Review

A review on annatto dye extraction, analysis and processing – A Food Technology Perspective

ABSTRACT

Consumer's preference to natural colours for edible purposes is the order of the day. Annatto dye also called as poor man's saffron is widely used in the food industry and this annatto is obtained from thin resinous aril portion of seeds of *Bixa orellana*, a tropical plant of great agroindustrial interest. Annatto (*Bixa orellana,* family Bixaceae) is a tropical plant of great agroindustrial interest. Bixin and norbixin are the main components of annatto colour which imparts red to yellow hue to food matrix. This annatto is the most sought after natural colorant in food industry in view of its availability, affordability and viability. It also widely finds use in cosmetics, pharmacy and dyeing purposes. An outline of recent developments in annatto dye extraction, efforts to improve the extract yield, stability aspects of annatto color in food products, potential viable methods to be employed for better economic prospects is warranted and will be useful to prospective entrepreneurs.

Keywords: Annatto; bixin; downstream processing; norbixin; stability.

1. INTRODUCTION

Bixa orellana L. is an ancestaral multiuse plant popularly known as Achiote or lipstick tree in view of its reddish – orange dye on its seeds because, Central and South American populations used these seeds to color their bodies and lips. *B. orellana* is the only species of *Bixa*ceae family [1]. The species name of this plant is named after the Spanish scientist conquistador, Franscisco de orellana. The dye obtained from a thin, highly coloured resinous coating of triangular seeds present in brown or crimson capsular fruit (Fig.1) is called as "annatto" colourant (E-160B). Annatto is known for its lack of toxicity, its high tinctorial value and its high range of colour- comprising of red, orange and yellow hues [2]. *B. orellana* is a tropical

shrub, native to south American countries and now its effective cultivation is reported in many parts of the world [3,4]. Historical evidences indicate its extensive distribution and cultivation initially in American tropics and subsequently its spread to Rest of the world [5]. Its seeds are composed of an ‘‘inner seed’’ with a shelled kernel containing oils, waxy substances, mineral ash and alkaloid compounds, a peel comprised of cellulose and tannins, and an outer cover containing pigments, moisture, and a small amount of oils [2]. Bixin, an apocarotenoid devoid of pro-vitamin A activity, is the main oil soluble pigment found in annatto [6]. Hydrolysis of the bixin methyl ester group yields the dicarboxylic acid, norbixin, which is an annatto pigment soluble in aqueous alkaline solutions [6,7].

Annatto has been applied to the production of various foods particularly the oil-soluble annatto colour used in dairy and fat-based products like butter, margarine, cheese, baked and snack foods [8], and also in pharmacy, dyeing of leather and cosmetics [9]. Annatto usage in traditional food preparations of Central and South American countries is known at least 200 years before its commercial extracts production started in Europe and US in 1870’s for coloring milk products mainly butter and cheese. As annatto color imparts yellow to red with varied hue index as it possess high tinctorial value, hence having significance in the food industry as a natural food grade colour, and stands second in rank among economically important natural food colourants [10,11], apart from its wide use in some regions of the world for non-food applications viz., to color textiles [12], fabrics and weapons [13]. Earlier review articles on *Bixa* provided a brief information about annatto chemistry [14], its extraction methods and formulations [15], pharmacology [16], its toxicology and processing [17] and analytical methods to analyze its

colour [18]. The quality of seeds and their geographical condition too had influence on annatto dye yield as evident from various reports wherein, the ones from Peru are the best with 3-4% bixin content. Though annatto is cultivated in many countries ultimately the colour content of the seed is an important factor for economics because it varies from 1% to 4% according to morphotypes (varieties), and also cultivation conditions and post harvest methods employed to separate seeds from capsules, drying etc. [4, 19]. In India, a sporadic report on this aspect indicates significant variation in bixin content of seeds that collected from different locations of Western Maharashtra region [20]. Recent studies and and a review on annatto provided a detailed and up-to-date facts and data about its food, ethanobotanical and diversified applications as well as its improvement through biotechnological interventions [4]. Similarly Albuquerque and Meireles [22] discussed about various trends in annatto agroindustry, bixin processing technologies and market. The present review deals with the recent developments pertaining to chemistry, extraction and processing methods along with pigment stability aspects of annatto.

2. CHEMISTRY AND PROPERTIES OF ANNATTO

Annatto is reddish orange in colour, usually soft, but hard and brittle when dry. It has a peculiar sweetish odour and a disagreeable saline bitterish taste. It softens in water, to which it imparts a yellow color, but does not dissolve. The principal pigment in annatto extract is bixin, which is contained in the resinous coating of the seed itself. Bixin was first isolated by Boussingault in1825. Its molecular formula ($C_{25}H_{30}O_4$) was established by Heiduschka and Panzer in 1917. Bixin is a half ester carotenoid and more precisely a diapo-carotenoid (Fig.2).

Historically bixin was the first carotenoid compound in which geometrical isomerism was encountered [23].

On the basis of their structural characteristics, bixin and norbixin are classified under carotenoid derivatives called apocarotenoids [24], but from their chemical structure point, they come under the category "chromophores with conjugated systems" [25]. Bixin and norbixin possess nine linear conjugated double bonds thereby making them to exhibit photoprotective effect [26]. Prior to these findings, development of the annatto pigment chemistry and stereochemistry had been reviewed [14,18].

Bixin is unique among naturally occurring carotenoids not only because of *9'-cis* structure containing carotenoid (oxygenated carotenoid like lutein and belongs to xanthophylls category) but also because the molecule has two carboxylic groups, one of which is a methyl ester. So it is chemically called as a monomethyl ester of a mono-cis polyene dicarboxylic acid. (methyl hydrogen 9'-cis-6,6'-diapocarotene-6,6'-dioate) with a molecular weight of 394.49. The methyl ester group gives it some liposolubility. The alkaline hydrolysis of this methyl ester group gives the water soluble salt of the dicarboxylic acid norbixin ($C_{24}H_{28}O_4$). Bixin is a highly unsaturated compound and its conjugated double bonds act as chromophores [27]. In alkali, norbixin acts as sodium or potassium salt which is water soluble and gets good tinctorial value in water based formulations. In practice, bixin and norbixin show better light stability than many other carotenoids colors but like other antioxidant carotenoids bixin and norbixin are both unstable in presence of atmospheric oxygen. Relative to other carotenoids, bixin and norbixin have good heat stability during food processing [18]. Cis-bixin which is about 80% in annatto pigment in solution; is partially converted into the *trans* isomer and a yellow degradation product

[28]. The extent of degradation depends on the temperature and the duration of the heating process and governs the red/yellow balance.

Cis-bixin is soluble in most polar organic solvents to which it imparts an orange color but is insoluble in vegetable oil. It may be readily converted to all-*trans* isomer due to its instability in the isolated form in solutions. *Trans*-bixin is the more stable isomer and has similar properties to *cis*-isomer but exhibits a red color in solution and is soluble in vegetable oil [29]. Properties of bixin and norbixin along with their *cis* and *trans* isomers were mentioned in a report by Reith and Gielen [7]. Presence of *trans*-bixin in annatto extracts was confirmed by Yukhihino et al. [30] though *trans*-bixin isomer is not naturally present, they are formed when they are extracted using any solvents. Significant contributions in identifying terpenoids in Bixa seeds was done by [31]. The major one is all-E-gerranylgeraniol (57%) which is a C_{20}-terpene alcohol in oleoresin from Bixa seeds. Apart from this, other minor terpenes such as farnesylacetone, geranylgeranyl octadecanote and geranylgeranyl formate, delta-tocotrienol and an apocarotenoid [31].

At high temperatures that used while processing some foods fortified with annatto dye, evidences were shown for degradation of annatto dye to form both coloured degradation products and the aromatic m-xylene and tolune, however this varied with the type of foods [32].

3. REGULATIONS

The reason for mild to moderate adverse effects of annatto pigment in some people is due to decomposed products of bixin (low color intensity upon storage) such as m-xylene, toluene, toluic acid etc [33] which are reported to cause neurological effects, dizziness, nausea etc [34]. In 90's, many investigations were focused to find out thermally degraded products in annatto products by various methods such as HPCL and GC [9, 35, 36].

Annatto seems to be important natural colorant for food and drug industries owing to its potential uses as a substitute for Tartrazine which is a synthetic colourant that is prohibited in many countries [37]. Later annatto is classified as a ‘ color additive exempt of certification’ by FDA of United States of America [38]. Any polar organic solvents can be used for the extraction of annatto pigment from the aril portion of the seeds that are matured and dried to obtain the moisture content at around 8-11%. As this oxygenated carotenoid ‘Annatto’ is the most consumed natural color additive in countries such as United Kingdom [39], and also due to its increased importance, a separate commission directive for the member countries of EEC [40] was framed, which briefs about certain criteria to be followed for the quality control measures due to its wide consumption. As per this commission directive, the solvent residues in the solvent-extracted annatto should not exceed 50mg/kg singly or in combination for the three solvents acetone, methanol and hexane; and for dichloromethane at not more than 10mg/kg [40]. In this regard standard specification regarding the different residues like solvents and also for some heavy-metals that are hazardous to human health [41, 42]. Though there are reports that mention that chloroform is the solvent that efficiently extracts the color from the seeds [6]; due to its utility as a food grade colorant, it can’t be used directly. As a result they had been tried in different solvent combinations for the efficient extraction. US-FDA also follows the specifications given by FAO/WHO [41,42]. Similarly, EFSA (European Food Safety Authority) also detailed the maximum limits for methanol, acetone, hexane and dichloromethane (DCM). According to Code CFR (Code of Federal Regulations (CFR), annatto extracts should not contain more than six solvents as residues such as DCM, ethanol, 2-propanol, hexane, acetone, ethyl acetate (EA), trichloroethylene. Though there are some uniformity in limits for methanol (50 ppm) among JECFA, EU and USA, there is variation for such limits for other

solvents viz., ethanol, 2-propanol and EA (50 ppm only in JFCFA), DCM (10 ppm in EU and USA). In 2006, Joint FAO/WHO expert Committee on Food (JECFA) categorized annatto extracts in to five categories which is mainly on the basis of major pigment and method of preparation, accordingly they are: Solvent extracted bixin as Annatto B, solvent extracted nor-bixin as Annatto C, aqueous processed bixin as Annatto E and alkali-processed nor-bixin but not acid precipitated as Annatto F. Subsequently in the year 2007, annatto extracts are classified into two classes as bixin-based which is INS No.160b (1) and nor-bixin based as INS No. 160b (2) by the 39th Codex Committee on Food Additives (CCFA). In a recent study by [43] solvent residues in annatto extracts were analysed using a static headspace Gas chromatography method, wherein, presence of six residual solvents in commercially available annatto extract products that consists of seven bixin based and 16 nor-bixin based products were analysed. This reports states that the levels of methanol and acetone in bixin products was more than specified limits of JECFA. Similarly in more than 50% of nor-bixin based samples acetone levels were more than specified limits of JEFCA. In the U.S. now Annatto dye is permanently listed as acceptable for use in foods, drugs and cosmetics and is exempt from certification under the following sections of the CFR: Foods - 21 CFR 73.30; Drugs - 21 CFR 73.1030; Cosmetics - 21 CFR 73.2030. in other countries Annatto is often referred to as CI Natural Orange 4, Bija, Rocou, Orlean, Achiote or by other international code numbers viz., CI# 75120, CAS# 1393-63-1, EU# E160(b).

4. DOWNSTREAM PROCESSING OF ANNATTO

Annatto extracts are obtained *via* extraction of the color from the seeds of the fruit of the *Bixa orellana L.* tree in one or more of the approved, food grade materials taken from a list that includes various solvents, edible vegetable oils and fats, as well as alkaline, aqueous and alcohol solutions [44].

Three main commercial processes viz., (i) direct extraction into oil to get more bixin fraction, (ii) direct extraction into aqueous alkali to get nor-bixin, or (iii) indirect extraction with solvents to get more bixin are used to extract the pigment from annatto seeds. [14]. The majority of annatto on sale was reported to be directly extracted particularly in United Kingdom [45]. Hot oil is used to facilitate isomerization of the naturally occurring 9'- *cis*-bixin to the relatively more soluble *trans*-bixin. The major coloring principles 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 suspension, or hydrolyzed in aqueous alkali to produce a solution of norbixinate.

Annatto pigment can be extracted from the annatto seeds by doing mechanical abrasion using any of the vegetable oils or diluted aqueous potassium hydroxide as a suspending agent

178 and they can be further processed as mentioned earlier [28]. Another process is by extracting
179 with one or more organic solvents or in combinations of solvents.

180 Microcrystalline bixin products of 80-97% purity are available for its use as an annatto
181 concentrates. This high concentrates is possible only by using solvent method of extraction.
182 Research updates have shown the use of different organic solvents like ethanol, chloroform,
183 chlorinated hydrocarbons, acetone, ethyl acetate, hexane, methanol or alcoholic sodium
184 hydroxide, etc., either alone or in combinations at different concentrations or ratio for the
185 efficient extraction and they are further concentrated by removing the solvents. Different
186 scientific groups have worked on the different solvent systems and their combinations for their
187 efficiency of extraction. Rama Murthy and Krishna Rao [46] had used ethyl acetate, acetone,
188 alcohol, chloroform and 1,2,-dichloroethane. Similarly acetone [47] and mixture of solvents like
189 ethanol and chloroform (75 : 25, v/v) were tried [48]. The commercial preparations of annatto
190 colours with organic solvents have the disadvantages of small concentrations of pigments and a
191 residual toxic solvent in the product.
192 Commercial extracts of oil-soluble annatto pigments are obtained from the seeds by several
193 processes: such as suspension in oil, mechanical processes and solvent extraction with
194 chloroform, dichloroethane and acetone [18].

195 Based on the above criteria, and by comparing the efficiency and economy with respect
196 to the annatto pigment, attempts have been made [49] to obtain an efficient downstream process
197 that increases the efficiency of elution of pigment from the seeds. Prior to this study, though
198 there were some reports [17,50, 51, 52] and patents with respect to the extraction at industrial
199 scale for commercial purpose; but there was no work that had been done on increasing the

extraction efficiency by using different solvent combinations or by using novel different methods like mechanical abrassion; sonication and magnetic stirrer based ones using solvents.

Of six different single solvents viz., DCM; Chloroform; Hexane; Methanol; Ethyl acetate and Acetone, used for extraction through mechanical abrasion, it was evident that; chloroform and dichloromethane gave good yield with 1.154 and 1.072 % annatto pigment content respectively [53]. Also, methanol and ethyl acetate gave a yield of 0.931 and 0.492 % respectively. Acetone and hexane were not suitable as a solvent for annatto pigment from seeds which might be due to the absence of a ring at the ends of the pigment. Similarly to find out the efficiency of the combination of solvents, all the six solvents were used to obtain two solvent combinations by permutation and combination methodology and those that gave synergistically higher extracting efficiency were taken for the performance of three solvent combination.

In the two solvent combination system at 1: 1 v/v ratio, chloroform when combined with dichloromethane, gave better yield (2.374 %) than when they are in single. This shows that in combination, chloroform and dichloromethane exhibit a synergistic effect of the eluting efficiency from the seed surface through abrasion. Also, uniquely, chloroform in combination with hexane gave 2.304 % of yield [53] which may be due to the fact that, hexane elutes the cyclic carotenoids and chloroform elutes the linear carotenoids and in combination, though the cyclic carotenoids are very little in nature in annatto, it adds cumulatively to the yield [53] .

When, chloroform is combined with methanol or ethyl acetate; the efficiency of chloroform is reduced and hence, yielded only 0.96 and 0.92 % for chloroform with methanol and chloroform with ethyl acetate respectively. Similar is the case for dichloromethane's combination with methanol or ethyl acetate with 2.108 and 2.083 % respectively. However, the

stability of consistency towards the extraction without altering the property appears to be much better in dichloromethane than chloroform [53] . Combination of methanol and ethyl acetate gave 1.979 % which is far better than the methanol or ethyl acetate in single which could not be explained. Based on the performance of the solvents in the two solvent combination system; as chloroform and dichloromethane combination gave better response hence could be selected to study three combination system by adding some combinations. Though chloroform and hexane gave better response, it was not recommended due to the fact that this is just a cumulative effect of the solvents than the increase in the eluting efficiency of a solvent by another. In that sense, another two solvent combination *viz*., methanol and ethyl acetate (1.979 %) gave better response than what they performed singly hence could be used. This shows that the two combinations chloroform & dichloromethane; and methanol & ethyl acetate had increased the annatto pigment yield by increasing the eluting efficiency synergistically [53]. From those two combinations, each component of a combination was combined with the other combination to give four combinations of three solvent combination system. They were, chloroform, dichloromethane, and methanol; chloroform, dichloromethane, and ethyl acetate; methanol, ethyl acetate, and chloroform; methanol, ethyl acetate, and dichloromethane. Among the combinations: chloroform, dichloromethane, and methanol; chloroform, dichloromethane, and ethyl acetate exhibited in a similar trend as like the single solvent of methanol and ethyl acetate respectively. However, only the yield is reported to be doubled due to the presence of chloroform and dichloromethane in those two combinations of three solvent systems. This means that, whatever the yield obtained by using methanol (0.931 %) and ethyl acetate (0.492 %) as a single solvent is proportionally increased (doubled) in case of chloroform, dichloromethane, and methanol (1.821 %); and chloroform, dichloromethane, and ethyl acetate (0.955 %) respectively [53] . In case of

the other two three solvents combinations, viz., methanol, ethyl acetate, and chloroform; methanol, ethyl acetate, and dichloromethane yielded 1.36 % and 1.778 % respectively. Which is less than any of the two solvent combinations and hence this cannot be efficient for the extraction of annatto pigment [53]. In general, of all the solvent combinations used, chloroform and dichloromethane combination efficiently extracted the annatto pigment from the seeds (2.374 %). Hence, to test the efficiency of the method of extraction; two methods of extraction viz., mechanical abrasion and magnetic stirrer based methods were tested [53] . The results showed that magnetic stirrer gave a yield of 0.194 % with the chloroform and dichloromethane solvent combination though the best one was by mechanical abrasion (2.374 %). This shows that, for efficient extraction of annatto pigment, it is not only the solvent system that matters, but also the method of extraction is important in such a way that there is a mechanical friction created between the seeds and the solvent system for the efficient extraction [53] .

Also vegetable oil based extracts were also made to avoid residual effects of solvents or other heavy metals for their use for food grade. It may be recalled; that FAO/WHO [41] framed the policies and regulations for the use of different solvents for extraction of annatto pigment that can be used for food grade with their acceptable residual values. There are different reports earlier for oil based extraction of annatto pigment [54] , wherein, color intensity and hue of the extract was recorded using a color measurement system, however, annatto pigment content was not quantified. Suspensions of annatto pigments in vegetable oil are more concentrated but can contain several degradation products due to the fact that high temperatures (>100 ºC) are used in the extraction process [6, 7] . 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 and oil suspensions of annatto usually contain 0.1 – 8% bixin.

Significant contributions have been made by researchers in India on annatto dye preparation. Bahl et al [55] prepared bixin and methyl bixin from seeds of *Bixa orellana,* which is mainly based on soxhlet based method wherein, ehthyl acetate was used for extraction of bixin and methanolic potassium hydroxide with dimethyl sulphate were used for methyl bixin respectively. Similarly, Murthi et al [54] demonstrated the efficacy of ground nut oil to extract annatto dye from seeds and suggested it as an alternate for the castor oil. A process optimization for bixin extraction from seeds of Bixa and its purification was reported by Koul et al [56] , which could yield upto 18.6% pure bixin. In another study, the different vegetable oil that have been used, viz., refined oil, castor oil and coconut oil; coconut oil gave best yield (2.897 %) and it was comparatively higher than the best solvent system (chloroform and dichloromethane). The yield of refined oil and castor oil were 1.737 % and 2.405 % respectively (Table 1). In case of oil extraction, the method of mechanical abrasion was used to increase the efficiency of extraction. Though Shuhama et al [57] had reported about the spouted bed dryer, the yield is not efficient and is a method of concentrating the pigment than the extraction methodology.

In a recent report, Chuyen et al [58] have demonstrated improvement in bixin extraction yield, and also extraction quality from annatto seed by modification and combination of different extraction methods. In this study 67.3% bixin yield was achieved by using acetone (Table 1). Even the combined extraction using sodium hydroxide solution (at 50^0C for 40 min) followed by soybean oil (at 100^0C for 20 min) resulted in 53.7% bixin yield. This study also showed the presence of very low levels of undesirable volatile compounds in the annatto extracts, when the entire extraction was carried out in absence of light.

In another study [22, 59], researchers have applied super critical carbon dioxide method as a pretreatment for defatting of annatto seeds. Subsequently bixin was extracted (22 mg/ gm of seeds) and economic evaluation of the process was shown as 300.00 US$/Kg of extract for the pilot plant with 2 vessels of 0.005m3 (Table 1).

Irrespective of the method of extraction either using oils or using solvents, bixin can be hydrolyzed into norbixin under specific conditions of temperature and pH, the dicarboxylic acid and saponified into the potassium salt of norbixin. At elevated temperature (>70 ^{O}C), annatto pigment gets degraded and form several products including a 17-C yellow compound known as McKeown's pigment [28]. Supercritical extraction with CO_2 could be a good alternative to avoid these problems [60]. Studies of Annatto pigment extraction have been carried out using supercritical CO_2 [61, 62, 63] and CO_2 modified with several entrainers (methanol, chloroform and acetonitrile) [64] . It was shown that the entrainers increased the efficiency of extraction. Supercritical CO_2 with different pressures and temperatures to extract natural food colors from annatto seeds was found to be advantageous for removing compounds from complex food systems than conventional methods [62] because of least thermal effects on products, high quality of recovered products, low energy requirement for solvent recovery, and high selectivity in the separation process [62] . Subsequently, Anderson et al. [64] reported supercritical fluid extraction with a combination of static and dynamic modes of extraction for extraction of nor-bixin . Supercritical fluid extraction of pigments from annatto seeds with CO_2 modified with ethanol showed high recovery of pigment [65] . Recently, micronization of natural bixin using super critical CO_2 as antisolvent is also being studied [66] . However, this supercritical carbon

311 dioxide fluid for the extraction of pigment from annatto seeds appears to be less feasible from
312 economics and efficiency aspects [63, 64, 65] .

313 Recovery of nor-bixin from a raw extraction solution of annatto pigments using colloidal
314 gas aphrons (CGAs) is reported [67] . Potassium norbixinate in annatto solution interacts with
315 surfactant in aphron phase leading to effective separation of nor-bixin and 94% recovery. There
316 are also reports on the production of annatto powder by spouted bed dryer method [57] and
317 mechanical extraction of bixin from annatto seeds by spouted bed method [68] . Solvent based
318 extraction using chloroform and dichloromethane yields 5-7% (w/w) of annatto with bixin
319 content of ~ 95% and alkaline treatment provides norbixin extract with 10% yield. Spouted bed
320 dryer method yields about 15-24% bixin.

321 Scientists of Central Food Technological Research Institute (CFTRI), Mysore, India
322 have developed downstream processing of annatto pigment [69] , particularly for isolation of
323 bixin (Indian Patent 737/DEL/2005) and also made a process for the formulation of spray dried
324 acid stable annatto dye (nor bixin) [69] . Another process for the production of 'Annatto Dye'
325 (Process code: CPS-1500), wherein the crystal like pure form of bixin was produced at CFTRI,
326 Mysore, which involves the batch type percolation technique using current extraction of annatto
327 seeds with selective solvents and further solvent recovery and vacuum dehydration of
328 concentrated dye to a crystal like form.

329 **5. ANALYSIS OF ANNATTO**

330 Once the pigment had been extracted, purified and concentrated; they have to be confirmed for
331 the presence of the pigment that has been extracted. With today's science and technology, there
332 are advanced techniques and analytical equipments to identify and confirm the presence of the

pigment that have been extracted for its purity and quality and also for its quantification. Recently, TLC analysis of food colourants from three morphotypes of *Bixa orellana* was carried out by Seal et al [70] ,wherein they tried to describe a simple solvent extraction method for the extraction of colorants from the three morphotypes. Unfortunately this study neither mentioned the details of morphotypes, it significance for different colours in the extracts, nor shown identity of the each of the three colour spots found in TLC. The extraction of annatto using solvents mixtures was shown to be efficient at least for separation of three distinct spots by TLC though the yield of the extract was not documented [70]. Bixin fraction of annatto pigment is lipophilic in nature, but the nor-bixin is hydrophilic. In view of this, crude extracts rich in bixin are often subjected to alkali treatment to get nor-bixin which is soluble in water, but the protonated form of nor-bixin formed after acid-precipitation and purification becomes insoluble.

With respect to bixin and norbixin – they can be identified and quantified using High Pressure Liquid Chromatography (HPLC), UV-VIS spectrophotometry (UV-VIS), Nuclear Magnetic Resonance (NMR), and Mass Spectrometry (MS). Details about various aspects of analytical methods employed for annatto dye analysis were reviewed [17,18]. In brief, they are:

5.1 UV-Visible spectrometry

The UV-Visible spectroscopy for annatto has been well studied and documented [6, 7, 71]. Historically, chloroform has been used as solvent for the spectrophotometric analysis of bixin and dilute sodium hydroxide (ca. 0.1M) for norbixin. The absorption maxima are 470 and 500nm for bixin.

5.2 HPLC analysis

Literature references on the application of HPLC to the separation of annatto colouring components are sparse. Early methods on HPLC analysis of annatto extract [72,73] reported the use of an isocratic reverse-phase system employing an ODS column and methanol/aqueous acetic acid mobile phase wherein the *cis*- and *trans*-isomers of both bixin and norbixin were separated within 10 minutes. But, the peaks of *cis*- and *trans*-bixin were not fully resolved and the shapes were generally poor. Later a method for the reverse-phase separation of bixin, norbixin and three curcuminoids using both isocratic and gradient elution systems, comprising of Zorbax ODS column and water/THF mobile phase was developed that gave better chromatographic separation [74]. However, only separation of the 'main' annatto coloring components was reported and no reference to stereoisomer separation was given. The analytical HPLC-photodiode array (PDA) method developed by Scotter et al. (1994) provided superior qualitative and quantitative data compared with UV-VIS spectroscopic methods [6, 73]for determining the colour content (as bixin and norbixin) in 21 commercial annatto formulations, particularly with respect to the coloured thermal degradation products [9, 29].

The method developed by Scotter et al. [29] has played a key role in the advancement of HPLC capabilities for the separation and characterization of norbixin and bixin isomers, and has been refined and adapted for the study of annatto stability and for the determination of annatto colouring components in colour formulations, foodstuffs and human plasma.

Although several methodologies have been reported for the HPLC analysis of annatto pigment it is not must that they have to give the same result always. The elution time for bixin and nor-bixin may vary depending upon the column type, length, diameter, ratio of the solvent system, brand of the instrument etc. Sometimes even after using the reported procedure, column

dimensions and solvent system it may not be possible to get the peaks at the same elution time. Therefore it is always better to compare the results obtained with standards for the same.

5.3 Mass Spectrometry

In common with other carotenoids, the MS spectra of bixin and norbixin are characterized by fragmentation leading to losses of toluene and xylene from the polyene chain and the structural significance of the intensity ratio of the ions related to the number of conjugated double bonds. Solid probe electron ionization (EI+) was used to confirm the structures of isolated and purified bixin and norbixin isomers [29]. Both the 9′-*cis*- and *trans*-isomers gave a molecular ion at m/z 394 (bixin) and m/z 380 (norbixin). In a later study, similar analytical conditions were used to characterize the 17-carbon major thermal degradation product of annatto [35].

The structure of bixin family of apocarotenoids was determined by EI+ and fast atom bombardment (FAB) MS [75]. Bixin structure was also studied using electrospray ionization (EI) and high resolution (HR) matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry [76]. TOF-MS with X-ray photoelectron spectroscopy was employed to ascertain the major carotenoid composition of *Bixa orellana* seeds [77-78]. The presence of bixin was revealed in the seed aril without any sample pretreatment from the detection of ions attributable to [M+2H] at m/z 396 with associated ^{13}C isotope analogues at m/z 397 and 398. In a related study, Bittencourt et al. [79]analysed extracts of *Bixa orellana* using TOF-MS as a means of characterising thermal effects. The spectrum was characterised by a large number of peaks generated by the principal ions and their multiple fragmentation patterns but also, more notably, by the presence of ions at m/z 790, 804 and 818, attributed to the presence of dimmers.

More recently, it has been shown that HPLC- PDA in combination with ion-trap elelctrospray mass spectrometric confirmatory analysis can be used to identify and measure norbixin and bixin in meat products using precursor ions [81].

5.4 GC Analysis

A method has been developed that uses ambient alkaline hydrolysis followed by solvent extraction and gas chromatography (GC), to analyse the annatto colour formulations for the presence of main aromatic hydrocarbon thermal degradation products m-xylene and toluene [36]. GC-MS is also used to analyse the volatile compounds present in water and oil soluble annatto extracts and this study revealed that annatto extracts contain odorants which has potential to influence the aroma of food [82]. The lipid fraction of annatto seeds has been analysed by GC-MS and they showed the presence of tocotrienols, mainly δ-tocotrienol, but no tocopherols [83]. GC-MS analysis studies on the seed oil of Bixa reported its chemical compostion [84]. 35 components were identified in *Bixa* seed oil of which the major ones are farnesyl acetate, occidentalol acetate, spathulenol and ishwarane. GC-MS has also been used to analyse the presence of bixin, norbixin and geranylgeraniol in supercritical CO_2 extracted annatto [62].

5.5 NMR Analysis

The earliest application of NMR in the study of bixin stereochemistry used low resolution (40 MHz) instrumentation to assign ^{1}H frequencies and deduce that the *cis* bond of the methyl analogue of 'natural or α-' bixin was in the 9′- (equivalent) position [85]. The high frequency shift of the proton assigned to H-8′was attributed to deshielding by the 11′-12′ alkene bond when compared to the *trans*- (or β-) isomer, which was confirmed via synthesis and more

423 detailed structural assignments [86]. Fourier transform (FT) NMR was used later to assign the
424 ^{13}C spectra of methyl *cis*- and *trans*-bixin using deuterated compounds, however no experimental
425 details were given and assignments were partly derived from spectra of carotenoids with similar
426 structural characteristics [87]. The ^{1}H FT-NMR spectrum of cis-bixin and cis-methyl bixin at 250
427 MHz has been reported but is limited to assignment of the terminal acrylate moieties [31].

428 The most comprehensive study to date on the determination of the structure of the bixin
429 family of apocarotenoids is by Kelly et al. [75], who utilized a combination of 1D and 2D NMR
430 techniques in conjunction with mass spectrometry and X-ray diffraction analysis. Chemical shift,
431 coupling constants and ^{1}H correlation data were examined alongside the ion abundances and
432 intensity ratios from standard electron impact (EI+) and fast atom bombardment (FAB+) MS
433 spectra, and bond measurement, cell dimension and degree of hydrogen bonding from X-ray
434 diffraction data to elucidate and compare the crystal structures of the *cis*- and *trans*- isomers of
435 bixin and methyl bixin.

436 **5.6 Other analytical techniques**

437 There are several less widely known techniques that have been used in the study of
438 annatto either alone or in combination with complementary techniques. These include infra-red
439 spectroscopy, where the characteristic strong absorption due to the C=O stretching frequency and
440 the complex bands due to C-O single bond characteristic of esters and carboxylic acids has been
441 exploited [7, 62, 88, 89]. Photoacoustic spectrometry in the UV, VIS and IR regions has been
442 used for the qualitative and quantitative analysis of annatto in commercial seasoning products
443 [90] and more recently in the determination of the triplet state energy of bixin [91]. X-ray
444 photoelectron spectroscopy was employed by Felicissimo et al. [78] to ascertain the major
445 carotenoid composition of *Bixa orellana* seeds and X-ray diffraction in conjunction with NMR

and mass spectrometry has been used to determine of the structure of the bixin family of apocarotenoids [75].

6. Stability of the annatto dye during processing of foods

The stability of the added annatto dye in foods is the most important parameter which is essential especially from quality and aesthetic point of view. Though bixin part of annatto pigment is highly stable compared to other carotenoids such as betacarotene, etc., which is mainly due to its apocarotenoid nature, various studies revealed that bixin too is susceptible to processing and storage conditions especially to high temperatures and light which leads to a loss in the color of the annatto added foods [92, 93]. Similarly the effect of water activity is reported to be having influence on bixin stability, wherein, bixin is more stable at intermediate and higher water activities [94].

It is imperative to have a knowledge of the structure of pigment molecules, stability against heat, light, pH and oxygen during processing of respective annatto dye added foods, especially in complex food matrices containing proteins or carbohydrates [95]. As industry requires, such information, noteworthy attempts were made by various researchers in this regard. Maga and Kim [96] studied the stability of oil based annatto formulations in extruded doughs, wherein some loss of added pigment was occurred. Similarly, Berset and Marty [92] carried out thermal stability studies in corn starch and found better stability of added dye, which indicates that the stability varies with the dough. The effect of various cooking temperatures, cheese processing conditions, emulsifying agents too had varied effects on annatto stability in cheese [97]. Annatto emulsions showed less stability upon heating than in solutions or suspensions. Incorporation of gamma-tocopherol along with annatto significantly improved the antioxidant potential and also

468 the added dye stability [98]. Ferreira et al [99] analysed the stability of commercial water-soluble
469 annatto solutions and found that there was gradual shift of redness to yellow shade in bixin at
470 high termperatures and nor-bixin too succumbed to some degradation.

471 Prabhakara Rao et al [100] have studied the storage stability of water –soluble annatto
472 formulations in orange RTS model systems wherein it had good stability compared to working
473 stock of the formulations. The oil soluble bixin and water soluble nor-bixin annatto preparations
474 with virgin olive oil polar extract were assessed in bulk olive oil and oil-in-water emulsions
475 stored at 60^0C for its antioxidant potential [101]. Norbixn with ascorbic acid, ascorbyl palmitate
476 and delta or gamma tocopherols exhibited improved antioxidant effect which is more than that of
477 phenolic antioxidants [101].

478 Similarly the studies on impact processing conditions on the stability of annatto dye incorporated
479 in some baked and fried snack foods indicates that high loss of colour in fried items as most of
480 the added annatto leached out into oil [102]. Apart from this, foods subjected to pressure cooking
481 showed more loss of added annatto than microwave cooking [102]. By using alpha- cylcodextrin
482 inclusion studies, Lyng et al [103] showed that the complexed form of bixin is more resistant that
483 free bixin to the damage caused by light and air and also showed better water solubility, which
484 are very important parameters for novel formulations.

485 While using commercial annatto oil-solutions for colouring foods, it is necessary to take a note
486 that the loss of color would be very high in case of dry powder than to the oleoresin under varied
487 storage conditions and it was recommended that the dye can be effectively stored in the oleoresin
488 form until its use for food formulations [50, 104]. In order to enhance its effective utilization in
489 food industry for wide range of applications a micronencapsulation of the annatto pigment with

chitosan by spray drying in different solvents was investigated by Parize et al. [105]. In sausages and meat products, sodium or potassium nitrite is widely used as curing agent for various purposes including imparting color to the sauce and meat. Partial replacement of nitrite by annatto as a colour additive in sausages under industrial conditions was studied which indicates the efficient retaining of added colour in samples containing 60% of annatto color [106]. In a study Rao et al [107] have shown the efficiency of water soluble annatto dye sugar powder formulation (5mg/kg and 30mg/kg) to obtain required color shades of sweetmeats such as jilebi and jangri which are well known Indian traditional foods. In a recent study, stabilization of a hydrophobic annatto dye by intercalation into organo-montmorillonite against irradiation with visible light was investigated [108]. Apart from this, the influence of microwave based method [109] on extraction and stability of annatto dye was studied.

7. New source of Bixin?

For several decades *Bixa orellana* was considered to be the only source of natural pigment bixin, but recently a group of scientists from VIT University, Vellore have claimed an alternative and competitive source for natural bixin production. Using comparative genome sequence analysis Siva et al [110] reported identification and functional characterized the bixin coding genes that present not only in *Bixa orellana* but also in *Crocus* and *Vitis*. Chromatographic studies based on TLC, FT-IR and GC-MS made the presence of bixin evident in these two organisms. However, further confirmation is warranted through molecular techniques.

8. Conclusion

During the last three decades various extraction and downstream processing methods in the form of publications, patents and processes were reported to produce either bixin or nor-bixin form of

annatto dye, and many of these are having their own advantages and impediments. The purpose of annatto dye utilization i.e. to impart colour to foods, as a cosmeceutical in body care products, as a dye in textiles, or to use in pharmacy as pharmaceutical and also as dietary supplement, has to be taken into consideration to choose the right method of extraction to obtain wide range of hue index with high tinctorial value, to get rid of solvent residues, and also to obtain good color stability. In fact, a good number of technologies that available as on today and various annatto dye formulations available in market are the outcome of all these remarkable investigations over the years. Most of the recent findings concerning to extraction yield improvement and purity of bixin and nor bixin [111, 112] are to be looked further to fine tune these technologies that are already in use. It is known that the method of extraction, matters a lot in annatto dye processing followed by retaining its stability in annatto added foods in food processing industry. Subsequent to the selection of appropriate technology there is a need to optimize the process with proper kinetics data which is essential to design the process development and to intend cost of manufacturing for economic evaluation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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841 **Figure 1:** Annatto yielding *Bixa orellana* plant. **A:** Whole plant; **B:** Flower; **C:** Fruit bunch; **D:**

842 Dehiscenced fruit with *Bixa* seeds

843 **Figure 2:** Chemical structure of Bixin and Norbixin.

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846 Table.1 Some of the important methods for annatto extract yield and purity

SNo	Type of method	Yield and purity	Reference
1	Extraction of seeds with Chloroform (1:5 ratio)	1.6% pure bixin crystals (seed weight basis), i.e. up to 59% of total pigments	[6]
2	Boiling with ethyl acetate	1.1% bixin crystals	[55]
3	Super critical CO_2 method	~1% of pigment	[62]
4	Super critical CO_2 method with a combination of static and dynamic modes of extract with CO_2	2.7 mg bixin per g.d.w.	[64]
5	Extraction and purification of bixin by mechanical agitation and solvent	18.6 % pure Bixin	[56]
	Spouted bed drying of aqueous extract of annatto	High yield of ultrafine powder of dye ~15%	[57]
6	Caster oil method	13.25% total dye	[50]
7	Colloidal gas aphrons method	3.26% (w/w) of norbixin in seeds Extract Yield 81% 94% recovery of norbixin	[67]
8	Super critical CO_2 extract with ethanol	45% recovery of bixin 13.7 g/dm3 extractable pigment	[65]
9	Extraction of bixin using super critical CO_2	The solubility of 93% pure bixin achieved	[63]
10	Spouted bed method	15-24% of bixin recovery	[68]
11	Solvent method using Chlroform and dichloromethane	5-7% total annatto dye (w/w)	[18]
12	Extraction with chloroform followed	norbixin yield of 10%	[17]

	by alkaline treatment		
13	Improved method for bixin extract and yield quality	Highly purified bixin	[58]
14	Downstream processing of annatto using solvent method	Pure crystals of bixin 3% (w/w) of crystalline dye Which contains ~1% of bixin (seed weight basis)	[69]
15	Spray dried method	High yield of norbxin	[17, 69]
16	Separation of norbixin from raw dye obtained from seeds	99% recovery of norbixin	[111]
17	Microwave assisted extraction of natural colorant from seeds of *Bixa orellana* with the aid of RSM and ANN models	Possibility of Efficient extraction of annatto dye	[109]
18	Aqueous two phase extraction method	Purification of norbixin	[112]

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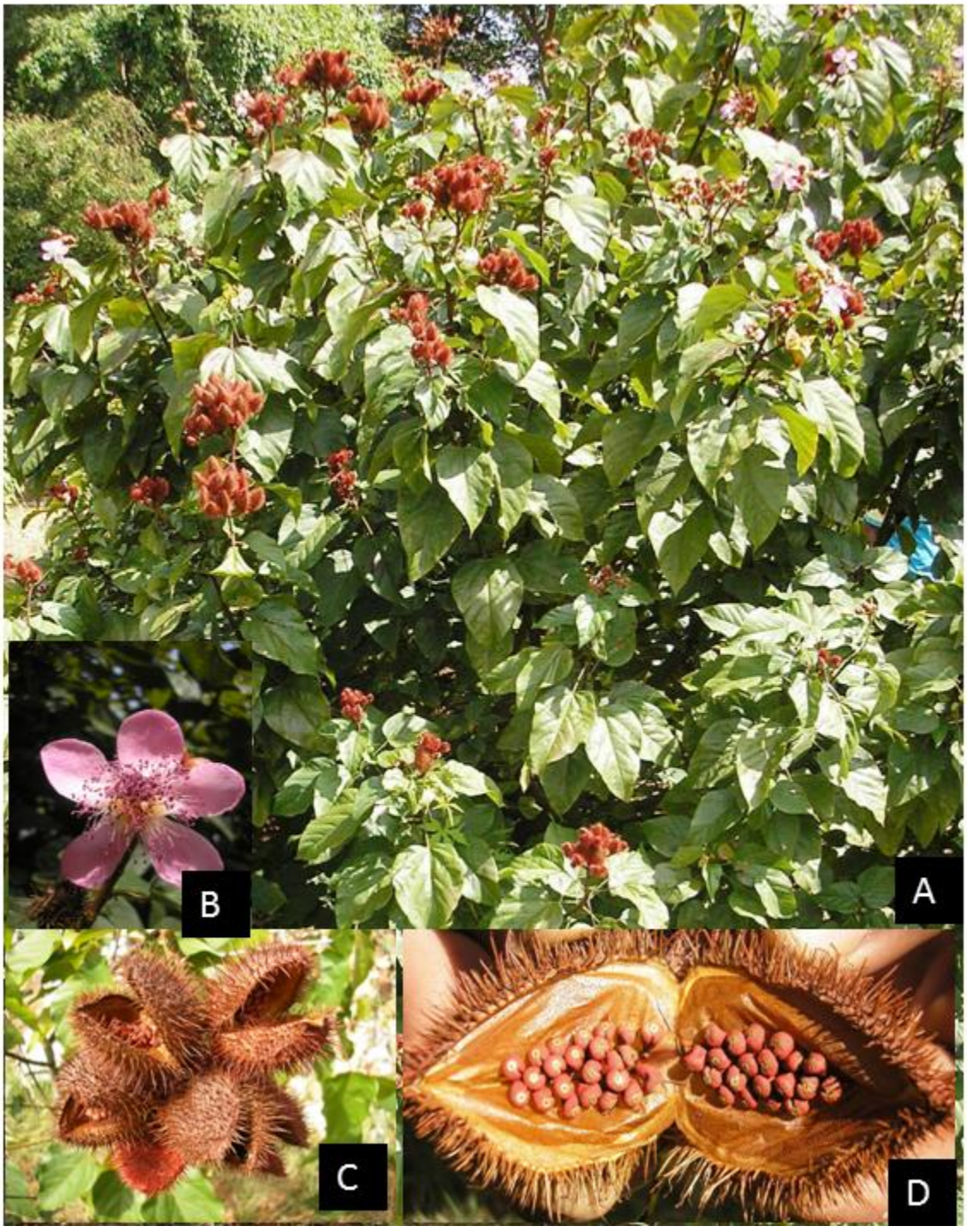

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850 Fig. 1

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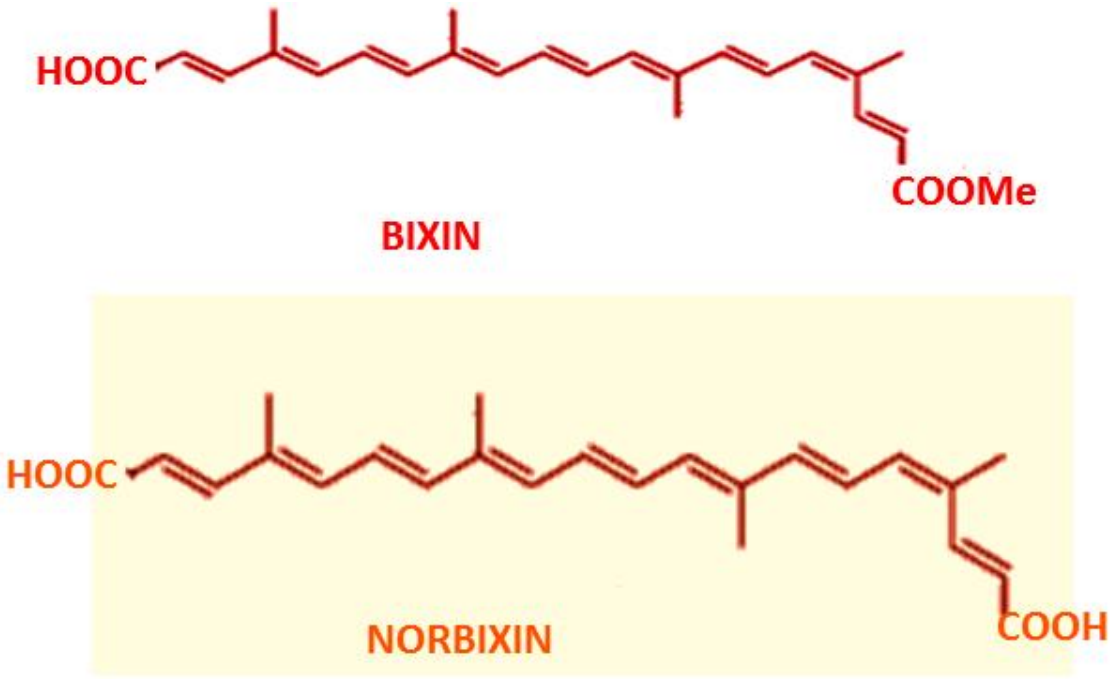

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855 Fig. 2