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Characterisation of the coloured thermal degradation products of bixin from annatto and a revised mechanism for their formation

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The main thermal degradation product of 9'-*cis*-bixin, the principal liposoluble colouring component of annatto food colouring, has been prepared, isolated and purified. Its structure has been confirmed as the *trans*-monomethyl ester of 4,8-dimethyltetradecahexaenedioic acid using HPLC with photodiode array detection, ^{1}H NMR spectroscopy and mass spectrometry. The compound has been shown to exist in different stereoisomeric configurations in solution and to be susceptible to hydrolysis, forming a range of compounds analogous to bixin and norbixin. The results lead to a revised mechanism for the thermal degradation of 9'-*cis*-bixin.

INTRODUCTION

Annatto extracts (E160b) are orange/red natural carotenoid colouring agents obtained from the seeds of the tropical shrub *Bixa orellanna* L. and have widespread use in the food industry for the colouring of many commodities. The major colouring component of annatto is the apo-carotenoid 9'-*cis*-bixin, the monomethyl ester of the dicarboxylic acid 9'-*cis*-norbixin. It is largely insoluble in vegetable oil, which is used to extract it from annatto seeds, but solubility is improved by heating. Commercially, this is achieved by heating a suspension of the seeds in oil to a maximum temperature of 130°C *in vacuo*. Under these conditions, 9'-*cis*-bixin undergoes isomerisation to the more soluble all-*trans*-isomer to produce oil solutions containing approximately 0·2–0·5% of pigment comprising a mixture of all-*trans*-bixin and 9'-*cis*-bixin in variable proportions, dependent upon extraction temperature and time. At these extraction temperatures, 9'-*cis*-bixin also undergoes a series of degradation reactions to produce a range of products coloured pale yellow to orange (Preston & Rickard, 1980). The main thermal degradation product of 9'-*cis*-bixin has been isolated and identified using paper chromatography and UV–Vis spectrophotometry as the yellow coloured 17-carbon polyene 4,8,dimethyltetradecahexaenedioic acid monomethyl ester (Fig. 1) (McKeown, 1963, 1965). The C17 species and other unidentified yellow coloured compounds have been reported to constitute up to 40% of commercial oil-soluble annatto products (Reith & Gielen, 1971). These degradation products are of commercial significance, since by controlling the degree of degradation, the orange/red to yellow colour balance of an annatto formulation can be adjusted. The formation of C17 and related compounds from 9'-*cis*-bixin is reported to be accompanied by the release of *m*-xylene and, to a much lesser extent, toluene, toluic acid and toluic acid methyl ester (McKeown, 1965), all of which are undesirable in preparations intended for food use.

In considering the use of annatto as a colouring agent, it is necessary to study the chemistry of annatto and to develop suitable methods of analysis to

- differentiate between directly and indirectly extracted annatto preparations,
- establish specific purity criteria for annatto preparations,
- determine the annatto content of foodstuffs in support of proposed regulations, and
- study the effects of storage and processing on annatto extracts.

To achieve these aims, reference standards of the main colouring components of annatto have been isolated and purified (Scotter *et al.*, 1994). A suitable reference standard of the main thermal degradation product of 9'-*cis*-bixin is also required, and this report describes the analytical methods and techniques used to prepare, isolate and purify this compound. Characterisation was by HPLC with photodiode array (PDA) detection, ^{1}H NMR spectroscopy and mass spectrometry (MS). A revised mechanism for the thermal degradation of 9'-*cis*-bixin is also postulated.

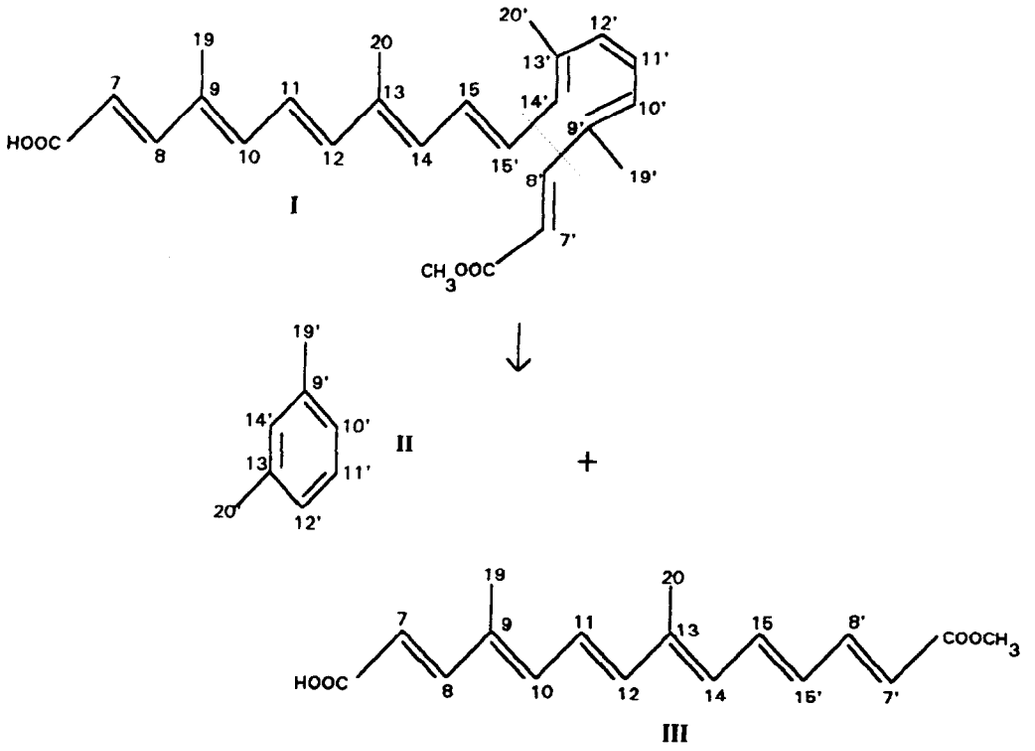

Fig. 1. Thermal degradation scheme for 9'-*cis*-bixin (I) to *m*-xylene (II) and C17 (III) according to McKeown (1963, 1965). IUPAC carotenoid nomenclature used throughout (though not used by McKeown).

MATERIALS AND METHODS

Materials

Cis-bixin was obtained from Chr. Hansen's Laboratory Ltd, Reading, UK. It was purified by recrystallising three times from acetone and dried in air. HPLC grade solvents acetone, chloroform, diethyl ether, ethyl acetate and methanol were obtained from Rathburn's, Walkerburn, UK. Far-UV grade acetonitrile was obtained from Fisons, Loughborough, UK. HiperSolv grade acetic acid was obtained from BDH, Dagenham, UK. Water was purified through a Milli-Q plus system (Millipore, Watford, UK). All other reagents were of a recognised analytical grade.

Preparation of C17

The monomethyl ester of 4,8-dimethyltetradecahexaenedioic acid (C17) was prepared according to the method described by McKeown (1963) with modifications. Three grams of 9'-*cis*-bixin was dissolved in 500 ml of *n*-butyl acetate and boiled under reflux for 16 h. The hot solution was filtered under vacuum. A small amount (*c.* 0·06 g) of an unidentified yellow/brown amorphous solid was obtained and retained for future investigation. The solvent was removed from the filtrate by rotary evaporation (80°C, 30 mbar) until a resinous residue persisted. The residue was dissolved in boiling glacial acetic acid (30 ml) and allowed to cool. The granular solid that precipitated out over 48 h was filtered off under vacuum, recrystallised three times from boiling methanol (250ml) and dried in a desiccator. Working up of the mother liquor filtrates afforded a total yield of 0·9 g. The product comprised well-defined small orange/red needles with a melting point of 209–212°C (cf. 213–215°C; McKeown, 1965).

Hydrolysis of C17

Approximately 0·01 g of C17 was dissolved in 50 ml of methanolic potassium hydroxide solution (10% w/v) and refluxed for 30 min. The mixture was allowed to cool and was diluted with an equal volume of water. A few millilitres of concentrated hydrochloric acid were added until the solution was acidic. The mixture was transferred to a separating funnel containing 100 ml of diethyl-ether: ethyl-acetate (1 : 1). The hydrolysis product of C17 was extracted into the organic phase. After separation of the phases, the organic layer was washed twice with water (50 ml), dried by passage through anhydrous sodium sulphate (*c.* 100 g), rinsed with ethyl acetate (50 ml) and the solvent removed by rotary evaporation (40°C, 30 mbar). The residue was dissolved in 50 ml of methanol containing a few drops of 10% (w/v) methanolic potassium hydroxide and retained for HPLC analysis.

Isomerisation of C17

Approximately 0·05 g of C17 was dissolved in boiling methanol (40 ml) containing 0·003 g of iodine and refluxed for 30 min. The solvent and iodine were removed by rotary evaporation (40°C, 30 mbar), the residue dissolved in 50 ml of methanol and retained for HPLC analysis.

Apparatus

UV–Vis spectrophotometry

UV–Vis spectrophotometry was carried out on a Perkin–Elmer Lambda 3 scanning spectrophotometer (Perkin–Elmer Ltd, Beaconsfield, UK). Sample solutions were measured in 1-cm matched quartz cuvettes. C17 (0·0019 g) was dissolved in chloroform to give a solution strength of $1{\cdot}9 \times 10^{-3}$ % (w/v). Two millitres of this solution was diluted to 10 ml to give a working solution of $3{\cdot}8 \times 10^{-4}$ % (w/v) C17. The absorbance and UV–Vis spectrum (300–500 nm) of the working solution were determined against a chloroform reference and the extinction coefficient calculated ($E^{1\%,\,1\,cm}$).

^{1}H NMR spectroscopy

^{1}H NMR spectra were obtained on a Bruker FT-250 operating at 250 MHz (Bruker Spectrospin Ltd, Coventry, UK). C17 was dissolved in deuterochloroform containing tetramethylsilane (0·03%) as a reference (Sigma Chemical Co. Ltd, Poole, UK).

Mass spectrometry

Solid-probe electron ionisation (EI) analyses were carried out on a VG 7070EQ mass spectrometer (Fisons Instruments, Altrincham, UK). The temperature programme was 40–350°C at 50°C/min with a data acquisition range of 600–35 daltons. The source was operated at a temperature of 200°C with 70 eV electron energy and 200 μA trap current.

Thermospray MS was carried out by direct injection into solvent flow. The solvent was acetonitrile: 0·1 M ammonium acetate pH 6 (65:35 v/v) delivered at 1 ml/min by an LKB 2150 pump (LKB Biochrom Ltd, Cambridge, UK). The solvent flow was coupled directly to a VG 12-250 quadropole mass spectrometer (Fisons Instruments, Altrincham, UK) via a resistively heated, crimped tip thermospray capillary interface. The capillary temperature was maintained at 200°C and the source temperature at 230°C. No discharge pin voltage was applied.

HPLC analysis

HPLC analysis was carried out using a Hewlett-Packard 1090M series II DR5 ternary pumping system with integral variable volume autosampler, column oven and model 1040 series II PDA detector with HP Pascal workstation (Hewlett Packard, Bracknell, UK). The column used was a 250 × 4·6 mm RPB 5 μm (Hichrom Ltd, Reading, UK) and the mobile phase consisted of 65:35 acetonitrile:aqueous acetic acid (0·4% v/v) delivered at 1 ml/min. The column was maintained at a temperature of 35°C. Detection was carried out at 435 nm with 60 nm bandwidth. All samples were filtered through a 0·2-μm membrane filter (Anotop 10, Whatman Scientific Ltd, Maidstone, UK) prior to analysis.

RESULTS AND DISCUSSION

UV–Vis spectrophotometry

The spectrum obtained for C17 in chloroform was consistent with that reported for C17 dimethyl ester (McKeown, 1963). Well-defined absorption maxima were found at 403 nm (max), 426 nm and 383 nm (shoulder). The $E^{1\%,\,1\,cm}$ extinction coefficients at 403 and 426 nm were calculated to be 2926 and 2734, respectively. No literature values are available for C17 extinction coefficients, but the values obtained are comparable to those reported for bixin in chloroform at 470 nm (3092) and 501 nm (2773) (Scotter *et al.*, 1994). There was no immediate detectable change in the absorption spectrum upon treatment of the chloroform solution of C17 with 1 drop of a dilute solution of iodine in chloroform.

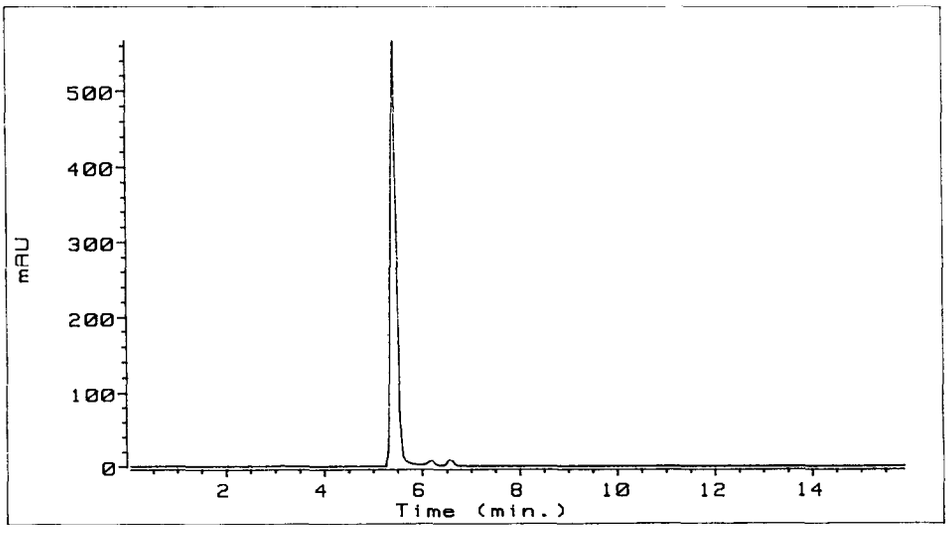

Fig. 2. Chromatogram obtained from a freshly prepared 40-mg/litre solution of C17 in HPLC mobile phase. Column, 5 μm RPB; mobile phase, acetonitrile:aqueous acetic acid (0·4%) (65:35); flow rate, 1·0 ml/min; detection, 435 nm with 60 nm bandwidth; injection volume, 25 μl.

^{1}H NMR spectroscopy

The spectrum obtained for a solution of C17 in deuterochloroform gave characteristic absorption bands at δ = 2·0 (doublet) due to olefinic CH_3- and at δ = 3·8 (singlet) due to -OCH_3. These are comparable to those obtained for bixin (Barber *et al.*, 1961). The fine structure of the multiplet bands absorbing between δ = 5·9–7·5 due to olefinic (vinylic) hydrogens were consistent with those obtained for *trans*-bixin. The signal due to the α-protons C17 (Hα) took the form of an overlapping doublet of doublets centred at δ = 5·9 with aa associated coupling constant of 14·1 Hz. The signal due to the β-protons (Hβ) was centred at δ = 7·3 and took the form of a pseudo-triplet of comparable intensity to the Hα signal and with an identical coupling constant of 14·1 Hz. However, unlike the Hβ signal obtained for *trans*-bixin, the Hβ signal for C17 was overlapped by a weaker triplet of unknown origin. This evidence suggests that the C17 prepared in this study is the all-*trans*-isomer and is relatively pure.

Mass spectrometry

The mass spectrum obtained for C17 using solid probe EI gave the expected molecular weight of 288. The spectrum also exhibited major ions characteristic of carotenoid fragmentation at $[M-106]^{+\cdot}$, $[106]^{+\cdot}$ (xylene), $[105]^+$ (methyltropylium) and $[91]^+$ (tropylium) (Enzell & Wahlberg, 1980). The thermospray spectrum of C17 exhibited the $[M + H]^+$ ion as the base peak, with other features such as sodium adducts at *m/z* 311 $[M + Na]^+$ and *m/z* 333 $[M + 2\ Na]^+$. No obvious attribution could be made to the lower mass fragments observed in the spectrum but there was evidence of the presence of bixin contamination at *m/z* 395 $[M + H]^+$.

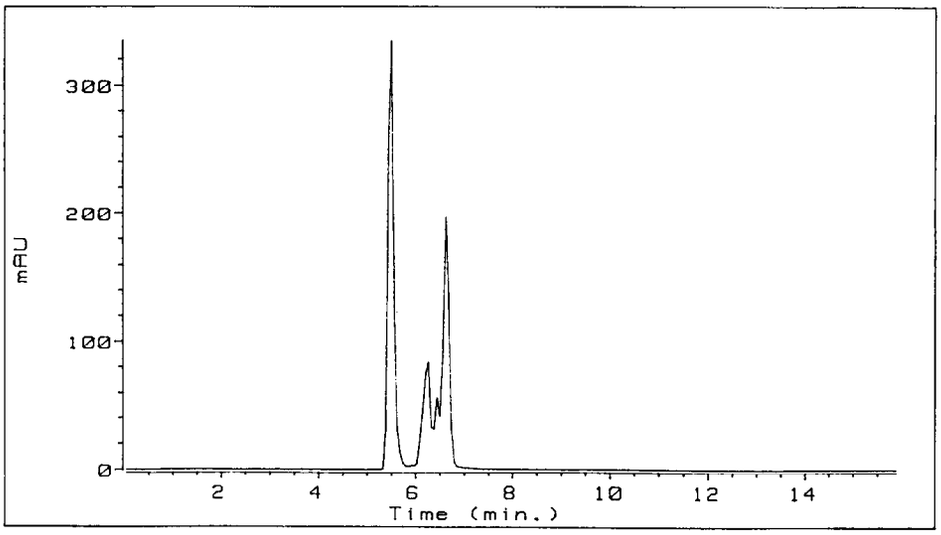

Fig. 3. Chromatogram obtained from a 40-mg/litre solution of C17 stored for several months at ambient temperature in HPLC mobile phase. Conditions as for Fig. 2.

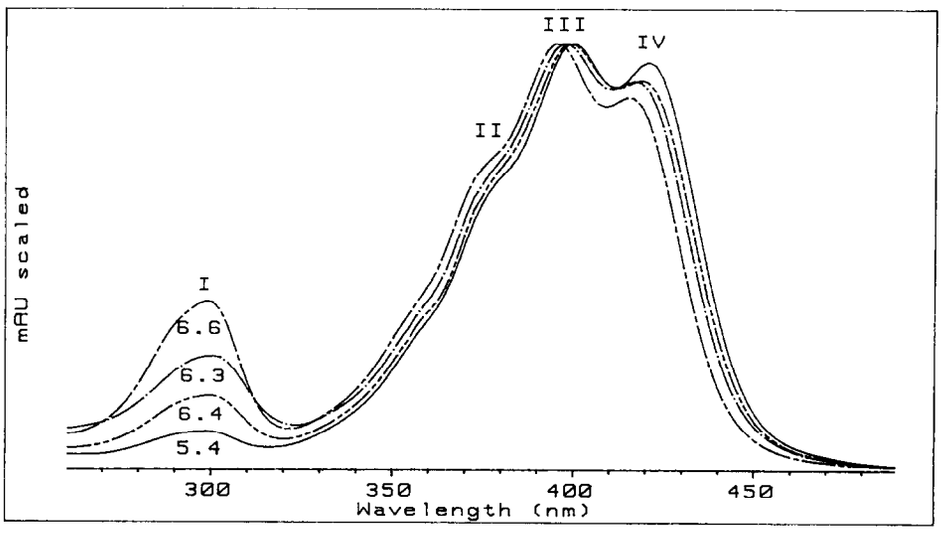

Fig. 4. HPLC–PDA spectra of C17 isomer peaks obtained from the chromatogram in Fig. 2. Peak retention times (min) and absorbance maxima I–IV are indicated.

Table 1. HPLC-PDA spectral data of C17 isomer peaks[a]

Retention time (min)	λ_{max} (nm)	$\Delta\lambda_{max}$ (nm)	REL I (%)	REL II (%)	REL (%)	IVIsomer
5·4	400	0	8	69	96	*trans*
6·3	396	4	27	76	91	9-*cis*
6·4	398	2	20	74	92	15-*cis*
6·6	394	6	45	78	88	13-*cis*

[a]Structural characterisation of *cis*-isomers is tentative. REL: relative absorption intensity of the three maxima I, II and IV, expressed as a percentage of the absorption at λ_{max} III.

HPLC–PDA

The chromatogram obtained from the HPLC analysis of a freshly prepared standard solution of C17 at 40 mg/l is shown in Fig. 2. From peak area measurements at 435 nm with 60 nm bandwidth, the isomeric purity of the main (all-*trans*-isomer) peak eluting at 5·4 min was 95·4%. Several smaller peaks were found to elute soon after the *trans*-isomer and were attributed to possible *cis*-isomers of C17. Figure 3 shows the chromatogram obtained from the HPLC analysis of a 40-mg/l standard C17 solution that had been stored for several months in HPLC mobile phase in a clear glass container in the laboratory. It was apparent that in the stored solution, *trans–cis*-isomerisation had taken place and proceeded towards an equilibrium state. This was manifested in a relative increase in concentration of the C17 *cis*-isomers with an associated decrease in concentration of the *trans*-isomer in the stored solution compared to the freshly prepared solution of C17. These findings were supported by data obtained from spectral analysis of the chromatographic peaks using the PDA detector (Fig. 4). The spectra are characterised by their absorbance maxima I–IV. Table 1 lists comparative spectral data obtained for each of the peaks. It should be emphasised that, whilst the spectral data support the structural assignments given for C17 *cis*-isomers, they are not conclusive.

The spectrum obtained for the main peak eluting at 5·4 min is consistent with that obtained for C17 using conventional wavelength scanning spectrophotometry. This spectrum exhibited the greatest λ_{max} (III) and lowest '*cis*-peak' (I) absorption intensity (at 300 nm) of the series. Analogous studies on the elution pattern of bixin and norbixin isomers under similar conditions

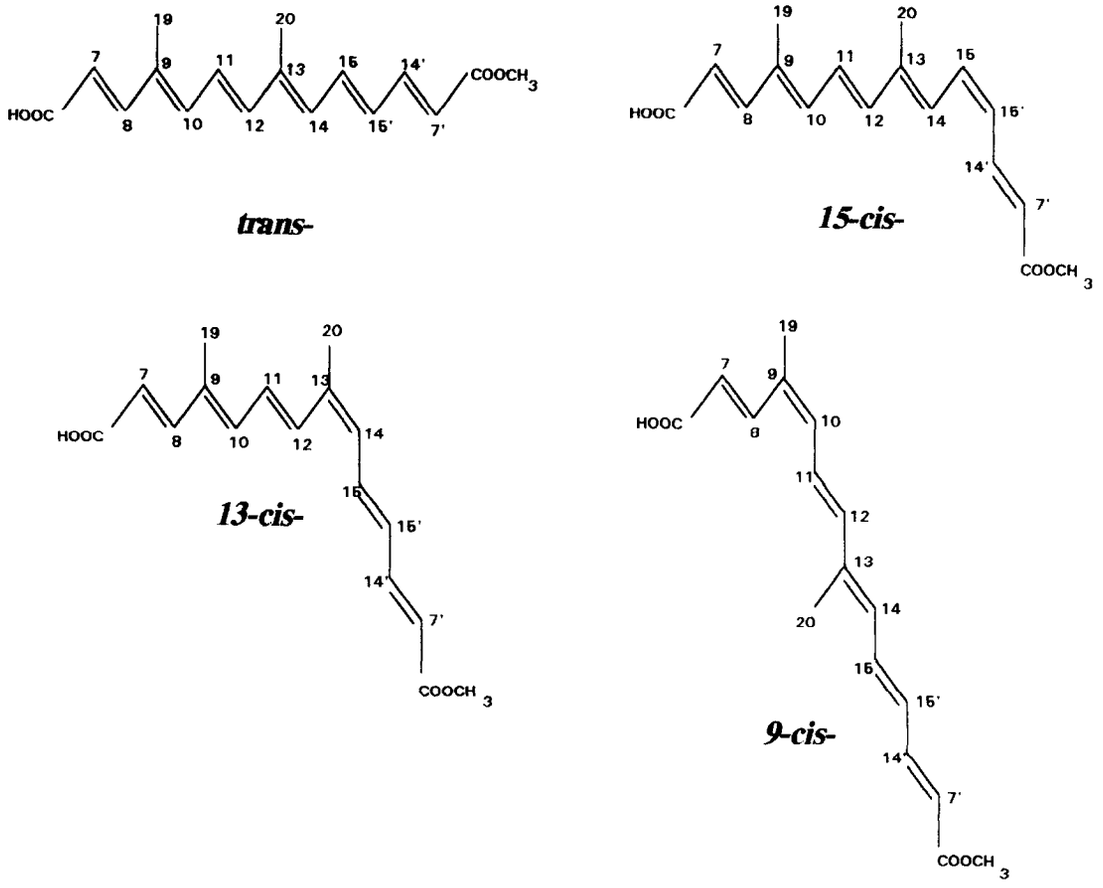

Fig. 5. Stereochemical structures of unhindered C17 isomers.

showed that it was the *trans*-isomer of a given series which generally eluted first (Scotter *et al.*, 1994).

Inspection of the $\Delta\lambda_{max}$ values in Table 1 shows that, for each of the *cis*-isomer peak spectra, the wavelength of λ_{max} absorption is, as expected, lowered from that of the all-*trans*-isomer. Assuming that C17 isomers follow the empirical rules for UV–Vis absorption of polyenes of carotenoid dimension, tentative identification of the various *cis*-isomers of C17 may be given. Thus, whilst the $\Delta\lambda_{max}$ value observed for the isomer eluting at 6·4 min is noticeably small at 2 nm, the corresponding values observed for the *cis*-isomers eluting at 6·3 and 6·6 min are consistent with mono *cis* configurations (Scotter *et al.*, 1994).

In studies on the spectral characterisation of β-carotene isomers, Pettersson and Jonsson (1990) have shown that the absorption intensity at λ_{max} III can be normalised and the relative absorption intensity (REL) of the three other maxima I, II and IV may be calculated to give REL(I), REL(II) and REL(IV) values, respectively. These values may be expressed as a percentage of the absorption at λ_{max} III (Table 1). According to the Pauling rules for steric hindrance in isoprenoid systems such as carotenoids (Zechmeister, 1960), only carbon double bonds 9, 13 and 15 in C17 are unhindered. Whilst from these rules it is predicted that three di-*cis*-isomers and a tri-*cis*-isomer may occur in the equilibrium mixture as a result of spontaneous or catalytic isomerisation, only small amounts are likely to be present, especially in the case of the tri-*cis*-isomer where energy requirements predict a very low probability of formation. Thus, the all-*trans*-isomers and mono-*cis*-isomers of C17 are likely to predominate in an equilibrium mixture. Since only four isomers were observed in measurable amounts in the chromatogram of isomerised C17 (Fig. 3), these have been designated as the all-*trans*-, 9-*cis*-, 13-*cis*- and 15-*cis*-isomers of C17. The stereochemical structures of the all-*trans*-isomers and unhindered mono-*cis*-isomers of C17 are given in Fig. 5. Preliminary studies using HPLC with gradient elution have revealed the presence of very small amounts of other *cis*-isomers of C17 (data not reported).

HPLC–PDA analysis of the alkaline hydrolysis product of C17 showed, as expected, a principal *trans*-iso-

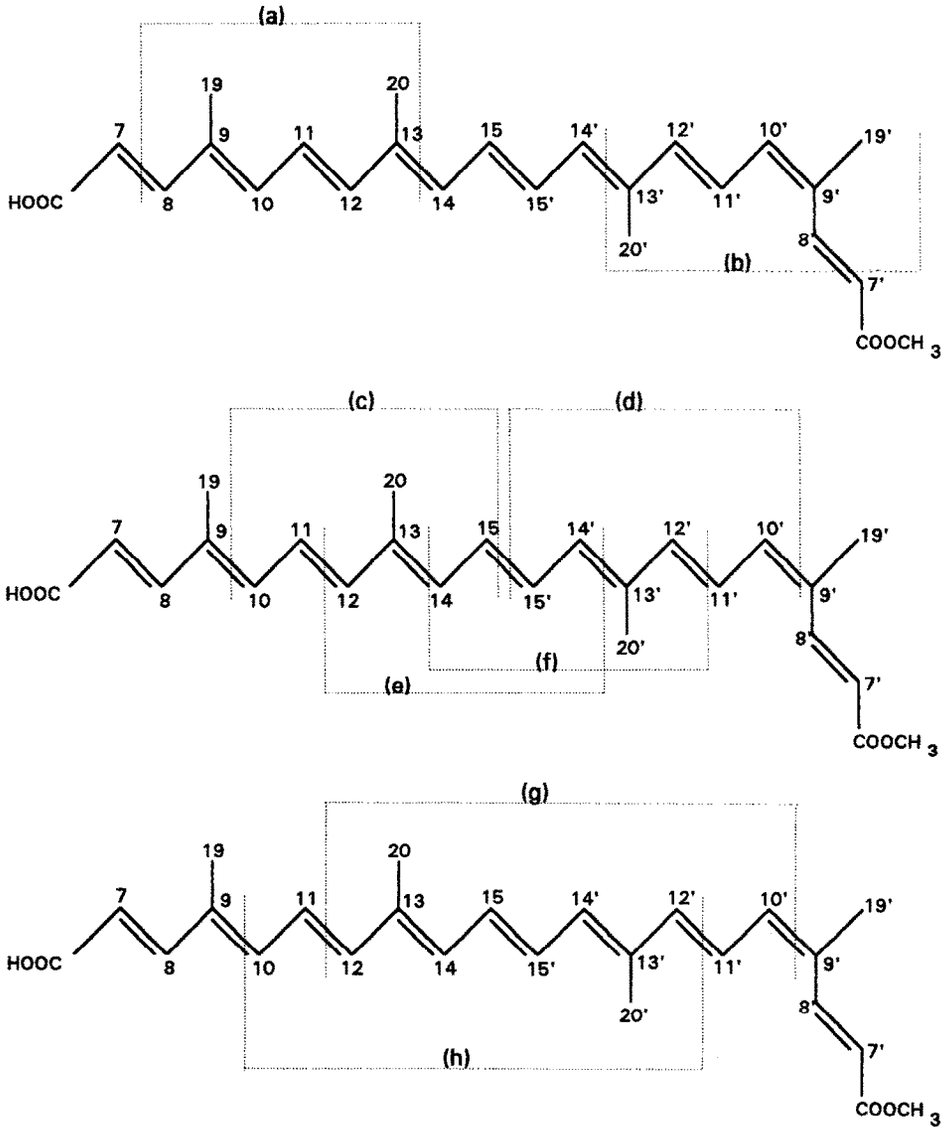

Fig. 6. Sites of elimination of in-chain units from 9'-*cis*-bixin; (a)–(b) *m*-xylene, (c)–(f) toluene and (g)–(h) dimethyldihydronaphthalene.

mer peak followed by a series of much smaller *cis*-isomer peaks similar in elution profile and relative magnitude to the C17 isomers eluting between 3 and 4 min. This demonstrated the formation of a series of 'free-acid' C17 isomers analogous to norbixin possessing greater hydrophilic character than the parent C17 compound.

HPLC–PDA analysis of the iodine-catalysed isomerisation product of C17 gave a similar series of peaks as the C17 solution which had isomerised on storage in HPLC mobile phase, but with approximately half the proportion of the 13-*cis*-isomer.

Mechanism for 9'-*cis*-bixin degradation

This paper has so far discussed only the thermal degradation of 9'-*cis*-bixin in terms of the formation of C17 isomers. It has been shown that during degradation not only is *m*-xylene formed (Hasselt, 1911) but traces of toluene and toluic acid also (Kuhn & Winterstein, 1932). It has been postulated (McKeown, 1963) that, since the terminal carboxyl groups in bixin (and methyl bixin) were found intact in the polyene degradation products, the degradation reaction must involve elimination from within the polyene chain and that the *cis* configuration of bixin is prerequisite. Moreover, all-*trans*-bixin was reported to be relatively stable under the conditions of the reaction and unlikely to isomerise prior to elimination, especially since C17 was reported to be formed rapidly during the initial stages of the reaction.

EI–MS has been used extensively to study the elimination of in-chain fragments from carotenoids, notably the formation of the ubiquitous $[M-92]^{+\cdot}$ and $[M-106]^{+\cdot}$ species and the often-occurring but less abundant $[M-158]^{+\cdot}$ ions (Enzell & Wahlberg, 1980). Investigations

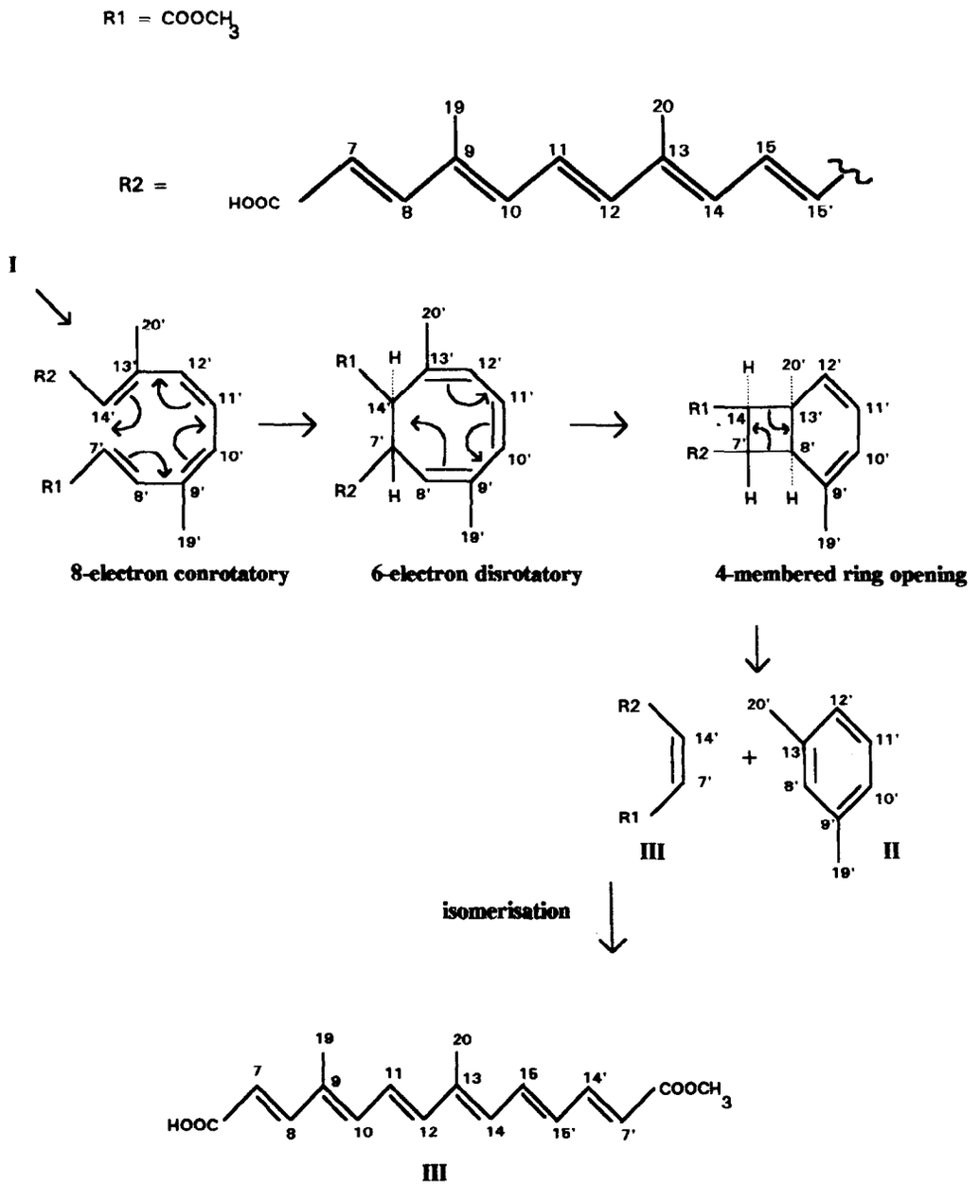

Fig. 7. Revised mechanism for the elimination of *m*-xylene (II) and the formation of C17 (III) from 9'-*cis*-bixin (I) via scheme (b).

employing labelled compounds have shown that the elimination of toluene (92), xylene (106) and dimethyldihydronaphthalene (158) are limited to certain parts of the polyene chain (Fig. 6). These and other studies have also demonstrated that the in-chain reactions occur without hydrogen shifts and thus involve scission of double bonds only (Budzikiewicz, 1974). The mechanism for the elimination processes, if stereochemical considerations are disregarded, has been reported to be not only applicable to EI-induced reactions but to thermally induced reactions as well, since the extent to which isomerisation of the carotenoid molecule and thermal elimination of in-chain units precede ionisation is unknown (Vetter *et al.* 1971). The mechanism for *m*-xylene elimination, as applied to 9'-*cis*-bixin, consists of three steps: an eight-electron conrotatory, a six-electron disrotatory electrocyclic reaction and opening of the four-membered ring (Fig. 7). It is apparent from Fig. 7 that a multi-*cis* configuration is prerequisite to the chain folding and elimination steps, facilitated by the initial 9'-*cis* configuration but is severely hindered by the thermally stable all-*trans* configuration. Thermodynamic considerations indicate that those elimination schemes which involve scission of the 9'-*cis* bond are most likely to predominate (McKeown, 1965), i.e. schemes (b), (d) and (g) in Fig. 6. Elimination of toluene (scheme (d)) and dimethyldihydronaphthalene (scheme (g)) would produce analogous 18-carbon and 13-carbon products, respectively (Fig. 8). Since it differs only by the presence of a methyl substituent, the 18-carbon product would be expected to exhibit very similar UV–Vis absorbance spectra to C17. However, it should be possible to differentiate between the two species using HPLC–MS. The 13-carbon product possesses only four conjugated olefinic bonds and would therefore be expected to exhibit absorption maxima at lower wavelengths than C17. The degradation of 9'-*cis*-bixin via scheme (b) shows the initial stereo-specific formation of the 13'-*cis*-isomer of C17. Since only the all-*trans*-isomer was isolated from the preparation mixture, facile isomerisation from the relatively unstable 13'-*cis*-isomer to the all-*trans*-isomer must have occurred during the purification process. Also, recrystallisation of the C17 from methanol could have reduced the content of any other *cis*-isomers to a very low level.

The possibility of further elimination of both *m*-xylene and toluene from C17 has been confirmed from the results of degradation experiments carried out on methyl and ethyl esters of C17 (McKeown, 1965), though the yields of the aromatic species were far lower compared to those from 9'-*cis*-bixin. Small amounts of the corresponding toluic acid esters were also recovered; however, the corresponding mechanisms of formation of these compounds is unclear.

In conclusion, the principal thermal degradation product of 9'-*cis*-bixin has been prepared, isolated and identified as the all-*trans* monomethyl ester of 4,8-dimethyltetradecahexaenedioic acid (C17). This compound has been shown to isomerise in solution to form

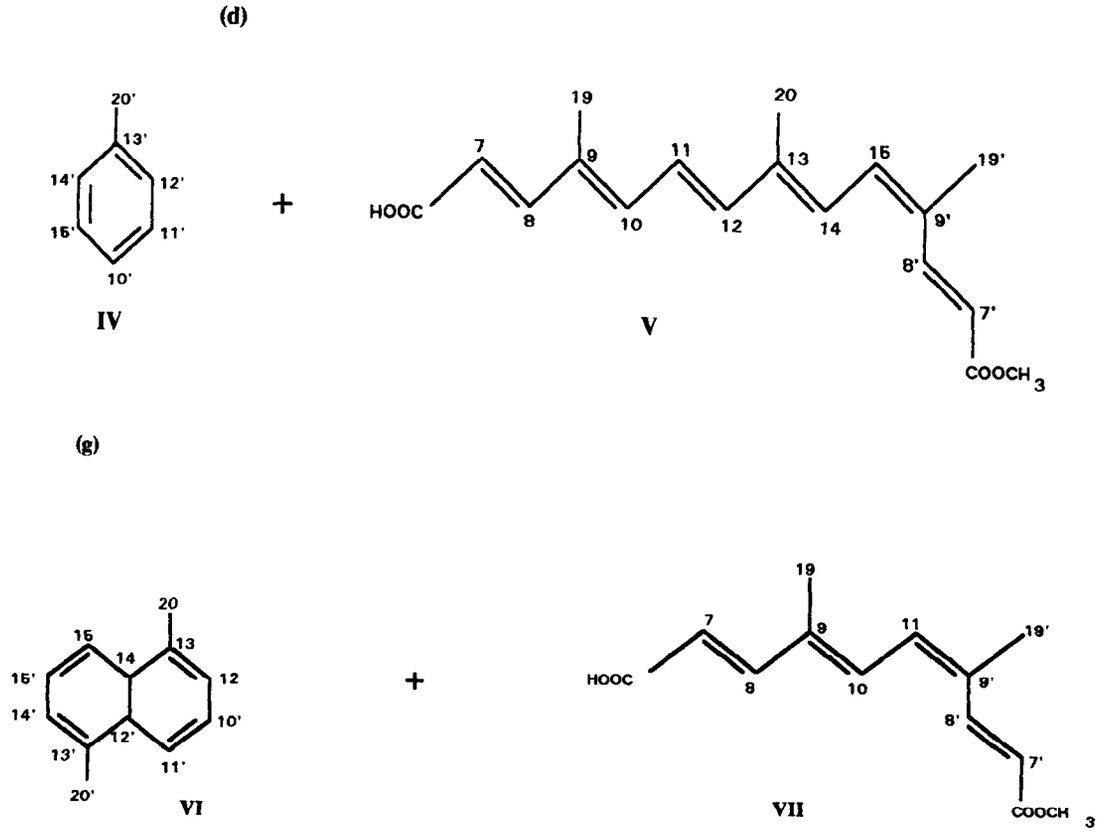

Fig. 8. Products from the degradation of 9'-*cis*-bixin via elimination schemes (d) and (g) in Fig. 6; (d) toluene (IV) and 18-carbon polyene (V); (g) dimethyldihydronaphthalene (VI) and 13-carbon tetraene (VII).

small amounts of *cis*-isomers and to be susceptible to hydrolysis, thus forming a range of compounds analogous to bixin and norbixin in terms of their chemical structures and chromatographic properties. The principal mechanism for the thermal degradation of 9'-*cis*-bixin has been postulated as a concerted electrocyclic process followed by the elimination of *m*-xylene and the formation of C17, which can degrade further by a similar mechanism. The results of this study will allow further research into the thermal stability of bixin and norbixin in model food systems and foodstuffs to be carried out.

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REFERENCES

Barber, M. S., Hardisson, A., Jackman, L. M. & Weedon, B. C. L. (1961). Studies in nuclear magnetic resonance. Part IV. Stereochemistry of the bixins. *J. Chem. Soc.*, 1625–30.

Budzikiewicz, H. (1974). Mass spectra of carotenoids — labelling studies. *Adv. Mass Spectrom.*, **6**, 155–65.

Enzell, C. R. & Wahlberg, I. (1980). Carotenoids. In *Biochemical Applications of Mass Spectrometry — First Supplementary Volume*, eds G. R. Waller & O. C. Dermer. J. Wiley & Sons, Chichester, UK, pp. 407–38.

Hasselt, J. F. B. van (1911). Etudes sur la constitution de la Bixine. *Rec. Trav. Chim. (Pays Bas)*, **30**, 1–47.

McKeown, G. G. (1963). Composition of oil-soluble annatto food colours. II. Thermal degradation of bixin. *J. AOAC*, **46**(5), 790–6.

Kuhn, R. & Winterstein, A. (1932). Thermischer Abbau der carotin-Farbstoffe (Über konjugierte Doppelbindungen, XXV. Mitteil), *Chemische Berichte.*, **65B**, 1873–80.

McKeown, G. G. (1965). Composition of oil-soluble annatto food colours. III. Structure of the yellow pigment formed by the thermal degradation of bixin. *J. AOAC*, **48**(4), 835–7.

Pettersson, A. & Jonsson, L. (1990). Separation of *cis–trans* isomers of α- and β-carotene by adsorption HPLC and identification with diode array detection. *J. Micronutr. Anal.*, **8**, 23–41.

Preston, H. D. & Rickard, M. D. (1980). Extraction and chemistry of annatto. *Food Chem.*, **5**, 47–56.

Reith, J. F. & Gielen, J. W. (1971). Properties of bixin and norbixin and the composition of annatto extracts. *J. Food Sci.*, **36**, 861–4.

Scotter, M. J., Thorpe, S. A., Reynolds, S. L., Wilson, L. A. & Strutt, P. R. (1994). Characterisation of annatto food colouring components using high performance liquid chromatography with photodiode-array detection. *Food Addit. Contam.*, **11**(3), 301–15.

Vetter, W., Englert, G., Rigassi, N. & Schwieter, U. (1971). In *Carotenoids*, ed. O. Isler. Birkhäuser, Basel, Switzerland, pp. 189–266.

Zechmeister, L. (1960). *Cis–trans* isomeric carotenoid pigments. *Progress in the Chemistry of Natural Products* (Vol. 18), ed. L. Zechmeister. Springer-Verlag, Vienna, pp. 223–39.