Analysis of Annatto (*Bixa orellana*) Food Coloring Formulations. 1. Determination of Coloring Components and Colored Thermal Degradation Products by High-Performance Liquid Chromatography with Photodiode Array Detection

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Twenty-one samples of commercial annatto formulations have been analyzed for color content (as bixin or norbixin) using a developed HPLC−PDA method and the results compared with those obtained using UV−vis spectrophotometric methods. HPLC−PDA provided superior qualitative and quantitative data, particularly with respect to the determination of colored degradation compounds. Two samples of norbixin of known production history were subjected to detailed HPLC analysis to identify possible differences in their colored and degradation component profiles. The samples differed significantly in their *all-trans*- and di-*cis*-norbixin isomer contents, which was indicative of their respective production histories.

Keywords: *Annatto; Bixa orellana; bixin; norbixin; coloring materials; additives; HPLC; photodiode array; spectrophotometry*

INTRODUCTION

Annatto extracts (E160b) obtained from the seeds of the tropical shrub *Bixa orellana* L. have widespread use in the food industry for the coloring of many commodities. The principal coloring component of annatto seeds is the liposoluble diapocarotenoid 9′-*cis*-bixin, which is the monomethyl ester of the water-soluble 9′-*cis*-norbixin (Figure 1). Annatto extracts are usually formulated to provide suitably stabilized forms in the color range red−orange−yellow for use in a wide range of foods such as dairy products, flour confectionery, fish, soft drinks, meat products, snack foods, and dry mixes. Annatto formulations are generally available as oil- or water-soluble solutions, suspensions, emulsions, encapsulated products, and spray-dried powders (with carriers). As with all other carotenoids, annatto extracts are susceptible to oxidative degradation. Color loss also occurs upon prolonged exposure to light, elevated temperature, and in the presence of sulfur dioxide (Preston and Rickard, 1980; Najar et al., 1988; Collins, 1992). The major thermal degradation products of annatto have been characterized as the yellow-colored isomers and hydrolysis products of the trans-monomethyl ester of 4,8-dimethyltetradecahexaenedioic acid (C17) (McKeown, 1963, 1965; Scotter, 1995).

Three main commercial processes are used to extract the pigment from dried annatto seeds. These are (i) direct extraction into oil, (ii) direct extraction into aqueous alkali, or (iii) indirect extraction with solvents (Preston and Rickard, 1980). The vast majority of annatto on sale in the United Kingdom was reported to be directly extracted (Hardinge, 1991). Hot oil is used to facilitate isomerization of the naturally occurring 9′-*cis*-bixin to the relatively more soluble *trans*-bixin. The major coloring principals produced by direct oil extraction are 9′-*cis*-bixin, *all-trans*-bixin, and C17. This method is generally employed to provide a color formulation suitable for fat- or oil-based products such as margarine. Direct aqueous alkali extraction produces alkali metal or ammonium salt solutions of 9′-*cis*-norbixin plus a small amount of the very poorly soluble all-trans isomer. Alternatively, the free acid form of norbixin can be precipitated with dilute acid, filtered, washed, and dried to produce a solid formulation. In the indirect extraction of annatto, the pigments are extracted from the seeds with solvent, which is subsequently removed. This produces highly concentrated extracts consisting mainly of 9′-*cis*-bixin along with much lesser quantities of *trans*-bixin and 9′-*cis*-norbixin. The solvent-extracted pigment may be used as a dry powder, milled with vegetable oil to produce a suspen-

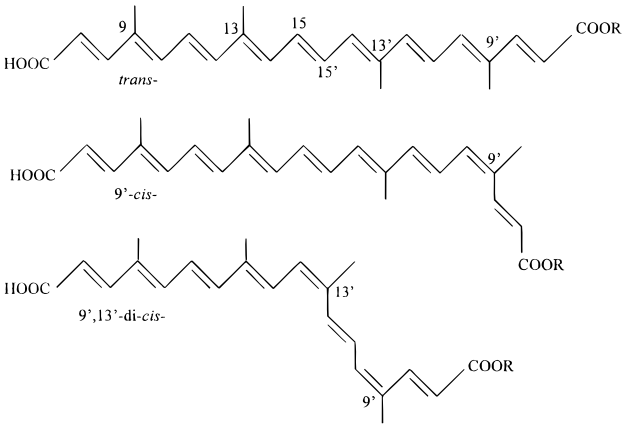

Figure 1. Chemical structures of some bixin/norbixin isomers. R = H, norbixin; R = CH_3, bixin.

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Table 1. Annatto Formulations Studied

sample code	type	form	production history and date when known
S1	bixin	dry powder[a]	indirect, solvent, ~3 years old
S2	bixin	dry powder[a]	indirect, solvent, ~3 years old
S3	bixin	crystalline	direct, hot oil, April 7, 1992
S4	bixin	crystalline	direct, hot oil, ~3 years old
S5	bixin	oil suspension/solution	direct, hot oil, ~3 years old
S6	norbixin	solution	direct aq alkali, July 31, 1991
S7	norbixin	granules	direct, aq alkali, April 7, 1992
S8	norbixin	granules[b]	direct, aq alkali, ~2 years old
S9	norbixin	powder[b]	direct, aq alkali, ~2 years old
S10	norbixin	granules	direct, aq alkali, ~2 years old
S11	bixin	encapsulated dry powder	N/A,[c] Nov 9, 1990
S12	norbixin	aqueous solution	direct, aq alkali, ~3 years old
S13	bixin	water dispersible liquid	direct, ~3 years old
S14	bixin	crystalline	indirect, solvent, ~3 years old
S15	norbixin	granules	direct, aq alkali, 2−3 months old
S16	bixin	crystalline	direct, hot oil, 2−3 months old
S17	norbixin	aqueous solution	indirect, solvent, Jan 21, 1993
S18	norbixin	aqueous solution	direct, aq alkali, Jan 21, 1993
S19	bixin	oil solution	N/A, Dec 1993
S20	norbixin	spray-dried on maltodextrin carrier	N/A, Dec 1993
S21	bixin	oil suspension/solution	N/A, Dec 1993

[a] Essentially the same product processed at different facilities. [b] Same type of norbixin but ground to different degrees. [c] N/A, information not available.

sion, or hydrolyzed in aqueous alkali to produce a solution of norbixinate.

In its response to comments received on its Final Report on the Review of the Coloring Matter in Food Regulations 1973 [Ministry of Agriculture, Fisheries and Food (MAFF), 1989], the U.K. Food Advisory Committee requested information comparing the unidentified components in directly and indirectly extracted annatto materials with a view to possible reclassification of their additive status. Annatto was reported to be the most commonly consumed natural color additive in the United Kingdom (MAFF, 1993) where the per capita daily consumption was estimated to be 0.065 mg/kg of body weight on the basis of pure coloring component, representing some 12.5% of the acceptable daily intake (ADI). The use of food colors in the United Kingdom is controlled by legislation implementing a European Parliament and Council Directive (EC, 1994), which sets the conditions under which they may be used. Annatto extracts may be used, singly or in combination, only in certain foods up to specified maximum levels (on a ready-for-consumption basis). Specific purity criteria for food colors, including annatto, are given in a separate Commission Directive (EC, 1995).

Current analytical data available on the annatto content of formulations are essentially nonspecific in that they do not provide details on the individual coloring components. Color content is generally expressed in terms of the major component, i.e. the 9′-cis isomer of bixin or norbixin. This is due largely to the shortcomings of available analytical methods, which generally employ direct spectrophotometric techniques and which may be prone to interference (McKeown and Mark, 1962). Current research on methods of analysis for annatto have focused largely on color formulations and their thermal stability, where developments in HPLC techniques have enabled the separation and determination of the major and minor geometric isomers of both bixin and norbixin, as well as their major colored thermal degradation products (Smith et al., 1983; Rousef, 1988; Scotter et al., 1994; Scotter, 1995).

The aim of this work was to develop an analytical procedure for the simultaneous determination of the principal color components, minor isomers, and colored thermal degradation products (C17-type polyenes) in annatto formulations.

MATERIALS AND METHODS

Samples comprised various commercial bixin and norbixin preparations in the form of dry powders (or granules), encapsulated powders, oil suspensions/solutions, and aqueous solutions. Samples were acquired over a three year period from several sources (Table 1).

Spectrophotometric Analysis for Bixin and Norbixin Content. Spectrophotometric measurements were carried out on either a Perkin-Elmer Lambda 3 or a Hitachi U2000 scanning spectrophotometer.

Oil-Soluble Annatto as Bixin (Smith et al., 1983). Approximately 0.1 g of sample was dissolved and diluted to 200 mL in 10% (v/v) acetic acid in chloroform. A 1.0 mL aliquot was diluted to 10 mL using a solution of 3% (v/v) acetic acid in chloroform to give a sample solution in 3.7% (v/v) acetic acid in chloroform. The absorbance of the diluted solution was measured at 505 and 474 nm in a 1 cm quartz cuvette against a reference solution of 3.7% (v/v) acetic acid in chloroform. The bixin content was determined by reference to $E_{1cm,1\%}$ extinction coefficients of 2790 (505 nm) and 3130 (474 nm).

Water-Soluble Annatto as Norbixin (Smith et al., 1983). Approximately 0.1 g of sample was dissolved and diluted to 200 mL in 5% (v/v) acetic acid in chloroform. A 1.0 mL aliquot was diluted to 10 mL with chloroform to give a sample solution in 0.5% (v/v) acetic acid in chloroform. The absorbance of the diluted solution was measured at 503 and 473 nm in a 1 cm quartz cuvette against a reference solution of 0.5% (v/v) acetic acid in chloroform. The norbixin content was determined by reference to $E_{1cm,1\%}$ extinction coefficients of 2290 (503 nm) and 2620 (473 nm).

Soluble Annatto Total Pigment, According to McKeown and Mark (1962). Approximately 0.1 g of sample was dissolved and diluted in 100 mL of chloroform. The absorbance of the solution was scanned over the wavelength range 400−600 nm in a 1 cm quartz cuvette against a chloroform reference. The wavelength of maximum absorbance (λ_{max}) in the region around 500 nm was determined and the absorbance measured at that wavelength and at 404 nm. The total pigment content expressed as bixin was calculated using the following formula:

$$\text{total pigment (\%)} = \frac{[(A_{max} + A_{404}) - (0.256A_{max})]}{282.6} \times \frac{100}{\text{sample wt (g)}} \times \frac{\text{dilution vol (mL)}}{1000}$$

where A_{max} is the measured absorbance at λ_{max}, A_{404} is the measured absorbance at 404 nm, the value 0.256 is the factor relating the absorbances at 404 and 501 nm for bixin in chloroform, and the value 282.6 is the absorptivity of bixin at 501 nm in chloroform.

Sample Preparation for HPLC Analysis. Between 0.01 and 0.10 g of sample, depending upon type, was weighed into a 50 mL volumetric flask. Samples S1−S4, S14, and S16 (Table 1) were dissolved in ∼3 mL of dimethylformamide (DMF) and brought to volume with acetonitrile. Samples S6, S11, and S12 were dissolved in ∼5 mL of water and brought to volume with acetonitrile. Samples S7−S10 and S13−S15 were dissolved in ∼5 mL of 0.1 M sodium hydroxide and diluted to volume with methanol. Sonication was used to aid dissolution.

Samples S17−S19 were treated as follows: 5 mL of sample was pipetted into a 100 mL volumetric flask and diluted to volume with 0.1 M sodium hydroxide/methanol (10:90). Samples S5, S19, and S21 were observed to contain substantial amounts of oil carrier and were treated as follows: ∼0.02−1.00 g of sample, depending upon strength of color, was weighed into a ground-glass stoppered test tube and dissolved in 5 mL of a solution of 0.5% (v/v) glacial acetic acid in DMF. Petroleum spirit (5 mL, 40−60 °C bp) was added, the tube stoppered, and the mixture shaken for ∼1 min. The upper petroleum spirit phase was transferred to a test tube containing 2 mL of 0.5% (v/v) glacial acetic acid in DMF. A second portion of petroleum spirit was added to the first test tube and the procedure repeated. The pooled petroleum spirit was extracted with the 2 mL portion of 0.5% (v/v) glacial acetic acid in DMF in the second tube and petroleum spirit layer then discarded. The DMF solutions were then pooled in the first tube. A gentle stream of nitrogen was bubbled through the DMF solution for several minutes to remove residual petroleum spirit. The solution was then diluted to an appropriate working volume with methanol.

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 (35 °C), and model 1040 series II photodiode array detector with HP Pascal workstation. The column used was a 250 × 4.6 mm RPB 5 μm (octyl-/octadecylsilane, fully end-capped 14% carbon loading; Hichrom, Theale, U.K.), and the mobile phase consisted of solvent A (acetonitrile) and solvent B (0.4% v/v aqueous acetic acid) delivered at 65% A:35% B at 1 mL/min. Detection was carried out initially at fixed wavelengths of 460 nm for bixin and norbixin isomers and 400 nm for C17 isomers. This was later modified to a single monitoring wavelength of 435 nm with a bandwidth of 40 nm. All samples were filtered through a 0.2 μm membrane filter prior to analysis. The system was calibrated using mixed reference standards of *all-trans*-bixin, 9′-*cis*-bixin, 9′-*cis*-norbixin, and *all-trans*-C17 prepared in the laboratory (Scotter et al., 1994). Calibration standards were prepared at ∼40−400 mg/l and contained butylated hydroxytoluene as antioxidant at ∼150 mg/L. Total coloring matter as percent bixin (or norbixin) was calculated from the total bixin (or norbixin) isomer peak area and expressed in terms of the main (i.e. 9′-cis-) isomer by interpolation from the calibration graph. The individual components in samples S17 and S18 were quantified by interpolation from the corresponding calibration graph of the reference material. When no reference materials were available, isomer contents were expressed in terms of the 9′-cis isomer.

RESULTS

Oil-Soluble Formulations. Table 2 compares the results obtained for the determination of bixin content in oil-soluble annatto formulations by spectrophotometric and HPLC methods.

Table 2. Bixin Content of Oil-Soluble Annatto Formulations

sample code	Smith method (bixin %)	McKeown method (pigment %)	HPLC method (bixin %)[a]
S1	9.0	12.6	9.2
S2	47.2	42.9	51.0
S3[b]	78.5	79.0	na[e]
S3[c]	38.9	39.1	31.6
S4	30.3	34.4	34.5
S5	3.2	3.7	1.8[d]
S13	0.2	na	0.08
S14	77.4	89.0	na
S16[b]	66.6	68.4	na
S16[c]	17.2	20.1	16.8
S19	0.8	0.5	0.25[d]
S21	4.0	4.7	3.8

[a] Expressed as total bixin isomers. [b] First analysis. [c] Second analysis after 15 months of storage. [d] Contained significant levels of C17: S5 = 0.06%, S19 = 0.16%. [e] na, not analyzed.

Table 3. Norbixin Content of Water-Soluble Annatto Formulations

sample code	Smith method (norbixin %)	HPLC method (norbixin %)[d]
S6	2.5	1.5
S7	37.7	43.6
S8	41.4	47.3
S9	40.1	30.2
S10	24.9	25.9
S11[a]	0.7	7.3
S12	0.6	3.3
S15	35.0	32.6
S17	3.3, (4.8)[c]	5.9
S18	*b*, (1.0)[c]	1.7
S20	16.4, (10.2)[c]	9.5

[a] Sample comprised an encapsulated matrix, and incomplete dissolution was observed for both methods. [b] Sample was too dilute for analysis by Smith method and formed an imiscible layer when a larger sample was taken. [c] Measured in 0.1 M NaOH at 453 nm using extinction coefficient $E_{1cm,1\%}$ of 2850 (Reith and Gielen 1971). [d] Expressed as total norbixin isomers.

Water-Soluble Formulations. Table 3 compares the results obtained for the determination of norbixin content in water-soluble annatto formulations by spectrophotometric and HPLC methods.

Detailed HPLC Analysis of Samples S17 and S18. Two samples (S17 and S18) of water-soluble annatto concentrate were obtained for detailed HPLC analysis of principal colors, isomers, and C17-type polyene degradation compounds. This analysis was undertaken specifically to identify any observable differences in HPLC profile between these two samples, which were of known production history. The results are given in Tables 4 and 5.

DISCUSSION

Analysis of Oil-Soluble Formulations. The Smith and McKeown methods for the analysis of oil-soluble annatto formulations provided quantitative estimates of the total pigment content but provided few qualitative data with respect to other isomers and C17-type polyenes content other than indicative spectral characteristics such as wavelength maxima. Differences in the results for total pigment content would be expected since each method uses a different calculation procedure. In the Smith method, no allowance is made for the absorp-

Table 4. Chromatographic and Spectral Data of Peaks in Samples S17 and S18

peak	retention time (min)	λ_{max} (nm)	dλ_{max} (nm) from trans-	REL(I) (%)	isomer[a,i] (tentative)
1	3.3	396	*c*	*c*	trans C17
2	3.6	458	7	*d*	mono-cis-[h]
3	5.3	448	17	9	di-cis-
4	6.4	465	0	9	trans-[e]
5	6.8	454	11	13	di-cis-
6	7.0	454	11	25	di-cis-
7	7.4	448	17	*d*	di-cis-
8	7.7	445	20	19	tri-cis- ?
9	8.1	442	23	24	tri-cis- ?
10	8.9	445	20	*d*	tri-cis- ?
11	9.6	459	6	11	mono-(9′)-cis-[e]
12	10.2	459	6	23	mono-cis-[f]
13	12.1	459	6	54	mono-cis-[g]
14	18.3	452[b]	*c*	*c*	unknown
15	19.8	412[b]	*c*	*c*	unknown

[a] Norbixin isomers unless stated otherwise. [b] Broad absorption maximum. [c] Not applicable. [d] Component at low level, not measurable due to noise. [e] Not tentative; compared to characterized standard. [f] Possibly 13′(or 13)-cis-. [g] Possibly central (15)-cis-. [h] Possibly 7′(or 7)-cis- (hindered ?). [i] No attempt has been made to discriminate between the configurations of di- and tri-cis-isomers.

tion due to nonbixin species, whereas the McKeown method applies a correction factor to allow for any absorbance due to C17-type polyenes. Nevertheless, the results given in Table 2 show reasonable agreement between the two methods. This indicates that the contribution of C17-type polyenes to the total absorbance was negligible in each of the samples analyzed. Samples S5 and S19 were subsequently found by HPLC analysis to contain 0.06 and 0.16% C17, respectively (normalized figures).

The results from the HPLC analysis of oil-soluble annatto formulations showed reasonable agreement with both spectroscopic methods, particularly the Smith method. This is not unexpected since the results from both the Smith and HPLC methods are expressed in terms of percent bixin, whereas the results from the McKeown method are given as percent total pigment. Moreover, some fundamental differences in the calibration procedures for the Smith and HPLC methods were apparent and are discussed below.

The results in Table 2 show that sample S3 underwent significant deterioration during ambient storage. When first analyzed, this sample contained ~79% bixin with good agreement in results between both spectroscopic methods (HPLC analysis was not carried out at this time). When reanalyzed some 15 months later, the norbixin content had fallen by half, due most likely to oxidative deterioration. This was not unexpected since the sample had been stored under ambient conditions in its original container. The results from both spectroscopic methods were again in good agreement and were slightly higher than the result found by HPLC analysis, indicating the presence of non-bixin absorbing species.

Analysis of Water-Soluble Formulations. Since the scope of the McKeown method does not include water-soluble formulations, only the Smith and HPLC methods were used. The qualitative and quantitative limitations of the Smith method are similar to those discussed above, and some disparity in results between samples analyzed by this method and by direct spectrophotometric measurement in 0.1 M NaOH was observed. Such disparity was thought to be mainly due to differences in the relevant calibration procedures for each method and the nature of the samples analyzed. The optical characteristics of annatto solutions show a marked dependency on acidity; hence, the solvent medium affects both the position of the maximum wavelength of absorption and the intensity. Smith et al. (1983) have reported that λ_{max} shifts of +4 and +7 nm and changes in absorption intensity of −2 and +19% were observed for solutions of bixin and norbixin, respectively, when the solvent medium was changed from chloroform to 5% acetic acid in chloroform. The greater changes observed in these parameters for the solution of norbixin are thought to be due to the presence of two free carboxyl groups, compared to bixn, which is monoesterified.

The Smith method had notable limitations in application, and some difficulties were experienced in the analysis of certain formulations. For instance, sample S18 was an aqueous ammoniacal solution (pH 13.6) of norbixin, which upon dilution for analysis according to the method protocol produced a solution that was too dilute to obtain a meaningful reading on the spectrophotometer. When a larger sample was taken, an immiscible aqueous phase was formed within the chloroform/acetic acid solution, thus preventing analysis. This sample, along with samples S17 and S20, was diluted in 0.1 M NaOH and analyzed by spectrophotometry. The norbixin content was determined by direct comparison of the absorbance obtained with an extinction coefficient ($E_{1cm,1\%}$ in 0.1 M NaOH of 2850) reported in the literature. The results obtained (Table 3) were generally comparable to those obtained by HPLC analysis with some exceptions.

Sample Degradation—Comparison of Samples S8 and S9. Samples S8 and S9 were solid norbixin formulations obtained from the same source material but ground to different extents, the former a porous granular solid and the latter a fine powder. Analysis by the Smith method showed that both samples contained ~40% norbixin, whereas analysis by HPLC gave results of 47.3 and 30.2% total norbixin, respectively. Sample S9 may be expected to show a greater extent of oxidative degradation during storage (due to a larger surface area) than the granular form, manifested in the presence of significant amounts of non-norbixin absorbing species as degradation products. This is supported by the relatively lower result obtained by HPLC analysis. In contrast, a higher result was obtained by HPLC analysis compared to analysis by the Smith method for sample S8 and several other samples, which cannot be readily explained. Notwithstanding the fact that isomerization and degradation can occur simultaneously, these differences in results may be due in part to fundamental differences in the methods with respect to calibration procedures, which could easily be overwhelmed by the presence of significant amounts of degradation products. Moreover, the Smith method uses an $E_{1cm,1\%}$ figure of 2620 at 473 nm in 0.5% acetic acid in chloroform, whereas the HPLC method is calibrated with purified 9′-*cis*-norbixin standardized against an $E_{1cm,1\%}$ figure of 2850 at 453 nm in 0.1 M NaOH (Scotter et al., 1994). While it might be expected that this would result in a consistent difference in results obtained by the two methods, the variability in sample type, integrity, and concentration across the range analyzed may be the overriding factor and illustrates further the limitations of spectroscopic methods.

Table 5. Major Norbixin Isomer Profiles of Samples S17 and S18

norbixin isomer	9′-cis	all-trans	mono-cis[e]	di-cis[e]	di-cis[e]	di-cis[e]
peak[a]	11[b]	4	13	5[c]	6[c]	3
S17						
concn (mg/L)	49500	900	225	d	8000	245
as % of total norbixin	84.1	1.5	0.4		13.6	0.3
S18						
concn (mg/L)	13400	1520	381	61	1134	108
as % of total norbixin	80.7	9.1	2.3	0.2	6.8	0.7

[a] As given in Table 4. [b] Not possible to resolve from peak 12. [c] Peaks 5 and 6 poorly resolved. [d] Appears as a shoulder on di-cis peak (6). [e] Arbitrary assignment, see Table 4.

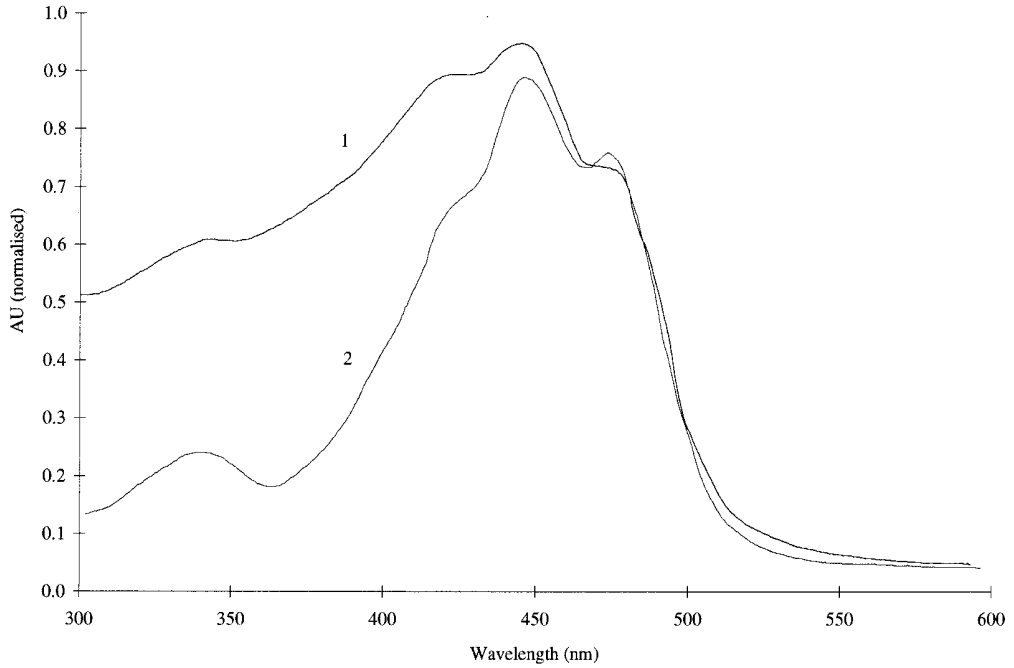

Figure 2. Comparison of spectra obtained from commercial norbixin sample S20 (1) and a 9′-*cis*-norbixin standard (2) in 0.1 M NaOH.

Spectral Distortion—Analysis of Sample S20. Figure 2 compares the absorption spectra obtained from the analysis of sample S20 and a 9′-*cis*-norbixin standard. The spectra show that while the locations of the wavelength maxima were the same for both the sample and the standard, the intensities of absorption at these maxima were different, resulting in a significant change in the corresponding absorbance ratios [the absorbance ratio λ_{max}(IV) 482 nm/λ_{max}(III) 453 nm was 0.89 for the 9′-*cis*-norbixin standard and 0.77 for sample S20]. Spectral distortion is particularly apparent in the region 350−420 nm, where the absorption maxima for C17-type polyene degradation products are known to occur. However, no water-soluble annatto samples were found to contain significant amounts of these compounds by HPLC analysis.

Detailed Analysis of Samples S17 and S18. Color Principals Content. Sample S18 had the distinct smell of ammonia and a pH of 13.6. In terms of the *total* norbixin isomer content (i.e. normalized values), samples S17 and S18 contained 84.1 and 80.7% 9′-*cis*-norbixin, respectively, as the principal coloring component. A 6-fold difference in the normalized levels of *all-trans*-norbixin and mono-*cis*-(not 9′-)norbixin was found between samples S17 and S18. Twice as much of the major di-*cis* isomer (III) of norbixin was found in sample S17 compared to sample S18, which contained twice the amount of total remaining norbixin compared to sample S17. Both samples contained only trace amounts of C17-type polyene degradation compounds. Preliminary method development for the determination of aromatic degradation products in annatto formulations using alkaline hydrolysis followed by solvent extraction and gas chromatography (Scotter et al., unpublished results) revealed that sample S17 contained ≈22 mg/L *m*-xylene, whereas sample S18 contained ≈1 mg/L *m*-xylene, and both samples contained <1 mg/L toluene. The method used was not fully validated; hence, the results are tentative. However they indicate a marked difference in *m*-xylene content between the two samples, which may provide further evidence of production history.

HPLC−PDA Analysis. Figure 3 shows the HPLC−PDA chromatograms obtained for samples S17 and S18, respectively. The four major coloring principals were identified along with some 11 other minor peaks exhibiting bixin/norbixin or C17-type polyene spectral characteristics. Tentative identifications of the peaks common to both samples are given in Table 4. Figure 4 shows the spectra obtained from the major isomer peaks of sample S18 and demonstrates how the isomers may be tentatively identified from their spectral characteristics, i.e. the wavelength of maximum absorbance and its relative absorption intensity compared to the "cis" peak around 350 nm [REL(I)]. Peak 4 corresponds to the trans isomer of norbixin, which displays the longest λ_{max} wavelength and the lowest REL(I) value, whereas peaks 11 and 13 display λ_{max} shifts (dλ_{max}) of 4 and 6 nm, respectively, from the all

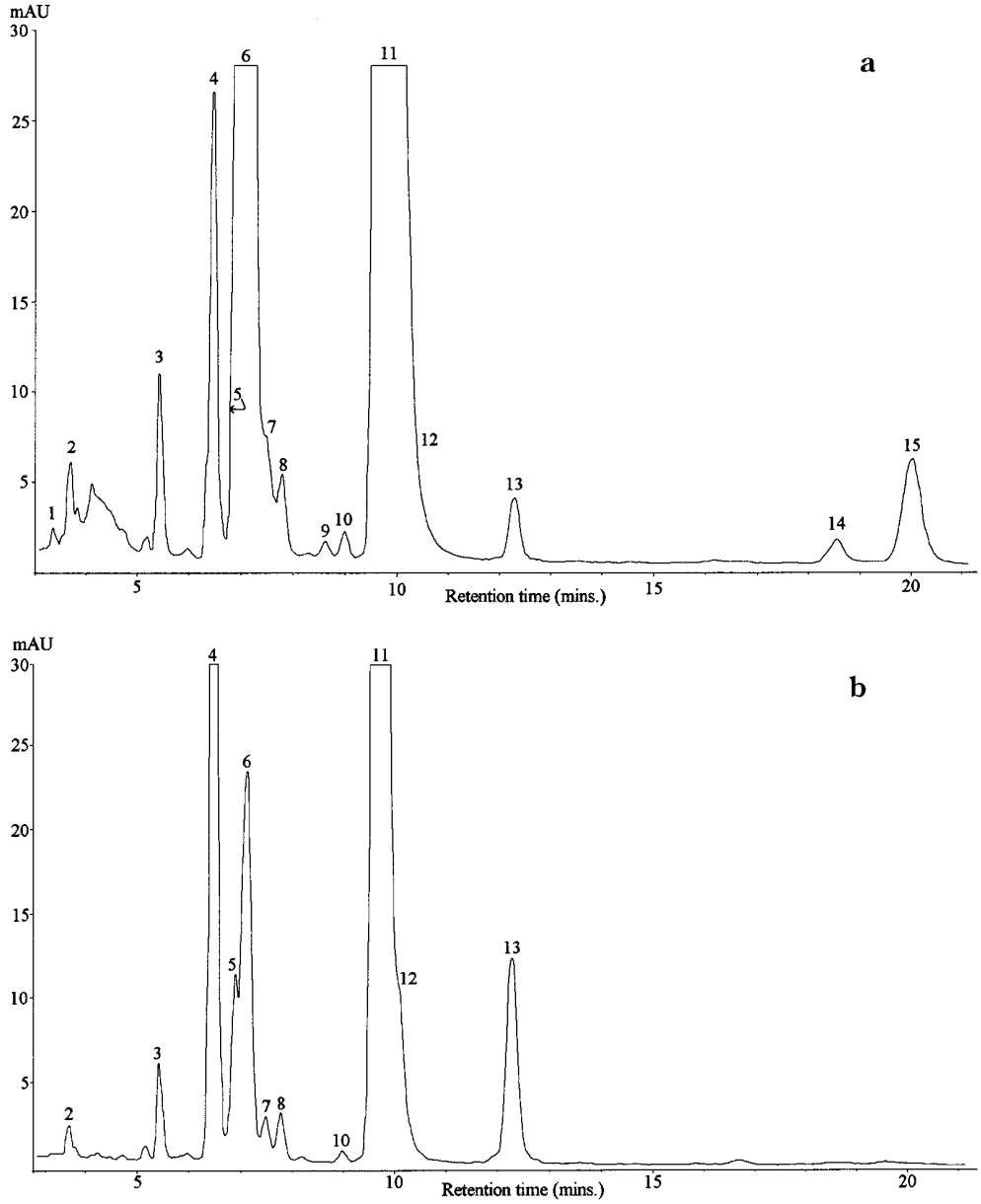

Figure 3. HPLC−PDA chromatograms of commercial norbixin samples S17 (a) and S18 (b). Peak numbers correspond to those given in Table 4.

trans isomer and are therefore mono-cis isomers. Peak 11 is the major 9′-*cis* isomer [REL(I) = 8%], which is confirmed by reference to purified 9′-*cis*-norbixin, whereas peak 13 displays a high REL(I) value of 57%, which is similar to the value reported for the 15-cis isomer of β-carotene (Pettersson and Jonsson, 1990). The λ_{max} values of peaks 5 and 6 are shifted 8 and 10 nm, respectively, from the trans isomer and are therefore likely to be di-cis isomers of norbixin. The REL(I) value of 22% obtained for peak 6 is similar to that obtained in previous studies for di-*cis*-norbixin (Scotter et al., 1994), whereas the corresponding value for peak 5, although lower (~10%), was difficult to measure due to signal noise.

Theoretical Considerations in Isomer Identification. While the formation of mono- and di-*cis* isomers of carotenoids from the all-trans configuration may be energetically favored, the probability of formation of higher cis isomers is predicted to be very low (Zechmeister, 1960). Since norbixin is a symmetrical molecule containing 9 conjugated double bonds, of which five are unhindered (positions 9, 9′, 13, 13′, and 15−15′), selection rules predict that 20 unhindered isomers could be formed (Figure 1). Furthermore, since each double bond (except the central 15) has a stereochemical equivalent counterpart, the probability of formation of a given cis isomer is effectively doubled compared to an asymmetric molecule such as bixin (O'Neil and Schwartz 1992).

Since norbixin is formed from the naturally occurring 9′-cis isomer of bixin, the formation of further cis isomers via the all-trans isomer may be circumvented, thus giving rise to a greater probability of formation of higher (i.e. di- and tri-) cis isomers, especially those containing a 9′-cis bond. While the effect of cis isomerization in the UV−vis absorption spectrum is characterized by $d\lambda_{max}$ and REL(I) values, the reliability of the structural assignments decreases rapidly when the $d\lambda_{max}$ value exceeds ~10 nm. Therefore, the peak

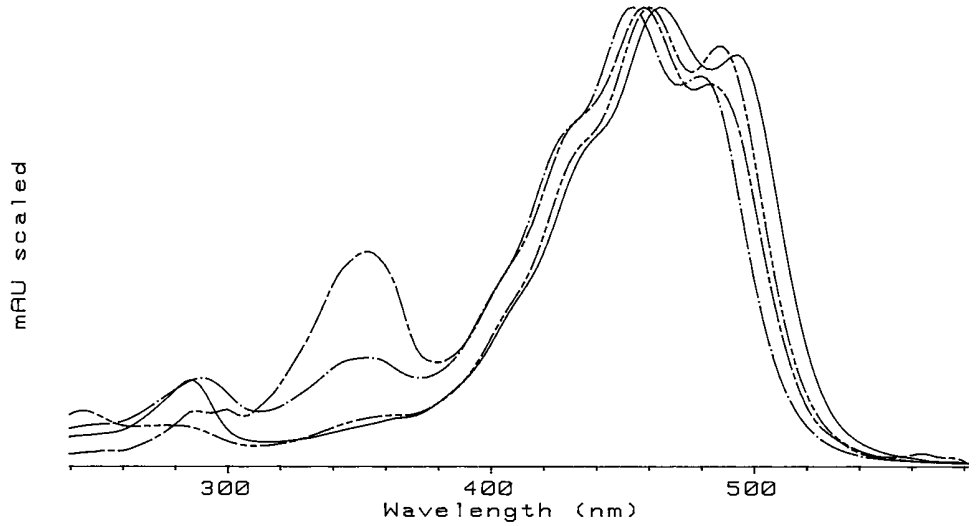

Figure 4. HPLC−PDA spectra of the major norbixin isomers in commercial sample S18: peak 4 (—); peak 6 (— · —); peak 11 (— -- —); peak 13 (— - —). Peak numbers correspond to those given in Table 4.

assignment of tri- and poly-cis configurations becomes highly tentative.

Relationship between Results and Sample Production Histories. Information obtained from the suppliers revealed that sample S17 was manufactured using an indirect process, i.e. solvent extraction followed by filtration, washing, removal of solvent, and alkaline hydrolysis, and sample S18 was manufactured by direct aqueous alkaline extraction. Since direct aqueous alkaline extraction may employ temperatures up to 70 °C, there is scope for the formation of *trans*-norbixin via thermally driven isomerization. The isomer profiles obtained by HPLC analysis support this since some 6 times as much *trans*-norbixin was found in sample S18 compared to sample S17 (normalized figures). The different extraction procedures may, however, give rise to different isomer profiles due to their differential solubility and/or stability in the extraction medium, which may be complicated by the effects of light and oxygen during extraction and handling and by the nature of the source material. The presence of C17-type polyene degradation products may be indicative of the use of higher extraction temperatures, but bixin may also undergo photocatalyzed degradation to produce products similar to those of thermal degradation. It is possible that such processes may therefore occur during solvent extraction of annatto prior to hydrolysis to norbixin. Furthermore, study into the mechanism of the thermal degradation of bixin (Scotter, 1995) has revealed that it may be possible for C17-type polyenes to similarly degrade to shorter chain analogues accompanied by the further release of *m*-xylene or toluene. This may explain the tentative presence of *m*-xylene and the absence of C17 polyenes in sample S17. The possibility that *m*-xylene may be present as a contaminant from an alternative route such as an impurity in the extraction solvent cannot, however, be ruled out.

It is not possible to draw firm conclusions about the relationship between the isomer and degradation product profile of annatto formulations and their production history from such limited analytical data. Although some differences in these parameters were apparent between samples S17 and S18, the analysis of further samples of annatto of known detailed production history is required to obtain sufficient data. Analysis of annatto formulations for aromatics content is to be reported subsequently and may provide further evidence for the differentiation between directly and indirectly extracted annatto formulations.

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