



ANNATTO EXTRACTS

Chemical and Technical Assessment

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1. Summary

The food colour annatto is obtained from the outer layer of the seeds of the tropical tree *Bixa orellana* L. The principle pigment in annatto, *cis*-bixin, is a carotenoid, which is contained in the resinous coating surrounding the seed itself. Processing is primarily done by abrading off of the pigment in an appropriate suspending agent for production of the native bixin from the seed. Processing may alternatively involve aqueous alkaline hydrolysis with simultaneous production of norbixin. Traditionally, water or vegetable oil is used as a suspending agent, although solvent extraction is now also employed to produce more purified annatto extracts. Microcrystalline bixin products of 80 - 97% purity have been developed as a response to the need for more concentrated annatto extracts. Annatto has been used for over two centuries as a food colour especially in cheese and the various forms are now used in a wide range of food products.

2. Description

Annatto is obtained from the outer layer of the seeds of the tropical tree *Bixa orellana* L. The principle pigment in annatto, namely bixin, is a carotenoid, which is contained in the resinous coating surrounding the seed itself. The major pigment present is *cis*-bixin; also present, as minor constituents, are *trans*-bixin, *cis*-norbixin and *trans*-norbixin (see section 4.1 on chemical composition). The annatto tree is native to Central and South America where its seeds are used as a spice in traditional cooking. In Brazil, and a number of other South American countries, substantial quantities of processed annatto seeds are sold in retail outlets often blended with other ingredients for addition to soups and meat dishes similar to the use of paprika seasonings in Europe.

Annatto seeds and extracts have been used for over 200 years in Europe and North America to impart a yellow to red colour to foods, especially dairy products such as cheese. Indeed, reference is made to the colouring of cheese with annatto, as far back as 1796 in England. In modern times, annatto has significant economic importance worldwide and it is one of the most frequently used natural colourants of the food industry.

The principle export form of annatto is as the seed, although to increase export values, several suppliers now also carry out partial processing / refining of annatto seeds into concentrates.

The main market for annatto is the USA, Western Europe and Japan, although there is also considerable inter-trade (in seeds) between the Central and South American suppliers.

INS number: 160b (annatto, bixin, norbixin)
Common synonyms: CI Natural Orange 4; CI 75120
Achiote, Achiotl, Achote,
Annotta, Arnatta, Arnatto, Arnotta,
Bija, Rocou, Roucou, Roucouyer, Roucoyer, Orlean,
Orleanstraugh, Terre orellana, Beni-No-Ki, Urucu, Urucum, L.
Orange

C.A.S. Number Annatto:	1393-63-1
Bixin:	39937-23-0 (<i>trans</i>)
	39937-79-5 (<i>cis</i>)
Norbixin:	542-40-5 (<i>trans</i>)
	626-76-6 (<i>cis</i>)

Annatto is native to tropical America, where it has been a traditional part of some foods for centuries, but is also cultivated in Asia and Africa, especially in areas where coffee is grown. The annatto plant, *Bixa orellana* L, is a tree that grows from two to five meters tall when mature. The fruits of the tree *Bixa orellana* L consist of a pod covered with fleshy spines, varying in size and shape. The inside of the pod is usually divided into two valves containing between ten to fifty small seeds. These seeds are covered with a resinous orange or red coating (arils) from which the commercial pigment is extracted.

Harvesting is manual whereby workers cut off the ends of branches that contain the seed pods. Such pruning of the branches seems to help increase seed production in the plant. The dried pods are threshed and winnowed to remove pod material and other detritus thereby releasing the seeds. The seeds subsequently are then dried and packaged for shipment.

Although annatto seed is harvested in many tropical countries including Bolivia, Ecuador, Jamaica, the Dominican Republic, East & West Africa (Kenya), India and the Philippines, it is Peru and Brazil that are the dominant sources of supply. The quantity of annatto seed harvested in the producing countries is estimated at 14,500 metric tonnes (Table 1). It is likely that around 7,500 tonnes of annatto seed are used annually as a food colour worldwide and, assuming an average colour content of 2%, this equates to 150 tonnes of bixin available for extraction. The remaining 7,000 metric tonnes of annatto seed is consumed locally in Brazil, Peru and Ecuador mainly as a spice / condiment.

Table 1. Estimated World Production (in Metric Tonnes) of Annatto seed*

PRODUCERS		IMPORTERS	
Brazil	5,000	(either as seed or its equivalent in extract)	
Peru			
Ecuador		North America	3,000
Colombia		Europe	2,500
Bolivia	3,000	Japan	1,500
		Other	500
Kenya			
Tanzania	2,500	Total	<u>7,500</u>
Guatemala			
Mexico			
Caribbean	2,000		
Ivory Coast			
Ghana	1,500		
India, Asia	500		
	<u>14,500</u>		
Of which:			
Domestic consumption**	7,000		
Available for export	7,500		
	<u>14,500</u>		

* Adapted from UNCTAD / GATT (1990), Wood *et al.* (1991), Green *et al.* (1995) and Dinesen (1999).

** within the producer countries.

3. Manufacturing

3.1. Manufacturing principle

Commercial production occurred in Jamaica in the 1790s. In Europe and the US, production of commercial extracts started before the 1870s, primarily to colour butter and cheese.

As the bixin pigment is found in the aril (outer waxy coat) of the seed, the processing includes abrading or raspelling (mechanical removal of the aril from the seed) of the pigment in an appropriate suspending agent followed by removal of the seeds. For the purposes of removing the pigment, it is not necessary to grind the seed. Processing methods may either be aimed at the production of the native bixin from the seed or may involve aqueous alkaline hydrolysis and simultaneous production of norbixin. Solvent extraction techniques are now also used as a means of production of annatto concentrates.

3.2. Detailed description

Annatto seeds may be processed in two fundamentally different ways. One process is mechanical abrasion using either vegetable oil or dilute aqueous potassium hydroxide as a suspending agent. Further direct processing of these extracts may then be undertaken (Figure 1). The other process is by extraction with one or more organic solvents. These two commonly used processing methods are outlined below.

3.2.1. Mechanical Abrasion:

3.2.1a. Extraction with refined vegetable oil

Oil processing usually involves mechanical methods using edible vegetable oil whereby the annatto seeds are massaged in warm oil (e.g. 70⁰C) to remove the pigment layer in a machine sometimes called a 'Raspeller'. The resulting suspensions contain bixin as the primary pigment at a concentration up to 8%. It is common to see annatto oil preparations presented commercially as suspensions containing between 4 - 8% bixin. There is evidence of some proprietary technology utilizing propylene glycol in place of vegetable oil as a suspending agent for Annatto seeds, however the volumes involved are small.

3.2.1b. Extraction with dilute aqueous potassium or sodium hydroxide
Mechanical methods with dilute aqueous potassium or sodium hydroxide involve the same principle, but mechanical abrasion is provided by stirring the annatto seed in mildly alkaline water at ambient temperatures. This dislodges and separates the pigment layer, which is then acid precipitated, filtered off and dried to give a granular powder with a bixin content of about 25%. This aqueous processed annatto can be further processed into a range of annatto products by dissolving or suspending it in oil.

In the original and traditional food recipes of Latin America, annatto seeds were heated with cooking oil, separated, and the coloured oil used for preparing rice, soups, and tortillas. A similar direct-processing system can be applied industrially by immersion of the seeds in vegetable oil to produce a bixin slurry, which can then be heated and filtered. The filtrate can be marketed as a colour for high-fat foods. Since bixin is soluble to a limited extent in vegetable oil, it is possible to obtain oil solutions of annatto whereby the final colour content is less than 1% w/w. Likewise, the use of alkaline propylene glycol instead of vegetable oil extends its application to both high-fat and low-fat foods and gives solutions of greater purity.

In all these cases, the process liquids, which contain up to 1.5% bixin or in some cases oil suspensions with up to 8% bixin are marketed directly as oil-soluble annatto food colour after standardisation of colour content. For aqueous applications (e.g. cheese colour) the processing of the annatto seed is done with aqueous potassium hydroxide or sodium hydroxide, which hydrolyses and saponifies the bixin on the seed yielding the water-soluble norbixin salt.

Direct processing usually involves steps to remove or emulsify seed oils and waxes that are carried over along with the pigment. These aqueous preparations commonly contain from 0.5 to 4.0 % w/w of norbixin. It is possible to use this type of preparation to prepare a water-soluble norbixin powder (by drying), which commonly ranges from 1 to 15 % in colour content. These types of annatto preparations are commonly used in foods that have a significant water phase.

Acid-precipitated norbixin concentrates with between 25 and 50% norbixin can be prepared by acid precipitation of norbixin from aqueous alkaline solutions, followed by filtration and drying into a granular powder. This article of commerce is increasingly replacing the direct extraction of water-soluble preparations from seeds in the end markets.

3.2.2. Solvent Extraction:

Microcrystalline bixin products of 80 - 97% purity have been developed as a response to the need for more concentrated annatto extracts. Extraction of annatto seed with organic solvents and subsequent solvent removal yields the bixin crystals, which are then processed by the manufacturers of food colours according to specific applications. Numerous patents and research reports cover a variety of organic solvents for producing concentrates, such as chlorinated hydrocarbons, mixtures of ethanol and chloroform, acetone, ethanol, ethyl acetate, hexane, methanol or alcoholic sodium hydroxide.

Table 3. Annatto Preparations

CATEGORY	DESCRIPTION	TARGET PIGMENT CONTENT	PROCESS DESCRIPTION
Annatto A	Solvent extracted bixin paste solvent removed.	Target ~20% Bixin	N/A. No longer an article of commerce
Annatto B	Solvent extracted crystallised bixin, dried crystals	Min. 80% Target ~ 90% Bixin	Seeds are washed with solvent to dissolve pigment. The extract is filtered to remove insoluble material. Subsequent processing involves removal of fats and waxes, solvent removal, crystallisation and drying. (Solvents used can be one or more of: hexane, acetone, ethanol, (alkaline) methanol, IPA, ethyl acetate). Residual solvent = <50ppm Fine powder with particle size <250 microns
Annatto C	Solvent extracted crystallised bixin, saponified, hydrolysed, acid-precipitated, washed and dried.	Min. 70% Norbixin Target ~80%	Seeds are treated as for the production of Annatto B. Aqueous alkali is added to the resultant powder, which is then heated to hydrolyse the pigment and cooled. The aqueous solution is filtered, and acidified to precipitate the norbixin. The precipitate is filtered, washed, dried and milled, to give a granular powder.
Annatto D	Oil processed bixin suspension (no solvent to be used)	Min. 10% Target ~ 15% Bixin	Annatto seeds are abraded in hot vegetable oil to remove pigment from the surface of the seeds. Crudely sieved to remove seeds.
Annatto E	Aqueous processed bixin, Dried into a granular powder	Min. 25% Target ~ 30% Bixin	Annatto seeds are abraded in cold aqueous alkali (potassium or sodium hydroxide) to remove pigment. The resultant suspension is acidified (sulphuric acid) to precipitate the bixin. The precipitate is filtered, washed dried and milled, to give a granular powder.
Annatto F	Alkali processed norbixin, acid-precipitated and dried into a granular powder. The pH of a 10% solution to be less than 5	Min. 35% Target ~ 40% Norbixin	Annatto seeds are abraded in cold aqueous alkali (potassium or sodium hydroxide) to remove pigment. Additional alkali is added to the resultant suspension, which is then heated to dissolve the pigment and cooled. Fats and waxes are removed. The aqueous solution is filtered, and acidified to precipitate the norbixin. The precipitate is filtered, washed dried and milled, to give a granular powder.
Annatto G	Alkali processed norbixin, mildly alkaline. Dried. (No acid precipitation step).	Min. 15% Target ~ 20% Norbixin	Annatto seeds are abraded in cold aqueous alkali (potassium or sodium hydroxide) to remove pigment. Additional alkali is added to the resultant suspension, which is then heated to dissolve the pigment and cooled. Fats and waxes are removed. The aqueous solution is filtered, and spray or tray dried. Potassium carbonate may be added.

4. Chemical characterization

Chemically, annatto pigments bixin and norbixin belong to the group known as carotenoids. Carotenoids are very widespread in nature, some of the better-known sources being carrots, shrimps, tomatoes and eggs. Bixin itself, is a C-25 diapo-carotenoid, and as such is oil-soluble, although sparingly so.

Annatto is almost unique amongst the carotenoid family in that it can act as a pigment in a number of different chemical forms. The predominant form is bixin, which is the methyl ester of the dicarboxylic acid norbixin. Annatto seeds contain *cis*-bixin (>80% of the total carotenoid content), mainly in the 9-*cis* configuration with smaller quantities of *trans*-bixin and *cis*-norbixin. Under specific conditions of temperature and pH, bixin can be hydrolysed into norbixin, the dicarboxylic acid and saponified into the potassium salt of norbixin.

At elevated temperatures (> 70°C), degradation reactions lead to the formation of several products including a 17-carbon yellow compound known as McKeown's pigment.

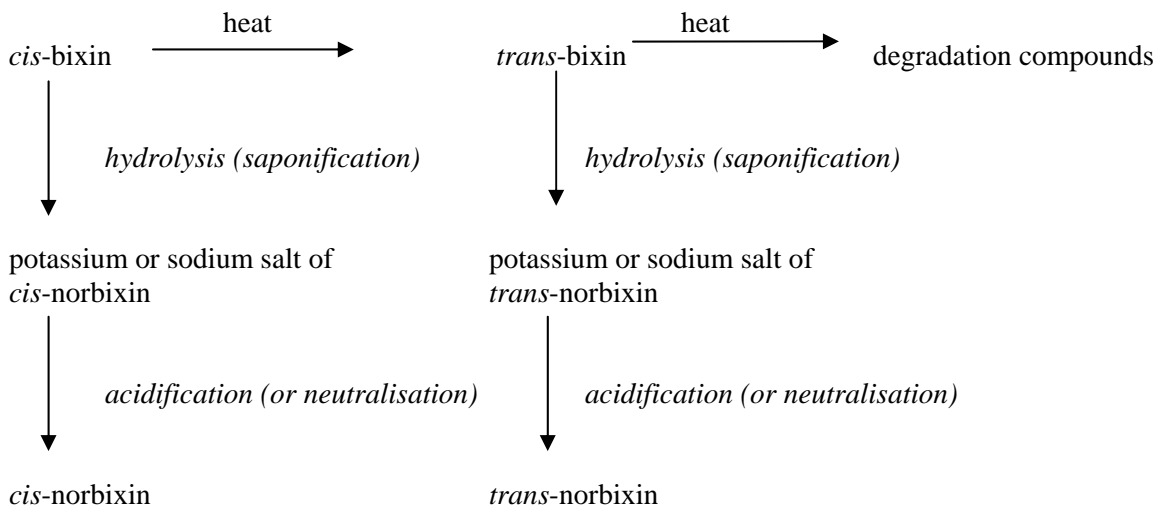
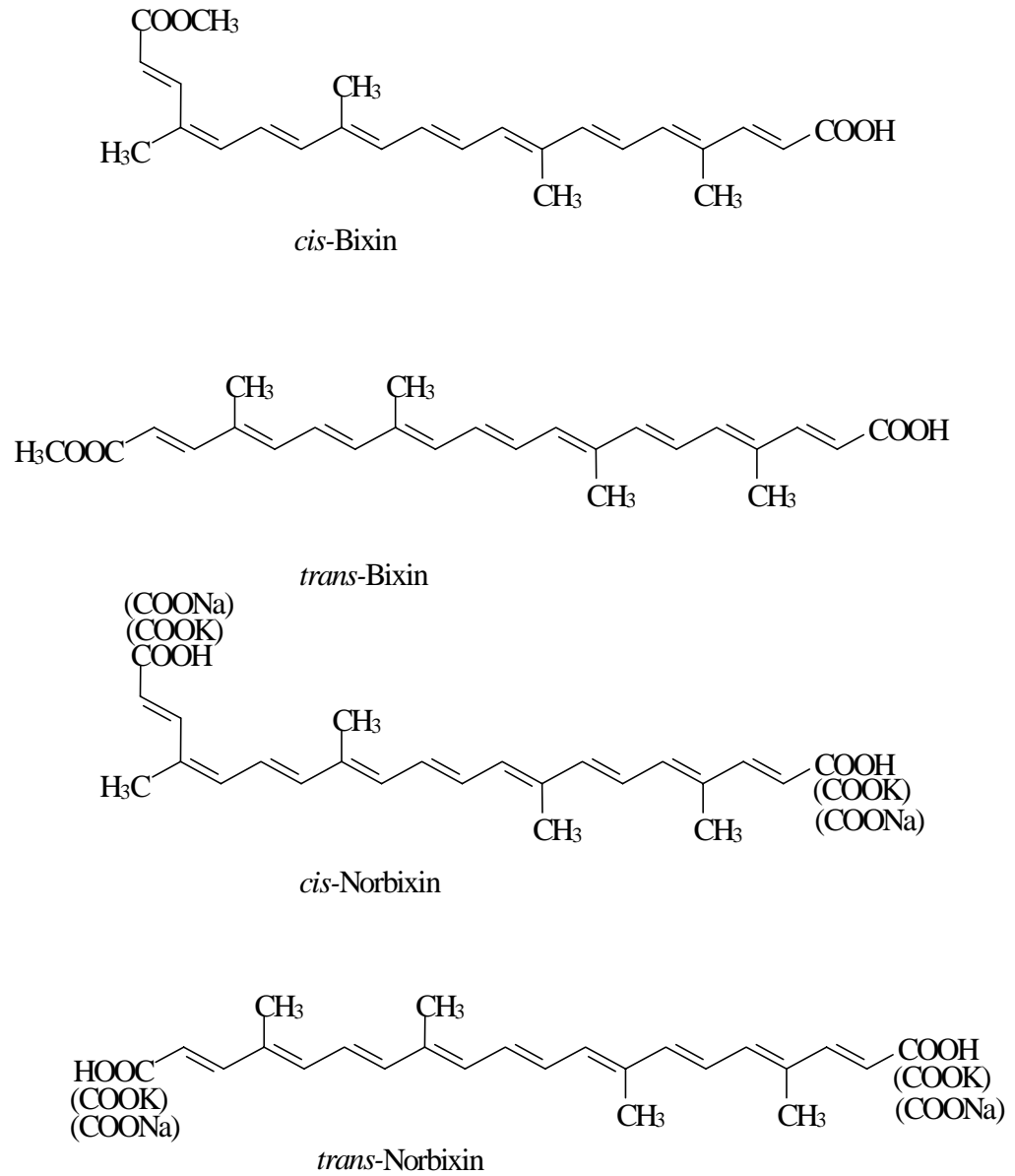


Figure 2. The inter-relationship of different annatto pigments.

The structural formulae of bixin (*cis*- and *trans*- forms) are shown in Figure 3. By hydrolysis of the ester group, the water-soluble norbixin (*cis*- and *trans*- forms) are formed. Figure 3 also shows the structural formula of norbixin.

Figure 3: The structural formulae of bixin and norbixin



4.1 Composition of the Food Additive

4.1.1. Pigment Fraction

Oil-soluble annatto consists of bixin, dissolved or suspended in oil. Water-soluble annatto consists of the dissociated form of norbixin in alkaline solution, usually potassium or sodium hydroxide. Emulsified annatto contains norbixin and/or bixin in association with an emulsifier. These chemical forms determine the colour shade in the food matrix.

Oil-Soluble Annatto:

The oil-soluble annatto colours exhibit different shades ranging from yellow to orange-red. Dissolution of bixin in oil changes the colour from orange to yellow. Further extended heating degrades the bixin into a more yellow C-17 compound. A range of refined food grade oils e.g. soybean oil, rapeseed oil, sunflower oil, etc. may be used to dissolve or suspend the bixin.

Oil solutions of annatto usually contain 0.05 – 1.0% bixin.

Oil suspensions of annatto usually contain 0.1 – 8% bixin.

Good quality oil with few free fatty acids, a low number of peroxides and a light colour is essential, as it otherwise may promote oxidation by chain reactions and thus create breakdown of colour. The advantages of strong suspension colours are better storage stability and economic factors.

Water-Soluble Annatto:

Water-soluble annatto colours consist of norbixin (usually as the potassium or sodium salt) and are commercially available as dilute liquid solutions (high pH) and powders.

Water solutions of annatto usually contain 0.1 – 4.0% norbixin.

Water-soluble annatto powders usually contain 1 – 15% norbixin

The extended use of annatto in industries other than the traditional dairy industry has increased the use of water-soluble annatto considerably.

Emulsified Annatto:

Addition of an emulsifier to bixin or norbixin results in colours, which may be miscible with both oil and water. These application forms are particularly suitable in products containing both a water and an oil phase. By appropriate choice of emulsifier, water-soluble bixin/norbixin with improved acid stability can be obtained.

Emulsified annatto are sold as liquids and usually contain 1 - 2.5% as bixin / norbixin.

For the 2002 submission, the chemical analyses of the different annatto preparations studied toxicologically were presented in detail in the following reports from HLS.

HLS Study No. ATE/001 – ‘Analysis of Annatto Test Materials’. This covers the materials used in the 7 day pre-palatability tests;

HLS Study No. ATE/009 – ‘Analysis of Annatto Test Materials’. This covers the materials used in the 28 day palatability studies, and 90 day studies on Annatto E and F.

HLS Study No. ATE/017 – ‘Analysis of Annatto Type B Test Substances’. Because of higher than usual levels of solvent residues in the Annatto B used in the 28 day study, a new batch of Annatto B was prepared from new samples supplied by the manufacturers. This was blended and used for the 90 day toxicology study.

These show that the preparations tested had the following composition:

Table 3: Analytical results for the test materials – 28 day palatability study (HLS Report ATE/009)

Annatto type	% Peak Area							
	% Bixin / Norbixin	Unknown	<i>trans</i> -Norbixin + <i>di-cis</i> -Norbixin	<i>cis</i> -Norbixin	Unknown	<i>trans</i> -Bixin	<i>cis</i> -Bixin	Total other
Blended B, Batch 2	84.7 (Bixin)	ND	0	4.17	0.19	0.76	94.59	0.29
Blended D, Batch 2	10.8 (Bixin)	ND	0	1.75	0.56	2.45	93.84	1.40
Blended E, Batch 2	27.2 (Bixin)	ND	0	4.16	1.15	2.41	90.03	2.25
Blended F, Batch 2	41.5 (Norbixin)	1.31	9.52	86.95	ND	0	0	2.22
Blended G, Batch 2	17.1 (Norbixin)	1.41	10.40	82.64	ND	0	0.06	5.49

Table 4: Analytical results for the test materials – 90 day toxicity study (HLS Report ATE/009 + ATE/017)

Annatto type	% Peak Area							
	% Bixin / Norbixin	Unknown	<i>trans</i> -Norbixin + <i>di-cis</i> -Norbixin	<i>cis</i> -Norbixin	Unknown	<i>trans</i> -Bixin	<i>cis</i> -Bixin	Total other
Blended B12	92.0 (Bixin)	ND	0.05	1.73	0.11	0.45	97.41	0.25
Blended E, Batch 2	26.0 (Bixin)	ND	0	4.16	1.15	2.41	90.03	2.25
Blended F, Batch 2	38.4 (Norbixin)	1.31	9.52	86.95	ND	0	0	2.22

Notes:

1. Total carotenoid expressed as Bixin (w/w), determined at 457nm.
2. Total carotenoid expressed as Norbixin (w/w), determined at 453nm.
3. Total other % = 100 – sum of named peaks %.
4. All figures reported as mean values.

4.1.2. Non-Pigment Fraction (Mass Balance Studies)

The qualitative and quantitative composition of the non-pigment fraction has been determined by comprehensive analytical work that attempted to achieve a complete mass balance (> 95%) of representative batches (Reading Scientific Services Ltd. (2005, 2006).

The following four products were investigated: annatto extract (solvent-extracted bixin) [B], annatto extract (aqueous-processed bixin) [E], annatto extract (alkali-processed norbixin, not acid-precipitated) [G], and annatto extract (alkali-processed norbixin) [F].

It was assumed in the study that the results for the solvent-extracted bixin [B] and the alkali-processed norbixin [F] would be applicable also to solvent-extracted norbixin [C] that was therefore not tested.

The samples were either tested directly or fractionated first by solvent extraction into hexane-soluble, acetone-soluble and acetone-insoluble material. The following tests were undertaken: protein (total nitrogen), ash (gravimetric), moisture (loss on drying), carbohydrate (colorimetry), sulphate and chloride (chromatography), sodium and potassium (atomic absorption spectroscopy), bixin and norbixin (spectrometry, HPLC), polyphenols (HPLC), fatty acids (GC), terpenes and other lipid-soluble compounds such as tocotrienols (HPLC, NMR), lignocellulose (NMR).

Appendix 1 summarizes the results for the four extracts studied. The non-pigment fraction of the less pure extracts (annatto extracts E, F and G) contains (beside bixin and norbixin) several well-known plant constituents: protein (1 - 6%), lignocelluloses (up to 15 %), fatty acids (up to 4%; probably as oil), polyphenols (up to 4%), inorganic salts (0.1 - 12%), waxes (up to 1.6%), and terpenoids (up to 25%). Moisture was determined to range from 4 to 90%.

Annatto extract E that is aqueous-processed, contained significant amounts of terpenoids (mainly geranyl geraniol and related di-terpenoids) at levels almost equal to the amount of bixin. Also annatto extract F that is processed using alkaline water contains geranyl geraniol at significant but not such high levels.

In contrast, the hexane-soluble fraction of extract B is considerably smaller and contains only residual levels of compounds that could not be quantified easily.

The lower levels of these compounds in annatto extract G are due to the fact that it is a liquid product that contains 90% water. On a dry weight basis its composition is similar to Annatto Extract F, however, with lower levels of geranyl-geraniol and other non-polar substances. Annatto F (norbixin as free acid) is produced by acid precipitation from the alkaline norbixin-rich hydrolysed extract, a step in which non-polar substances probably co-precipitate.

The presence of geranyl geraniol and closely related substances such as geranyl geranene (all C₂₀-terpenoid) is in line with the recent elucidation of the biosynthesis of norbixin (a C₃₀-terpenoid) that is itself derived from lycopene by oxidative cleavage (Giuliano *et al.* 2003). Lycopene, a C₄₀-terpenoid, is formed in several steps from two molecules of geranyl-geraniol-pyrophosphate.

Until now, only very limited data on the composition of the non-pigment material of annatto preparations were available. Several carotenoids, fatty acids (oil), and resins with a bitter taste were mentioned (Hager, 1972).

The quantitative/qualitative data for four different annatto extracts presented in this submission show that those components which form the coat around annatto seeds are still present in the commercial extracts. The pericarp (fruit wall) is derived during fruit development from plant cells; therefore the presence of lignocellulose, proteins, fatty acids and other cell components is to be expected.

The extract processed with organic solvents (Annatto B) does contain lower amounts of non-polar, fat-soluble compounds. Its manufacturing process involves a solvent-extraction step that separates the bixin quite efficiently from these constituents.

4.2. Possible impurities (including degradation products)

At elevated temperatures (> 70°C), degradation reactions lead to the formation of several products including a 17-carbon yellow compound known as McKeown's pigment. *trans*-Bixin is a thermal degradation product.

4.3. Analytical methods

Thin Layer Chromatography

Activate a TLC plate (e.g. LK6D SILICA GEL 60 A (layer thickness: 250 µm, size: 5 x 20 cm)) for 1 hour at 110 C. Prepare a 5% solution of the sample in 95% ethanol and apply 10 µL to the plate. Allow to dry and develop using a mixture of n-butanol, methylethylketone and 10% aqueous ammonia (3:2:2 by volume) until the solvent front has ascended about 10 cm. Allow to dry. Bixin and norbixin appear as yellow spots with R_f values of about 0.50 to 0.45, respectively. Spray with 5% sodium nitrite aqueous solution first, then spray with 0.5 mol/L sulfuric acid and the color of the spot immediately decolorizes.

High Performance Liquid Chromatography

Sample Preparation.

Dissolve 25-50 mg of sample in 50 mL of a suitable solvent. Because sample solubility may vary considerably between oil-soluble and water-soluble extracts, no single solvent is suitable for all preparations. Oil-soluble formulations (high bixin content) will usually dissolve in 3-5 mL of dimethylformamide with subsequent dilution to 50 mL in acetonitrile. Water-soluble formulations (high norbixin content) will usually dissolve in 5 mL of 0.1 M NaOH solution with subsequent dilution to 50 mL in methanol. Either preparation should be thoroughly dispersed with sonication and filtered through 0.45 µm pore PTFE membrane filters before injection into the HPLC system.

HPLC mobile phase preparation.

To prepare 1.0 vol% acetic acid, combine 10 mL of acetic acid with 1.0 L of HPLC grade water. This aqueous solution can be premixed with acetonitrile (65 parts acetonitrile: 35 parts acetic acid solution) or mixed by the HPLC pumping system as specified below.

Instrumentation and settings.

Use an HPLC with a UV/visible wavelength range absorbance detector set for the following conditions.

Column:	mixed C8/C18-bonded phase, 250 mm x 4.6 mm, 5 µm particle size (C18-bonded phase column will give similar results with slightly longer retention times)
Mobile phase:	isocratic, 65 vol% acetonitrile / 35 vol% acetic acid solution
Flow Rate:	1.0 mL/min
Detector wavelength:	460 nm
Injection Volume:	10 µL
Run Time:	40 minutes

Retention time may vary due to differences in e.g. columns and dead volume in the system. By comparing known validated standards with those obtained, identity of peaks can be determined and quantified. Accurate identification and quantification of isomers requires calibration of the HPLC method with pure, individual pigment standards. These may optionally be prepared as described in Scotter *et al.* (1994, 1998).

Method of Assay

Oil-soluble and oil-dispersible annatto composed mainly of bixin:

Transfer 0.1 g to 1 g of the sample, accurately weighed, into a 100 mL volumetric flask, add 10 mL of tetrahydrofuran and mix to dissolve pigment. Dilute to volume with acetone, and mix. Transfer a 1 mL portion of the solution into another 100 mL volumetric flask, and dilute to 100 mL. Measure the absorbance A of this solution at the peak wavelength (about 487 nm). Adjust the sample concentration as needed to obtain an absorbance of 0.2 to 1.0.

$$\% \text{Total carotenoid (expressed as bixin)} = \frac{A}{3.090} \times \frac{100,000}{\text{sample weight (mg)}} \times 100$$

Water-soluble and water-dispersible annatto composed mainly of norbixin:

Transfer 0.1 to 1 g of the sample, accurately weighed, into a 100 mL volumetric flask, dissolve in 0.5% potassium hydroxide, dilute to volume and mix. Transfer a 1 mL portion of the solution into another 100 mL volumetric flask, and dilute to 100 mL. Measure the absorbance A of this solution at the peak wavelength (about 482nm):

$$\% \text{Total carotenoid (expressed as norbixin)} = \frac{A}{2.870} \times \frac{100,000}{\text{sample weight (mg)}} \times 100$$

4.4. Rationale for proposed specifications

According to the manufacturers' submission in 2002, the ADI led to restrictions in use of annatto and a consortium of colour manufacturers (AIG/IACM) performed new toxicological studies, with a view to requesting an increase in the ADI. A number of annatto extracts were prepared that are representative of extracts currently used in food, but which focus on those containing higher than previously tested contents of bixin or norbixin. These materials were designated as:

- Annatto B – solvent-extracted bixin containing 92% bixin;
- Annatto C – solvent-extracted norbixin containing 91.6% norbixin;
- Annatto D – oil-processed bixin containing 10.8% bixin;
- Annatto E – aqueous-processed bixin containing 27.2% bixin;
- Annatto F – alkali-processed norbixin containing 41.5% norbixin;
- Annatto G – alkali-processed norbixin, sodium and potassium salts containing 17.1% norbixin.

Four of these extracts, Annatto B, C, E and F were selected for toxicological testing. Annatto B and C had the highest concentrations of bixin and norbixin respectively. Both Annatto D and Annatto E are bixin based preparations obtained from the seed by mechanical abrasion and the suspended annatto particles are effectively the same but in different suspending media. Consequently testing of Annatto E would be expected to cover the toxicology of both Annatto E and D. Annatto F and G were also regarded as alternatives containing mainly norbixin. Annatto F is the free acid norbixin while Annatto G is the sodium and potassium salts of norbixin. It is not generally required to test both a free acid and its salts, since these would normally be toxicologically equivalent except for the added sodium or potassium, and Annatto F was more palatable to the animals. The studies on Annatto C were sponsored by San-Ei, Gen F.F.I.,Inc, and were performed separately at a different laboratory.

The current proposal suggests that due to the difficulties for risk assessment and risk management, it is desirable that ADIs for annatto preparations are expressed on a pigment basis. In line with the proposed adoption of two separate ADIs for bixin and norbixin, two separate specifications for Annatto extract (bixin) and Annatto extract (norbixin) respectively would allow proper use and control of annatto extracts. The proposed specifications are based on the temporary specifications adopted by JECFA in 2003. Their relationship to them is as follows.

Proposal 2005	JECFA Specifications 2003	Synonym (AIG Code)
Annatto extract (bixin)		
Solvent-processed extracts	Annatto extract (solvent-extracted bixin)	B
Not less than 75 % pigment (expressed as bixin)	Not less than 85% pigment (expressed as bixin)	
Total pigment must contain not more than 5 % norbixin	Pigment must contain not more than 2.5% norbixin	
Aqueous-processed extracts	Annatto extract (aqueous-processed bixin)	E
Not less than 20% pigment (expressed as bixin)	Not less than 25% pigment (expressed as bixin)	
Total pigment must contain not more than 7 % norbixin	Pigment must contain not more than 7% norbixin	

Proposal 2005	JECFA Specifications 2003	Synonym (AIG Code)
Annatto extract (norbixin)		
Solvent-processed extracts	Annatto extract (solvent-extracted norbixin)	C
Not less than 70% pigment (expressed as norbixin)	Not less than 85% pigment (expressed as norbixin)	
Alkali-processed extracts	Annatto extract (alkali-processed norbixin)	F
Not less than 15% pigment on a dried basis (expressed as norbixin)	Not less than 35% pigment (expressed as norbixin)	
	Annatto extract (alkali-processed norbixin, not acid precipitated)	G
	Not less than 15% pigment (expressed as norbixin)	

Both proposed specifications were changed, redundant information was removed, the language used was checked for consistency and some proposals for simplification are made.

The following changes to single tests are of a major nature and therefore separate justification is provided:

Issue	Comments and justification for change
<i>Decreased assay levels</i>	<p>In 2003 the JECFA chose assay values which were higher than the ones requested in AIG's submission. They were obviously much closer to the level of bixin/norbixin reported for the corresponding batch used in the 90-day rat study.</p> <p>In order to cover the variability of the material of commerce, it is proposed to apply lower assay values. Since the non-pigment fraction is of no specific toxicological concern, this decision would not compromise the safety of the products.</p>
<i>Increased norbixin levels in bixin extracts</i>	<p>In 2003 the JECFA chose "impurity" values which were obviously very close to the level of norbixin reported for the corresponding batch used in the 90-day rat study.</p> <p>In order to cover the variability of the material of commerce, it is proposed to apply higher values. Since there is no specific toxicological concern for norbixin if present at the proposed levels, this decision would not compromise the safety of the products.</p> <p>The chromatographic method for the assay is also suitable for the determination of norbixin.</p>
<i>Definition includes carrier use</i>	<p>Some of the carotenoid specifications contain this entry which assures that formulated products are acceptable if the colour preparation used for them complies with the JECFA specifications</p>

Annatto extract (alkali-processed norbixin, not acid precipitated)

In 2003 JECFA did not allocate an ADI for this material (Annatto extracts G) because the material had not been tested biologically in a 90-days rat study like the other four materials. However, AIG had taken the decision not to test this extract because its composition was thought to be very close to the one of Annatto extract (alkali-processed norbixin) (Annatto F), the main difference being that G was a salt whereas F was a free acid (the latter is prepared from the former by acid precipitation).

Since this assumption was confirmed by the findings from the mass balance study (see Table 5, page), this material can be integrated into the norbixin-rich extracts for which the safety database is sufficient to apply the ADI. Due to the physico-chemical properties of the salt and the fatty acid no difference between both products with respect to absorption and bioavailability is to be expected: under the acidic conditions of the stomach and the alkaline pH of the smaller intestine they will both occur in the same dissociated form of norbixinate.

5. Functional use

5.1. Technological function

Annatto is used as a food colour in various foods depending on the nature of the extract (see 5.2).

5.2. Food categories and use levels

The following applications relate to the global use of annatto, and are not restricted to those EU approved categories listed in Annex IV of European Parliament Council Directive 94/36/EC.

Oil-based annatto preparations are commonly used to colour foods with high fat content, such as processed cheese or margarine/shortening. They are also used extensively in bakery products, biscuit fillings, popcorn and snack foods, sauces, dressings and cream desserts.

Emulsified annatto colours, in general, may advantageously be used in such products as processed cheese, ice cream, soup, confectionery and dairy products.

The more acid stable emulsified annatto finds its use in such applications as juices, liqueurs, transparent jellies and gelatinous desserts.

Water-soluble annatto has traditionally been used for colouring of cheese, but is now used in many other applications.

As the basic water-soluble annatto is not acid-proof (precipitation below pH 7), it is normally not used in clear acidic solutions (clear soft drinks without pulp), but it is very suitable in acidic foodstuffs having a matrix or solid structure.

Some present applications of the water soluble annatto colour include sausage casing, sausages, puddings, tomato sauce, breakfast cereals, butter milk desserts, chocolate fillings, smoked fish and pet food.

The basic nature of annatto extracts makes it suitable in products where the pigment is absorbed by protein and/or starch. These characteristics are extremely valuable for use of annatto in products like cheese and breakfast cereals.

As the water-soluble annatto also exists in a powdered form, applications may be extended to powdered products like instant desserts, dip mixes, health food powders and fibre tablets. These products will become yellow or orange yellow when reconstituted.

Solvent-extracted annatto is used in a variety of ways. Solvent extraction is used to produce annatto of greater than 90% purity. The solvent extract is then subsequently dissolved or suspended in oil, aqueous alkali with or without a carrier such as propylene glycol to yield the final application form. This type of product is very useful when formulating colourants with special functions. Good examples would be acid and brine-stabilised annatto, food products where light tolerance and long shelf-life is required and special colour blends containing annatto and other natural colourants, such as curcumin, paprika and carmine.

6. Reactions and fate in foods

Annatto, being carotenoids, is sensitive to oxidation in foods. This oxidation is exacerbated by the presence of light and heat. The addition of antioxidants can help prevent the loss of the annatto in foods prone to oxidation. Norbixin will precipitate at low pH and can react with metallic salts in water to give a haze. Norbixin can complex with proteins to give a more light-stable product that is redder in colour. Sulfur dioxide above 1000 ppm will cause norbixin to fade.

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Appendix 1: Mass balances for four annatto extracts

Fraction / component	Annatto extract (solvent-extracted bixin) [B]	Annatto extract (aqueous-processed bixin) [E]	Annatto extract (alkali-processed norbixin)[F]	Annatto extract (alkali-processed norbixin, not acid precipitated) [G]	
	Percent w/w	Percent w/w	Percent w/w	Percent w/w	Calculated: percent w/dry w
Hexane solubles	2.4	26.8	12.6	0.75	
Geranyl geraniol	Not detected	8.4	10.0	0.75	7.7
Aliphatic hydrocarbons (wax)	Not quantifiable	1.6	Present	-	-
Tocotrienols	<0.01	3.4	0.02	<0.01	<0.1
Other terpenoids by difference incl geranyl geranene	Not detected	13.4	Not detected	-	-
Aromatic component	Not quantifiable	tr	Present	-	-
[Acetone solubles]					
Bixin	87	29.2	-	-	-
Norbixin	-	0.9	39.0 (9-cis)	1.8 (9-cis)	18
Norbixins	2.0		8.0 (others)	0.5 (others)	5
Bixin isomers	4.0	-	-	-	-
Unknown bixins	-	1.1	-	-	-
Fatty acid esters	< 0.01	4.1	1.9	<0.01	<0.1
Polyphenols	Not detected	4.0	Not detected	Not detected	-
Moisture	0.1	9.4	4.1	90.6	-
Acetone insolubles	<1	20.4	34.5	7.4	
Protein	0.9	5.6	6.2	0.5	5
Ash	0.1	4.9	12.1	3.2	33
Carbohydrate	0.1	0.3	0.7	1.5	15
Lignocellulose	<0.1	9.6	15.5	1.5	15
TOTAL	95	95.9	>95	>99	>99