use in spices. However, amongst other non-permitted dyes bixin was
1141 detected in 18 of 893 samples of spices, sauces and oils by UK enforcement

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3 1142 laboratories during 2005-2006 as part of the UK Imported Food Programme (Food
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5 1143 Standards Agency, 2006). This has led directly to a need for analytical methods
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7 1144 capable of detecting very low levels of annatto in food ingredients and commodities in
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10 1145 which it is not permitted, driven not only by the enforcement of regulations on a
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12 1146 national scale (disseminated through the EU Rapid Alert System; EU 2008) but also
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14 1147 by the need for the food manufacturing industry to ensure compliance, especially in a
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16 1148 proactive manner and through the adoption of a 'zero tolerance' approach as applied
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18 1149 to the monitoring of illegal dyes such as the Sudan Red group. Established HPLC
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20 1150 methods capable of detecting bixin or norbixin at ca. 0.1 mg/kg in samples using UV-
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22 1151 VIS or diode-array technology are not sufficiently sensitive. LC-MS/MS methodology
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24 1152 is the obvious candidate but sufficiently detailed methods in peer-reviewed
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26 1153 publications have not been forthcoming to date. Nevertheless, it is generally
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28 1154 considered amongst analytical chemists working in this area that LC-MS/MS is
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30 1155 capable of detecting bixin at ca. 0.01 mg/kg in certain commodities, but this is heavily
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32 1156 dependent upon the degree of signal suppression caused by matrix effects. This can
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34 1157 give rise to false negative results using a screening approach, which in turn identifies
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36 1158 a need for suitable extract clean up regimes, and guarding against ion suppression by
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38 1159 using the method of standard addition
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48 1161 *Future aspects*

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50 1162 There is a clear requirement in the future for the development and validation of highly
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52 1163 sensitive methods of analysis for annatto in food commodities and other food
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54 1164 ingredients, driven by the need to ensure compliance with food quality regulations and
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56 1165 especially in the light of the pursuit of suitable alternatives to synthetic food colours.
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59 1166 An in-depth understanding of the chemistry and stability of annatto is therefore
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3 1167 requisite and brings clear benefits to the production of annatto, and to the formulation
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6 1168 and application of food colouring to a wide range of food commodities. Greater
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8 1169 understanding of the processes of degradation may also benefit studies in the areas of
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10 1170 food safety, particularly in risk assessment, and biomarkers of exposure such as
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12 1171 circulating (plasma) levels of norbixin. Here, complementary analytical techniques
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15 1172 such as HPLC-PDA, LC-MS/MS and NMR will play a vital role in the detection,
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17 1173 confirmation and measurement of comparatively low levels of bixin and norbixin
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20 1174 isomers.
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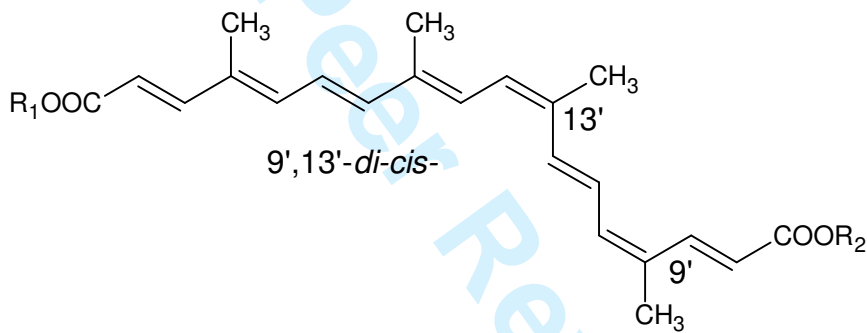
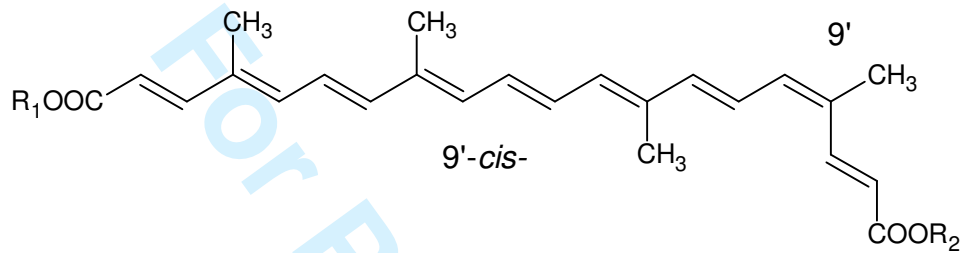
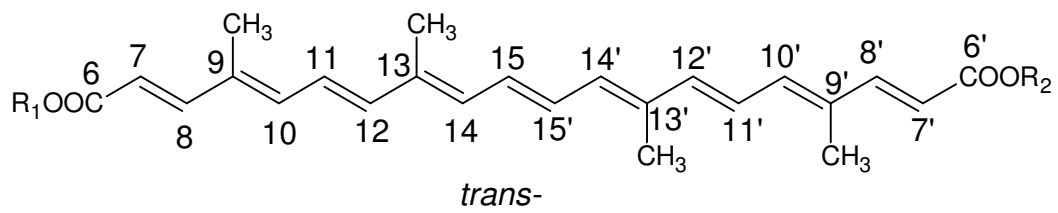
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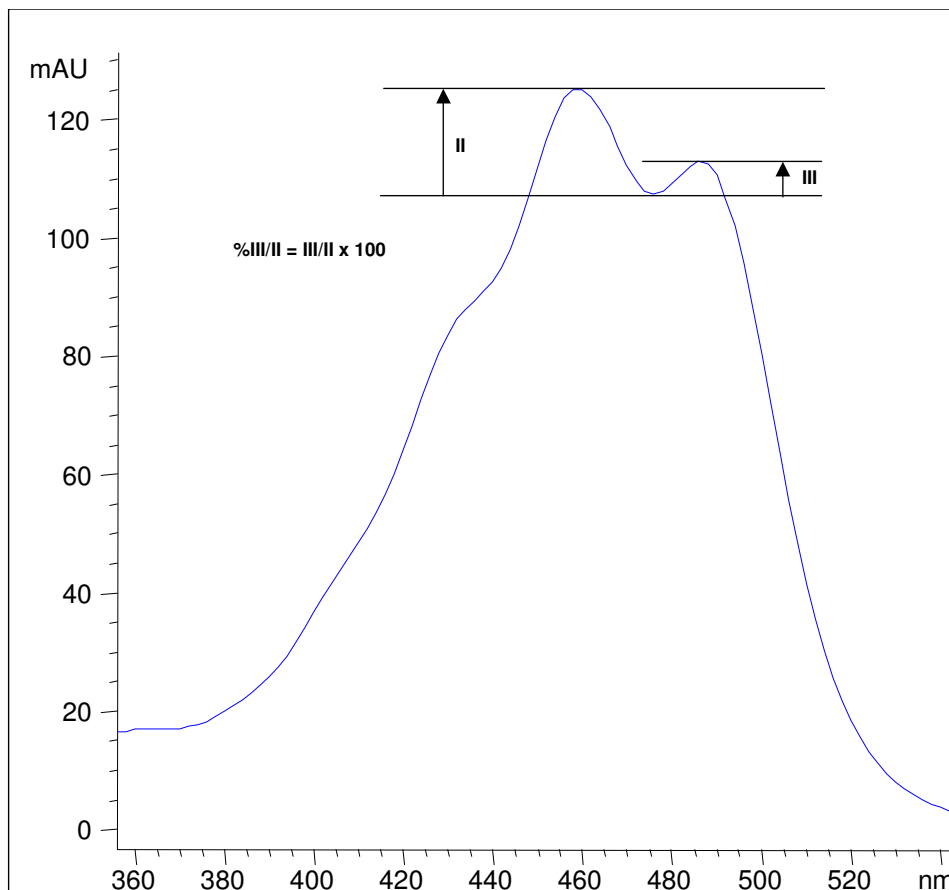
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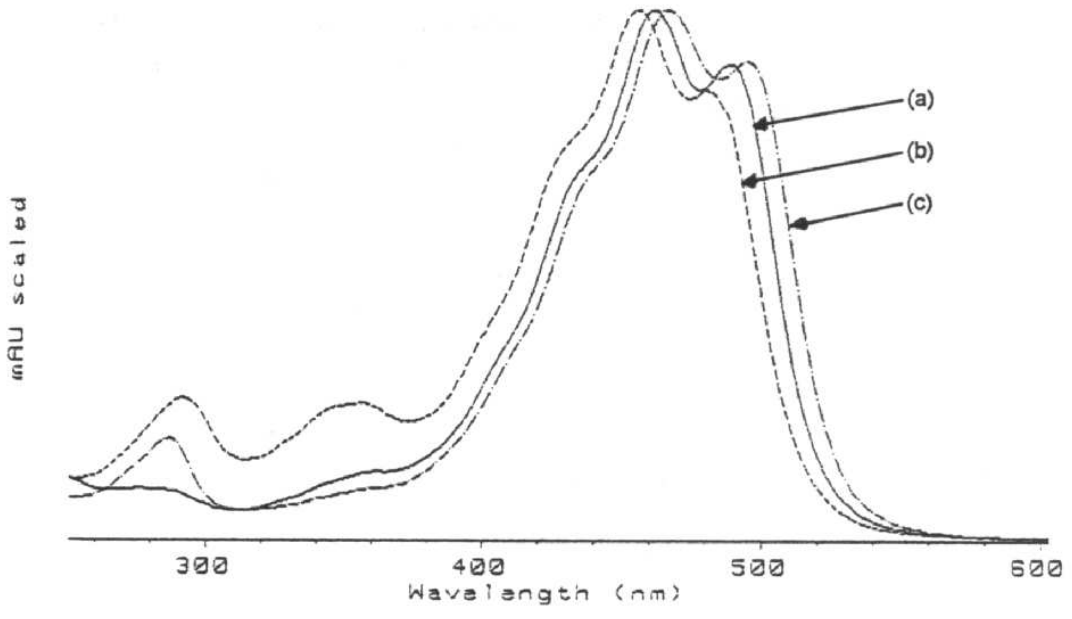
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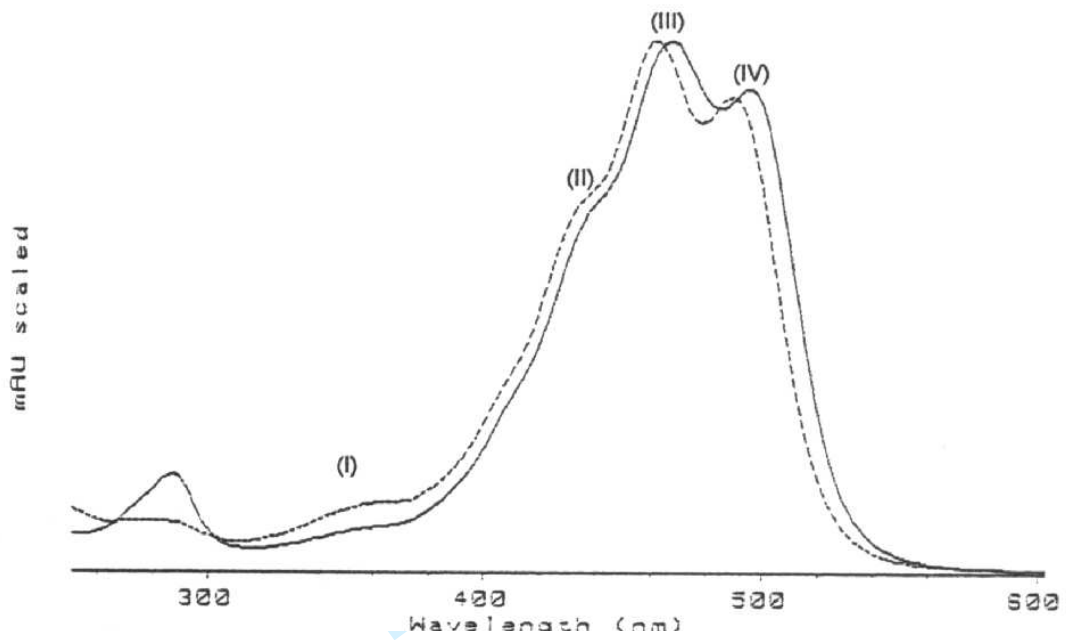


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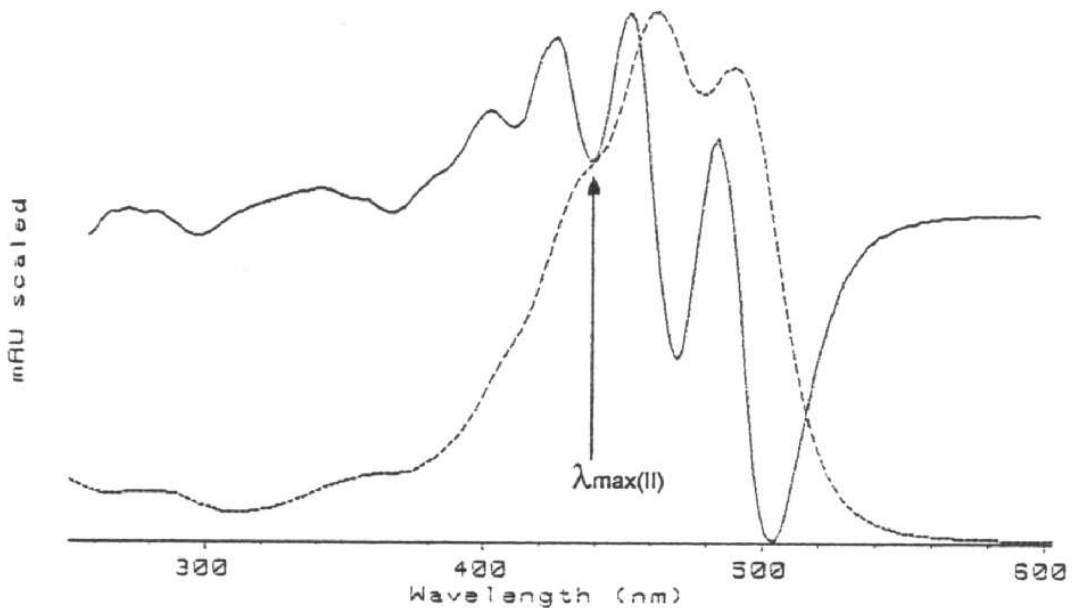


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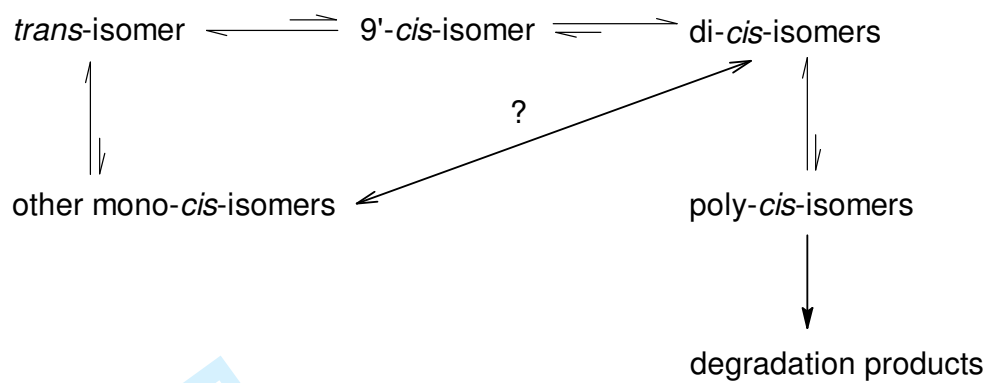


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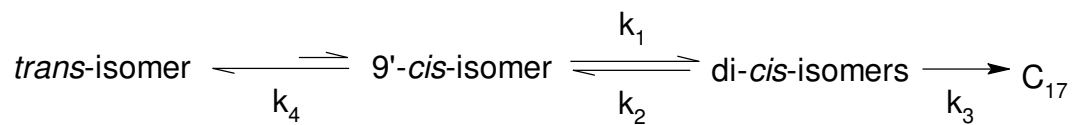
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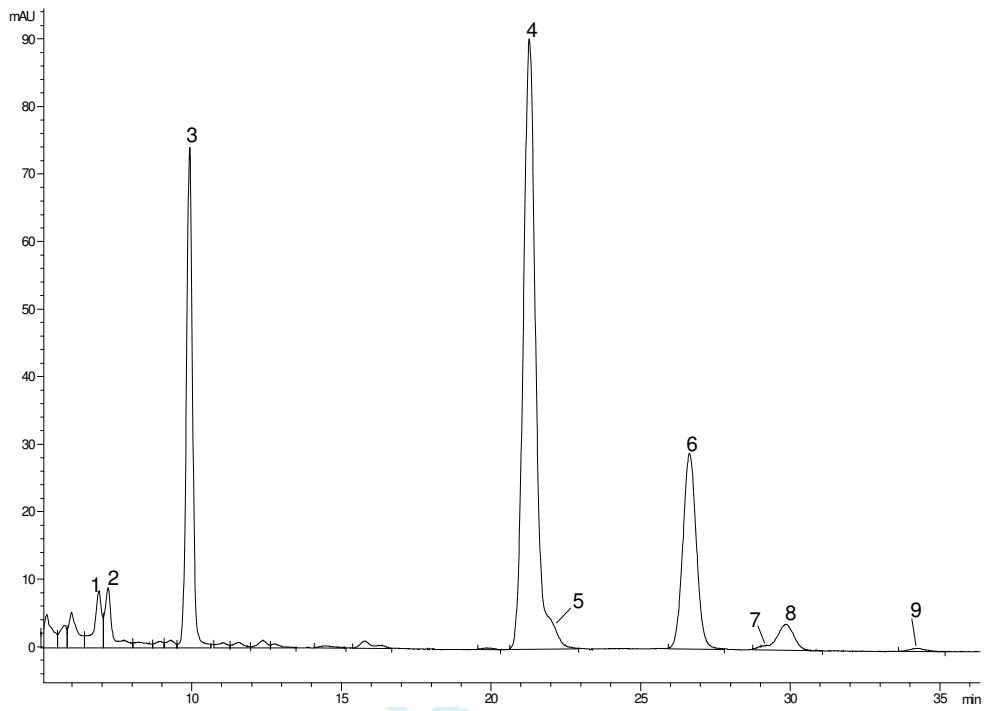
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Legends for figures

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).

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Figure 8. HPLC separation of bixin and norbixin isomers. Conditions: Column HRPB C₈/C₁₈ 250 x 4.6mm, 5µm; Mobile phase Acetonitrile: 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 ₂ O	Tirimanna, 1980
Bixin and norbixin commercial formulations	Silica gel GF	PE:Et ₂ O:HOAc (85:15:2.5)	Preston and Rickard, 1980
Ether extracts of foods	Silica gel	(1) CHCl ₃ :HOAc (9:1) (2) Et ₂ O:IPA (9:1)	Corradi and Micheli, 1981
Annatto seeds	KC18 reverse phase	MeOH:H ₂ O (70:30)	Chao et al., 1991
Bixin and other carotenoids	Silica gel GF	(1) HEX:ACE (10:9) (2) DCM:Et ₂ O (9:1) (3) PE: BZ (1:1) (4) PE	Mínguez-Mosquera, Hornero-Méndez and Garrido-Fernández (1995)
Annatto 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, CHCl₃ = Chloroform; DMF = N,N-Dimethylformamide; EtOAc = Ethyl acetate; EtOH = Ethanol; Et₂O = 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, 5µm	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, 5µm	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	<i>Cis/trans</i> bixin and norbixin	Supelco LC-18 250 x 4.6mm, 5µm	MeOH: 2%HOAc (9:1) isocratic 1 ml.min ⁻¹	UV-VIS 500nm	Lancaster and Lawrence, 1995
Foods	Bixin, norbixin and carminic acid	Supelco LC-18 250 x 4.6mm, 5µm	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, 10µm	ACN: 0.08% CF ₃ CO ₂ H (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, 5µm	ACN: 0.4% HOAc (65:35) isocratic 1 ml.min ⁻¹ 35°C	UV-VIS PDA 455 x 10nm	Scotter et al., 2002
Cheese	<i>Cis/trans</i> bixin and norbixin	ODS2 C ₁₈ 250 x 4mm, 5µm	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, 3µm	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, 5µm	A: MeOH: H ₂ O: TEA (90:10:0.1) B: MTBE: MeOH: H ₂ O: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, 5µm (2) ODS2 C18 150 x 4.6 3µm	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, 3µm	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	<i>Cis/trans</i> norbixin	Beckman C18 250 x 4.6mm, 5µm	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