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Stability of organic food colorant extracted from *annatto* seeds on food matrix

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Abstract
This study was conducted at the laboratory of Food Technology and Rural Industries, Bangladesh Agricultural University and Department of Chemistry, BAU, Mymensingh, Bangladesh. It was concerned with the extraction of annatto extract, a well-known natural food colorant, and the effect of solvents, food matrix, temperature and light on the stability of the color. During extraction, the reflux method gave the highest yield (10.1%) while the soxhlet method yielded 7.1% of dyes, Water soaking method yielded 8.93% of annatto color during 18 hour soaking period. The best background color was found at a concentration of 500 ppm. The annatto color was found quite stable in food matrix. Absorbance of annatto color exhibited almost unchanged over all sugar solution regardless of concentration. Absorbance reading and heating showed a linear relation with negative slope but with low value of slope of regression line. Annatto color was found highly sensitive to light. Oxidation was rapid within first 2 hours of incubation. There is minor change in the intensity of annatto color with the change of pH. Beyond the pH 3.8, color intensity as measured by absorbance reading showed almost close to control.

Keywords: annatto, seed, extraction, stability

1. Introduction

1.1 Developments of natural color

The past 10-15 years have been a distinct move towards naturals, especially flavours and colors (Cathy, 1999) [3]. The move is particularly pronounced in the UK, Scandinavia and the northern part of continental Europe. Many consumers associate natural products with superior quality and a good, natural-looking color in a food or beverage will signal high quality whilst a washed out or artificially bright product can give the opposite impression. Also, in relation to colors the fact that they are derived from well-known sources such as beetroot, grapes, cabbage and paprika, makes the consumers feel safer and thus recognition and acceptance are easier. The general health trend revealed that consumers are increasingly aware of functional foods. Many different nutrients are today applied in functional foods and more will be added over the coming years. Some of these are natural color pigments, which have only recently been recognized for their possible health effects.

Those pigments that are presently acknowledged for their nutritional properties are a number of carotenoids and anthocyanins. Natural caroteniods include cararotenes, lutein and lycopene and have been recognized as antioxidants that are linked to the prevention of degenerative diseases (Seddon *et al*., 1994; Hankingson *et al*., 1992 and Giovannuci, *et al*., 1995) [13, 9, 7]. It is fact that epidemiological evidence of the nutritional benefits of fruit and vegetables points to a range of carotenoids rather than a single carotenoid providing these benefits.

Another consequence of the health trend is the rising interest in and demand for reassurance concerning product quality, food safety and production methods. The most visible result of this is the increase in organic products on the market, where the total organic penetration of the EU food and beverage market is expected to be 9.2% in 2004 (Hugo and Siddika, 1999) [10]. The demands on the natural colors industry will not only be for organic colors but also for a generally higher level of information with regard to production methods, specially HACCP, as well as traceability of all ingredients.

In general, competition is increasing within the food and beverage industries and greater pressure is put on new product development. In their search to differentiate product developer are looking at new ingredients options and natural colors are one of many possibilities. Simultaneously the natural colors industry is required continiously to bring forward new coloring opportunities to match the increasing demands of their customers. Future developments are expected to concentrate on improvements of well-known technologies within the formulation and processing of existing color pigments. Besides the new technologies solutions, natural color manufacturers are also looking at a number of new pigment sources. One of the limitations in developing totally new color formulations is the lengthy and costly safety testing and regulatory approval process. Therefore, 'untapped' sources of raw materials that conform to current regulations give valuable options to develop new color products.

Color is one of the most important attributes of foods considering quality indicator and acceptability. The common practice for coloring food is to use synthetic azo-dyes, which is considered low in cost and high in stability. The recent research revealed that food colored with synthetic dyes associated with numerous health affects specially hyperactivity in children (McCann *et al*., 2007) [11]. The health-benefit of natural pigments have been focused by many works, especially those of anthocyanins and

carotenoids, whose antioxidant properties have been extensively studied (Azeredo, 2009) [1]. For these reasons people have increasingly avoided synthetic colorants, preferring natural pigments, which are considered to be harmless or even healthy. These requirements compelled numerous regulation changes worldwide. The current market for all food colorants is estimated US $1 billion, with natural pigments responding for only one fourth of the total. However, the market for synthetic colorants has been tended to decline in favor of natural ones (Fletcher, 2006) [6]. Nature produces a variety of compounds adequate for food coloring, such as water soluble anthocyanins, batalains, and carminic acid, as well as the oil soluble carotenoids and chlorophylls. Chlorophylls, carotenoids, anthocyanins and betalains are the principal groups of pigments that present in fruits and vegetables. The chlorophylls are green, the carotenoids yellow, orange or red, the anthocyanins blue or red and the betalains red or yellow. Among the common colorants, carotenoids are probably the best known of the colorants and certainly the largest group of pigments produced in nature with an annual production estimated at 10 billion tons. Most of these are fucoxanthin produced by algae in the ocean and the three main pigments, lutein, violaxanthin and neoxanthin in the ocean (Scotter, 2009). Over 600 carotenoid compounds have been reported. Research have been continuing to search alternative source of carotenoid, which is easy to extract and cheap.

1.2 Annatto

Annatto is the seed of the bush, *Bixa orellana*, which is mainly found in Central and South America and in East Africa. Traditionally, annatto seeds are also used as a spice, often blended with other ingredients before addition to soups and meat dishes. The seeds grow in large clusters of capsular fruits that upon harvesting are dried in the sun, cracked open and taken out by hand (Fig. 1).

Fig 1(a): *Bixa orellana* tree with fruits

Fig 1(b): Annatto seeds

The bixin and norbixin are the principal color of Annatto (Fig 2). Bixin are the apocarotenoid and nobixin is resulted from bixin when the methyl group is removed. The basic color pigment is bixin, a natural carotenoid that is found in the thin resinous coating of the seed. It can be extracted in different ways to yield oil-soluble extracts, oil suspensions or water-soluble extracts.

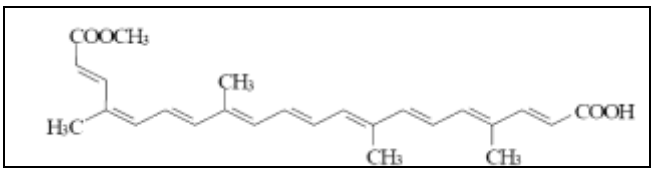

Fig 2(a): Chemical structure of bixin

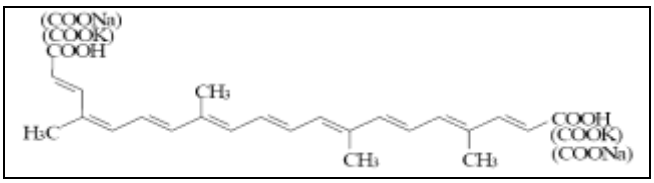

Fig 2(b): Chemical structure of norbixin

Oil-soluble extracts are made by extraction of annatto seeds with hot vegetable oil, resulting in products with low color content, typically 0.1–0.2% bixin, as bixin is poorly soluble in oil. By suspending the undissolved color pigment in vegetable oil or fat base the content of bixin can be increased by up to 5%. Oil- soluble extracts and oil suspensions are yellow-orange and suitable in products with a high quantity of oil present, i.e., margarine and butter, salad dressings and extruded snacks.

By extracting the annatto seeds with water and potassium hydroxide, bixin is converted into the water-soluble pigment norbixin. Water-soluble solutions typically contain 0.5–4% norbixin, and powders with concentrations as high as 15% can be achieved through further spray-drying. Bixin and norbixin can also be blended to produce colors applicable in both water and oil-based foods. Typical applications for norbixin products are cheese, ice cream and water ice, bakery, sugar confectionery and beverages, where they give a yellow-orange color. Both norbixin and bixin are reasonably stable to heat, whereas light stability is best if norbixin is bound to protein, i.e., in cheese. Norbixin may precipitate at low pH and cannot be mixed with products containing calcium. However, it is possible to formulate norbixin products that are stable at low pH.

1.2.1 Extraction and yield of annatto dye

According to Bhoomika (2006) [2] five different methods, i.e. oil extraction, aqueous alkali extraction, plain water extraction and solvent extraction using acetone and chloroform as solvent were tried to extract the dye from annatto seeds. The solvent extraction method resulted in the highest dye yield (3.2%), followed by aqueous alkali extraction (3%). The least was in plain water extraction (0.9%). The dye yield in different accessions ranged from 101.29 to 255.65 g/tree with an average of 148.62 g/tree.

1.2.2 Stability of natural dye

The common molecular features which give rise to absorption in the visible region, i.e., a conjugated double bond system, electron withdrawing and donating constituents and in the case of tetrapyrroles reactivity and oxidation state of the central metal will be crucial to the reactivity and stability of food colorants. The main practical

aspects need to be considered in relation to food processing are oxidation, pH, thermal degradation and additives.

1.2.3 Oxidation

It is well established that unsaturated fatty acids undergo oxidation, via a radical reaction mechanism. Carotenoids undergo similar reactions and indeed do this so readily they can act as antioxidants in food materials. This antioxidant ability of carotenoids derives from their ability to form a resonance stabilized free radical. In certain controlled conditions chemical oxidation of carotenoids can give rise to epoxide formation and isomerization of this to a furanoxide (Wong, 1989) [14]. The epoxide formation has been shown to occur in canned fruit juice and can give rise to considerable loss of color. This loss of color can be accounted for by the reduced resonance stabilization of the product, there is basically a loss of two conjugated double bonds, one in the 6 membered ring and alternate to this (Wong, 1989) [14].

1.2.4 PH

Since carotenoids are not soluble in water there is no need to consider pH effects for this group of food colorants. Of the other groups of food colorants it would expect pH effects to be greatest where the molecule contains ionizable groups. The most obvious group here is the anthocyanins which have the flavylium cation structure with a charged oxygen atom. In low pH (pH_1) the color of anthocyanins is red (AH+, Fig 3) as the pH is increased the anthocyanin may undergo two possible pathways (1) deprotonation to result in a blue quinoidal compound (A) or (2) hydration to result in a chalcone (C).

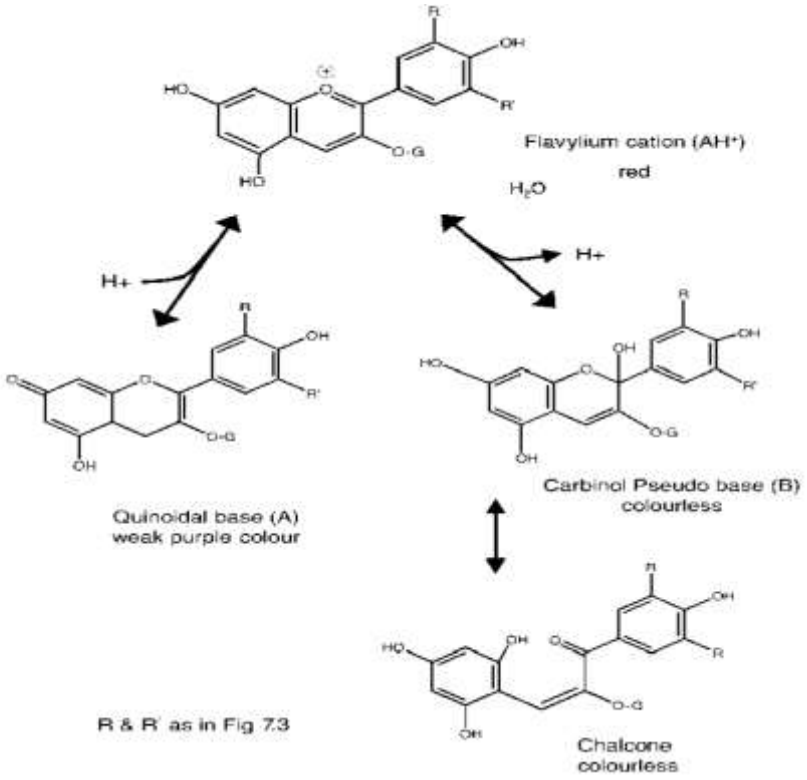

Fig 3: Effect of pH on anthocyanin color and structure

Thermal stability

In relation to food products thermal stability might be considered in different broad temperature ranges: refrigeration to ambient for various storage conditions; 60 to 120ºC to cover boiling, pasteurization, sterilization and 180 to 220ºC for oven cooking, grilling and frying. It must be remembered that '*in vitro*' studies on the rate of reaction of individual compounds may not be a true reflection of their behavior in complex food systems. Although increasing the temperature increases the rate of reaction as given by the Arrhenius equation (Eqn 1), it may also change the course of the reaction.

Arrhenius equation:

$$k = Ae^{-Ea/RT}$$

$$lnk = lnA - Ea/RT \quad \text{Eqn} \quad (1)$$

where k = rate coefficient (min^{-1})
Ea = Activation energy ($J\ mol^{-1}$)
A = constant (min^{-1})
R = Gas constant ($J\ mol^{-1}\ K^{-1}$)

Dyrby *et al*., (2001) [5] studied the effect of heat (20ºC to 80ºC) on anthocyanin extracts from a range of plant materials (red cabbage, blackcurrant, grape skins, and elderberry) and in different media (buffer, carbonated soft drink). Over the 6h period of their study they found that the data could be fitted to first-order rate law as found by others (Cemeroglu, 1994) [4].

Both norbixin and bixin are reasonably stable to heat, whereas light stability is best if norbixin is bound to protein, i.e., in cheese. Annatto is somewhat unstable to light and oxygen but, technically, it is a good colorant. Norbixin may precipitate at low pH and cannot be mixed with products containing calcium. For this drawback, annatto color has limited application in coloring of soft drinks.

Keeping this view in mind attempts have been taken to assess the stability of norbixin extracted by water and potassium hydroxide.

The specific objectives are

- to assess extraction method on the yield of annatto color;
- to assess color intensity in relation to color concentration;
- to assess color stability in synthetic sugar concentrate;
- To assess stability of color in response to heat, light and pH.

The natural colors market is currently growing twice as fast as that of synthetic colors. A detailed review on reason of general increase of the use of natural ingredients, types of natural and synthetic color, chemistry, factors affecting on the stability, use, common sources of natural color and annatto as color, its source, types and use has been carried out.

2. Materials & Methods

The present study was conducted in the Food Technology Laboratory of Department of Food Technology and Rural Industries and Chemistry Laboratory of Department of Chemistry, Bangladesh Agricultural University, Mymensingh, Bangladesh, in the year of 2009 to 2010.

2.1 Materials

2.1.1 Raw Materials & Chemicals

Annatto seeds & phosphate buffer

2.1.2 Instruments

Spectophotometer, micropipette, tips & vials.

2.2 Methods

2.2.1 Extraction of dye

Pigment extraction was solely conducted in the Department of Chemistry under the supervision of Dr. Hari Pada Seal, Bangladesh Agricultural University. Annatto color was extracted from the seeds of annatto using soaking and soxhlet & reflux methods. Distilled water for soaking method and ethyl acetate for soxhlet & reflux methods were used as solvents. Only the water soluble pigments water used for stability studies.

2.2.2 Sample preparation and analysis

Bixin color sample was supplied in concentrated form. Before determining absorbance several dilutions were made. Dilutions having absorbance spectra within 0.4 to 0.8 were chosen as standard dilution for accurate measurement of absorbance in spectrophotometer. Spectrophotometric scan of bixin color revealed that the highest spectra were shown at 503 nm. For entire work color analysis were conducted by measuring absorbance of sample at 503nm and absorbance of distilled water at 503nm was used as blank.

2.2.3 Color intensity in relation to concentration

With increasing concentration absorbance of colored solution is also increased. In spectrophotometer absorbance of colored samples became stable beyond a certain level of concentration, which is the main drawback of determining absorbance of photometric color determination. Experiments revealed that the solution concentration having absorbance range of 0.4 to 0.8 resulted in less error in spectrophotometer. To determine the color intensity, supplied bixin samples were diluted in distilled water. Five dilutions were made having concentrations of 100, 250, 500, 1000 and 2000 ppm. Absorbance of the diluted samples was taken at 503 nm by the spectrophotometer.

2.2.4 Stability test in synthetic sugar solution

For assessing the stability of bixin color in food matrix, synthetic sugar solution was prepared of 1x, 2x and 2.25x strength as shown in Table 1. Samples of bixin color at a concentration of 500ppm were made using synthetic sugar solution of different strength and the spectrophotometer reading of prepared samples were taken at 503nm.

Table 1: Synthetic sugar solution in relation to sugar concentration

Sugar strength	Sugar concentration (g/L)
1x	12
2x	24
2.25x	54

2.2.5 Temperature effect on the stability of bixin color

To assess the temperature effect, eight vials containing 500ppm bixin color prepared using distilled water were incubated at four different temperatures. The incubation temperatures are 4 ^{0}C, 25 ^{0}C, 50 ^{0}C and 100 ^{0}C. Every two samples were incubated in each temperature for 30 min. After 30 min the temperature of incubated samples were adjusted to the room temperature and absorbance was determined at 503nm by spectrophotometer.

2.2.6 Effect of light on the stability of bixin color

For judging the effect of light on the annatto extract the solution having concentration of 500 ppm was treated under direct sunlight for different period of time. After keeping the samples under sunlight for 2h, 5h and 7h, the absorbance reading of different samples were taken in a spectrophotometer at 503 nm as prescribed before. A control sample was taken as standard, which was not treated under sunlight to compare the effect of light on color of annatto extract.

2.2.7 Effect of pH on the stability of bixin color

To evaluate the effect of pH on the intensity/stability of the annatto color, sodium citrate buffer was used to adjust the pH of color solution. Considering the pH of soft drinks, buffer of pH 2.8, 3.2, 3.6, 3.8 and 4.0 were prepared. The annatto extract was dissolved in each buffer at a concentration of 500ppm. After preparing color samples in buffer, they placed rest for 15 min and then the absorbance reading was taken at 503nm by the spectrophotometer as described before.

3. Results and discussion

3.1 Extraction of dye

Annatto color extraction was conducted in the Department of Chemistry, BAU. The water and chemical extraction methods were conducted for extracting pigments. For chemical extraction, reflux and soxhlet methods were carried out using ethyl acetate. The percent yield of pigment by water soaking, reflux and soxhlet are shown in Table 2. The reflux method gave the highest yield (10.1%) while the soxhlet method yielded 7.1% of dyes. In the soaking method, seeds were soaked in water for different time

periods (6, 12, 18 and 24 hour). The highest yield (8.93%) was obtained in the 18 hour soaking period. Compared to Bhoomika (2006) [2] studies, who carried out five different methods, i.e. oil extraction, aqueous alkali extraction, plain water extraction and solvent extraction using acetone and chloroform as solvent, the present study yielded higher dye from annatto seeds.

Table 2: Percent color extraction by three different methods

Methods	Percent yield
Water soaking	8.93
Reflux method	10.10
Soxhlet method	7.10

According to Bhoomika (2006) [2], the solvent extraction method resulted in the highest dye yield (3.2%), followed by aqueous alkali extraction (3%). The least was in plain water extraction (0.9%). The dye yield in different accessions ranged from 101.29 to 255.65 g/tree with an average of 148.62 g/tree. This difference might due to purification of pigments.

3.2 Color intensity test

With increasing concentration absorbance of colored solution is also increased. In spectrophotometer absorbance of colored samples became stable beyond a certain level of concentration, which is the main drawback of determining absorbance of photometric color determination. Experiments revealed that the solution concentration having absorbance range of 0.4 to 0.8 resulted in less error in spectrophotometer reading.

The results of color intensity with respect to color concentration have been shown in Fig 4.

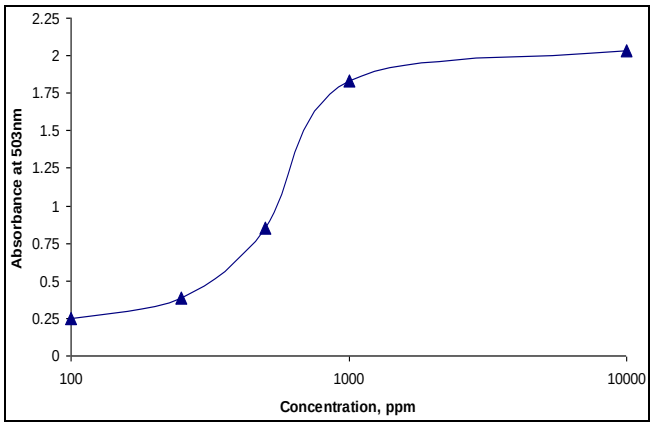

Fig 4: Effect of dilution on the absorbance of color

As can be seen in Fig 4, sample dilution below 1000 ppm and above 10000 ppm did not have any effect on the absorbance of color i.e., there were almost no change in absorbance reading.

3.3 Stability test in synthetic solution

Sugar is one of the important ingredients of processed foods and beverages. The content of sugar in various process foods varies from low to high. In case of beverages, the total soluble solid content varies from single strength to 5 to 6, which corresponds to 65 to 67%. Considering this aspect, the effect of sugar concentration on the stability bixin color was carried out and the results are shown in Fig 5.

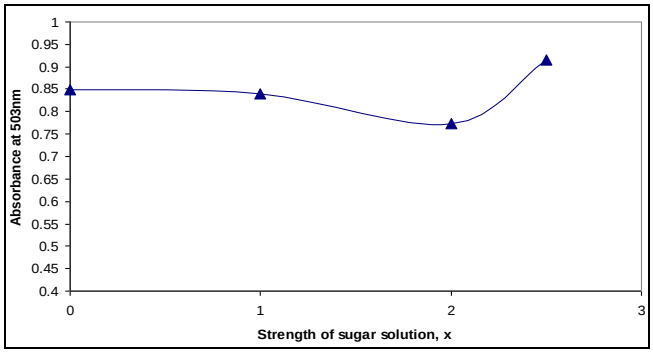

Fig 5: Stability of bixin color in concentrated sugar

As shown in Fig 5, absorbance of annatto color exhibited almost unchanged over all concentrated sugar solution. Absorbance reading in single strength sugar was found almost the same as that of control. In 2x strength, it was slightly decreased but in 2.25x again it was increased. This might due to the increase of solutes in the solution.

3.4 Effect of temperature

The application of natural pigments in coloring foods are limited due to week color intensity, presence of impurities which adversely interact, unstable during storage and low stability during heating. Among them instability during heating was marked as serious factors of using natural color in foods because most of the processed foods need to be sterilized or pasteurized during packaging. For these reason the effect of heating on the stability bixin color were assessed and the results are shown in Fig 6.

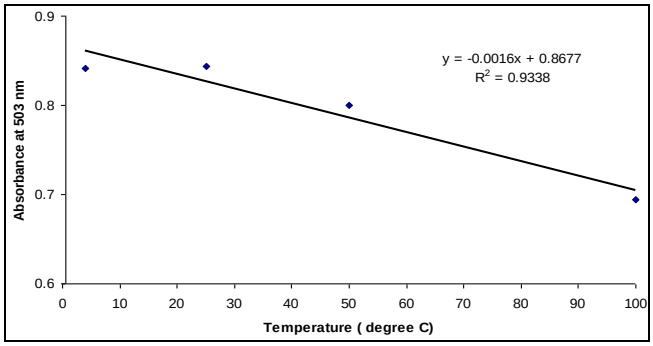

Fig 6: Effect of heating on the stability of annatto color

As shown Fig 6, absorbance reading and heating showed a linear relation with negative slope, which indicated that with increasing heating temperature color intensity of bixing are getting to be lost. From low temperature to room temperature color showed quit stable and beyond the room temperature color intensity was started to be lost. It is noticeable that the slope of the curve is very low (0.0016), which indicated that the kinetics of color loss was very low.

3.5 Stability of annatto color in response to light

The major causes of carotenoid loss are due to the oxidation of the highly unsaturated carotenoid structure. The important oxidation that occurred in caroteniod containing color is autooxidation, photooxidation and coupled oxidation. Among them photooxidation produced by oxygen in the presence of light affects seriously of the stability of annatto color. To assess the stability of bixin color under direct sunlight was carried out and the results are shown in Fig 7.

Fig 7: Oxidation of bixin color under direct sunlight

Fig 7 showed that annatto color was highly sensitive to light. Oxidation was rapid within first two hours of incubation. Within this time color loss was clear linear and after two hours annatto color was oxidized to almost a colorless end-products and up to 7 hours change seemed to be stable. It is assumed that this color loss was due to oxidation and photo oxidation was taken placed during incubation under direct sunlight (Gross, 1991) [8].

3.6 Effect of pH on the stability of bixin color

Effect of pH is highly concerned for caroteniods, which are soluble in water. PH effect to be greatest of those food colorant, which contain molecules having ionizing groups. The bixin and norbixin are the principal color of annatto. It is expected that the norbixin might be effected by pH and the experiments to assess its effect on the stability of norbixin are shown in Fig 8.

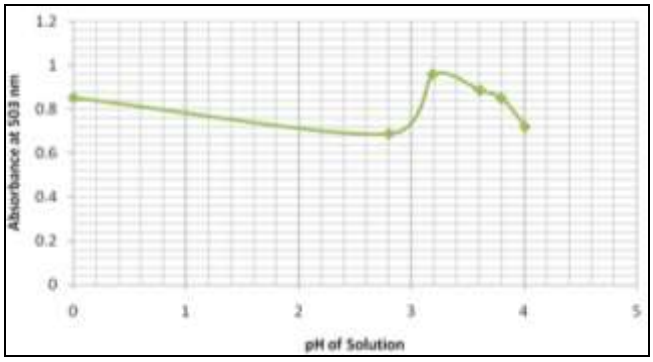

Fig 8: Effect of pH on the stability of annatto color

As shown in Fig 8, there is minor change in the color intensity of annatto color with increasing pH. It is interesting that color change suddenly increased within the pH 3.25 to 3.8. But beyond the pH 3.8, color intensity showed absorbance reading almost close to control. In low pH (pH 1) the color of anhocyanins is red as the pH is increased the anthocyanin may undergo two possible pathways; one deprotonation to result in a blue quinoidal compounds or second hydration to result in a chalcone (Wong 1989) [14].

4. Summary and Conclusion

This study was conducted in the Laboratory of the Dept. of Food Technology and Rural Industries and Department of Chemistry BAU, Mymensingh, Bangladesh. Annatto extract, an well-known natural color, were isolated and the effect of solvents, food matrix, temperature and light were studied.

The water and chemical extraction methods were conducted for extracting pigments. The reflux method gave the highest yield (10.1%) while the soxhlet method yielded 7.1% of dyes. In the soaking method, seeds were soaked in water for different time periods (6, 12, 18 and 24 hour). The highest yield (8.93%) was obtained in the 18 hour soaking period.

The best background color was found at a concentration of 500 ppm. Sample dilution below 100 ppm and above 1000 ppm did not have any effect on the absorbance of color i.e., there were almost no change in absorbance reading.

The annatto color was found quite stable in food matrix. Absorbance of annatto color exhibited almost unchanged over all concentrated sugar solution. Absorbance reading in single strength sugar was found almost the same as that of control. In 2x strength, it was slightly decreased but in 2.25x again it was increased.

Although absorbance reading and heating showed a linear relation with negative slope, the kinetics of color loss was quite low and it was indicated by low slope of regression line. From low temperature to room temperature color showed quit stable and beyond the room temperature color intensity was started to be lost.

Annatto color was found highly sensitive to light. Oxidation was rapid within first two hours of incubation. Within this time color loss was clearly linear and after two hours annatto color was oxidized to almost colorless end-products.

There is minor change in the color intensity of annatto color with increasing pH. It is interesting that color change suddenly increased within the pH 3.25 to 3.8. But beyond the pH 3.8, color intensity as measured by absorbance reading showed almost close to control.

In conclusion, detail kinetics of color loss due to light and temperature needs to further study and how these losses could be protected is also essential to study.

5. Acknowledgement

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