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Stability study of an aqueous formulation of the Annatto dye

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Abstract

Annatto is a natural dye extracted from the pericarp of *Bixa Orellana* L. seeds. The main coloring agents of this dye are bixin and norbixin. In this study, it was evaluated the effect of light and temperature on the stability of an aqueous formulation of norbixin. Both studies were carried out in controlled conditions. Photostability studies were conducted exposing samples in different concentrations under irradiated for 6 hours using a xenon lamp at 1000 W/m^2. The effect of temperature was assessed by analyzing the samples exposed at 30±2ºC during 12 months, in order to simulate the natural storage condition. Norbixin concentration during storage after exposure to various conditions was measured by spectrophotometry at 455 nm. This was correlated with color, which was measured by sensorial analysis, where perceptible changes in color were identified. Results from forced photostability studies showed that samples at high norbixin concentration (5.58%) did not suffer decomposition. The decay of norbixin promoted by temperature was fitted a linear model, and a significant change of color was observed at 12 months when the remaining norbixin concentration was 4.42%.The findings showed that high concentrations are a protective factor against photo-degradation, and the shelf life of norbixin in aqueous formulation was 12 months at 30ºC.

Introduction

Annatto is one of the world's oldest natural dyes used to give color to ranging from red to yellow. It is obtained from the pericarp of *Bixa Orellana* L. seeds. The carotenoids bixin (methyl ester) and norbixin (carboxylic acid) are the main coloring agents of this dye. The bixin is liposoluble and norbirxin is soluble in alkaline media. Both of these carotenoids have strong coloring capacity, e.g. a solution of norbixin at 1% is sufficient to color 16 tons of cheese. In fact, annatto is categorized by the FDA as a colorant exempt from certification and suitable for use in foods, drugs and cosmetics (Giridhar *et al.*, 2014).

Due to their stability and low cost, synthetic colorants have come to constitute the first option for processed food products; however the consumers have become aware of the adverse health effect associated to them besides that legislation on the use of synthetic dyes have increased restrictions in different countries. (Shahid and Mohammad, 2013). For example, Annatto is considered to be a potential alternative to replace tartrazine, which is banned in many countries (Giridhar *et al.*, 2014).

Carotenoids have a system of conjugated double bonds that make them susceptible to degradation reactions promoted by temperature, light, oxygen and pH (Scotter, 2009). The stability of bixin was reported by Najar *et al*. in 1988, who studied the effect of light (at 1380 or 430 lux), air, nitrogen, benzoyl peroxide and ascorbyl palmitate at 24º±1°C in the degradation of chloroform solution of bixin. They found that bixin is extremely sensitive to light. Likewise, Prentice Hernandez and Rusig, (1999) reported that the half-life of bixin, in drink colored with annatto, varied from 462 days in the dark to 6 days when it was exposed to visible light. Balaswamy *et al*. (2006) studied the stability of bixin in annatto seeds, oleoresin and powder. They found that bixin is decomposed faster under the form of dye powder than oleoresin. Since bixin decomposes at high temperatures (Scotter *et al*., 1998), there is a growing interest in exploring the stability of bixin during the processes of food production (Prabhakara *et al.*, 2005; Prabhakara *et al*., 2007; Balaswamy *et al*., 2012).

As regards to norbixin, recent studies have been conducted with the purpose of studying its stability. Prabhakara *et al*. (2002) analyzed annatto in a liquid formulation at concentration of 0.224g/L and 30.6mg/L. They found that light and temperature accelerate the degradation of norbixin. Parvin *et al.* (2011) found that the degradation of norbixin follows first order kinetics in aqueous solutions (500 ppm) at 70-100ºC, whereas Ferreira *et al.*, (1999) reported a second order kinetic in solution (0.71%) at 90ºC-

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140ºC. Overall, there is more information on the stability of bixin that norbixin (Parvin *et al.*, 2011).

The existent information could generate concerns about the use of this dye in industrial process, and could not allow to take advantages of its high antioxidant capacity and low toxicity. It is a common practice in the industry to employ ready-to-use-mixes in order to facilitate incorporation into the food matrix. They are concentrated formulations containing solubilizing agents. Annatto is dispensed in such mixtures for use food industry process because of its low hydrosolubility. Since degradation of annatto dye in storage conditions depends to a large extent on the formulation where the dye is incorporated (Giridhar *et al*., 2014), it is necessary to determine the stability of the annatto dye in ready-to-use-mixes under study conditions that simulate the storage and distribution conditions. Moreover, this can be used as a model to study the stability of norbixin at high concentrations.

This study is looking for contributing to the understanding of the complex stability of annatto and elucidating the proper conditions for its industrial use. In light of that, this study focuses on the evaluation of the shelf life of a concentrated formulation of norbixin through forced photo-stability studies, and long term stability study. Also, sensory analysis was used to determine the norbixin concentration, at which the formulation loses the quality of its color.

Materials and Methods

The formulation of annatto natural dye (Annatto 0101) was supplied by the company C.I. Organic Evolution S.A.S. It is specially designed to be used in aqueous or emulsified matrices in the food industry. Its principal coloring agent is norbixin with a concentration of 5.58% w/v.

Forced photo-degradation study

Annatto 0101 was evaluated at 5.58, 0.5, 0.05 and 0.005% w/v (prepared by dilution with purified water). These solution were put into test tubes (10 mL) and placed in a solar simulation chamber (Solarbox 2000e) equipped with a xenon lamp. The xenon lamp is recommended in Guidelines by the International Conference on Harmonization (1997), to simulate the solar radiation that reaches the Earth's surface. The samples were irradiated at 1000 W/m^2 for 6 hours. Independent samples were protected from the light with aluminum foil and taken to the same irradiation chamber where the temperature reached 40ºC.

Storage conditions for long term stability study

For this study, 250 mL containers of opaque white polyethylene (the same material used to market the product) were used to store the sample in chamber with controlled humidity and temperature. The temperature was 30± 2°C and the relative humidity was 75%±5% hr in accordance with climatic zone IVB of the guidelines emitted by World Health Organization (WHO, 2009). The storage period was 12 months, during which physical alterations, such as the separation of phases or formation of particles, not were observed on the samples.

Norbixin assessment test

According to Ghidouche *et al.* (2013) and Cardarelli *et al.* (2008), the degradation of a dye can be evaluated by analyzing its absorbance using a spectrophotometer. This absorbance later becomes the concentration of the pigment. In this work, the remaining norbixin concentration was evaluated instrumentally with the spectrophotometer and sensorially to determine the perceptible difference in color, or in other words, the tolerance threshold.

Spectrophotometric evaluation

Spectrophotometric evaluation was carried out to determine the concentration of the principal component (norbixin) during the storage time. Absorbance was registered at a wavelength of 455 nm. The concentration of norbixin was calculated from a calibration curve using a norbixin standard and preparing solutions in a range of $1x10^{-4}$ - $3x10^{-4}$ %w/v with deionized water. The following equation was obtained:

$$\text{Concentration (\%w/v)} = \frac{\text{Absorbance} + 0.0194}{2222}$$

The sample was prepared by taking an aliquot of 136.2 mg (in order to improved reproducibility in the analysis, aliquots were weighed, due to the high viscosity of the sample, previously it was determined the sample density. This was transferred to a 100 mL volumetric flask, it was diluted with deionized water and stirred until complete dissolution in an ultrasonic bath. Then successive dilutions were made using deionized water to obtain a solution whose concentration fits within range of the calibration curve.

Sensory analysis

Sensory analysis was performed to find the perceptible difference in color or the tolerance threshold and was determined by the paired comparison method, with a panel composed of nine trained female judges. Five samples were prepared at decreasing concentrations to simulate different

levels of degradation of the dye, as is shown in Table 1. The sample with the greatest concentration was established as the Reference and the other four samples were compared with it. Reference corresponds to formulation Annatto 0101 recently prepared.

Immediately before carrying out the sensory analysis, the samples were diluted (1g en 100 mL of water), and 10 ml of the diluted sample were transferred into test tubes. Each sample was compared visually with the reference and it was sensorially determined whether there exists a perceptible difference ($P<0.05$) or a similarity between them.

The tolerance threshold was determined in terms of norbixin concentration and correspond to the sample of highest concentration where the judges found color difference with respect to the reference. This threshold indicates that the shelf life of the formulation has expired because there are a noticeable change in color with respect to the reference (recently prepared sample).

Determination of shelf life

To determine the time in which the product lost its quality as result of degradation, the remaining norbixin concentration was monitored during the storage (12 months). Finally, the remaining concentration was plotted against time and the regression line was calculated. The shelf life is given by the intersection of tolerance threshold and the 95% one-sided confidence limit for the regression line.

Results and Discussion

Forced photo degradation study

It has been reported that norbixin is light sensitive e.g. Parvin *et al*., (2011) found that an aqueous solution of 500 ppm illuminated under direct sunlight had a $t_{½}$ of 28 hours while in the dark the $t_{½}$ was 462 hours. Unfortunately, the irradiation conditions used in that study were not easy to reproduce. In this sense, a forced photo-stability study was undertaken to obtain a profile of the norbixin sensitivity to light. It was found that the effect of light is greatly influenced by the concentration of norbixin. In diluted solutions the photolytic reaction advanced significantly, while in concentrated solutions the process was slow as depicted in Figure 1. At concentration of 5.58% w/v the decomposition process did not exceed 0.5%, this was a very low level of degradation considering that the sample was subjected to an irradiation of 1000W/m2 for 6 hours in a transparent glass recipient, suggesting that the formulation did not reach high levels of decomposition when exposed to light under natural storage conditions (Singh and Bakshi, 2000). Sensitivity to the concentration in photolytic reaction has also been found in other substances. It has been observed that in concentrated solutions a screen effect occurs, the light is absorbed by molecules that are close to the surface of the sample, stopping the light from going through the solution and affecting the molecules in the interior (Bhalekar *et al*., 2008).

Table 1. Sensorial analysis by paired comparison

Samples	Number of trials	Concentration (%w/v)
A2 vs A1	24	5.58 vs 5.20
A2 vs A4	24	5.58 vs 4.70
A2 vs A5	27	5.58 vs 4.32 *
A2 vs A3	24	5.58 vs 3.90*

* There is significant difference ($P<0.05$)

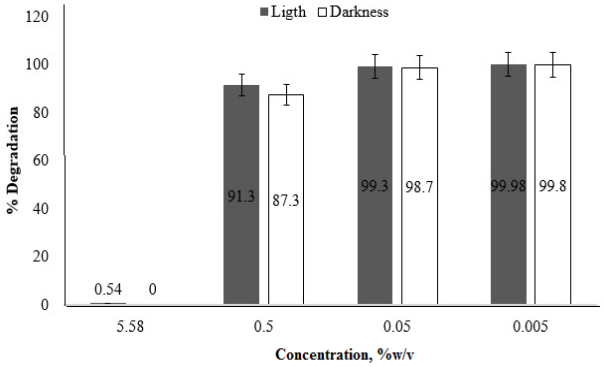

Figure 1. Photo-degradation of norbixin solutions in different concentrations, exposed to solar simulator at 1000 Wm^{-2} for 6 hours

Prabhakara Rao *et al.* (2002) found that norbixin is most stable against light in lower concentrations. They compared the degradation of solution of norbixin (0.224 g/ L) in water with a formulation of norbixin (30.6 mg/L), the decomposition was close 98% and 10%, respectively, both samples were exposed at ambient temperature and light, and packaged in transparent glass bottle, for a period of 90 days. The authors concluded that the presence of other additives (sugar, citric acid) in the formulation affects the stability. However, although this formulation was stable to light, the author also stated that presented physical instability, formed a fine precipitate. Probably, the high photo-stability observed obeys to a fact that the molecules in solid state are more stable to light.

Long term stability study

In this study was important to identify what attribute was susceptible to change during storage, as well as the level at which that quality attribute remain within acceptance criteria. Therefore, a theoretical review of the degradation products formed during the decomposition was carried out.

Table 2.Determination of shelf life of the norbixin formulation

Time moths	Concentration, %w/v*	95% Confidence limit Lower	95% Confidence limit Higher
0	5.58	5.5	.8
2	5.37	5.3	5.5
5	5.24	5.0	5.2
6	5.14	4.9	5.0
7	4.79	4,8	4.9
8	4.70	4.7	4.8
9	4.73	4.6	4.7
10	4.64	4.5	4.6
11	4.52	4.4	4.5
12	4.34	4.3	4.4

*Average values of three replicates

The scientific literature reports that carotenoids are degraded by isomerization reactions, giving rise to other colored compounds. It has been reported that aromatic compounds like m-xylene, toluene and dihydronaphthalene, which affect human health, only are formed at temperatures greater than 100ºC (Scotter *et al*., 2001). Bearing this in mind, storage stability for the annatto formulation will be given by its functional attribute, specifically its ability to provide color.

In order to define the acceptance level, a sensory analysis was carried out to judge the perceptible change in color, the result was correlated with the concentration (measured by absorbance). Table 1 shows the statistical analysis of the sensory evaluation of the samples used in this test. According to the judges, significant differences were not found with respect to the reference in the samples with concentrations of 5.2 and 4.7%. In contrast, in the samples with concentrations of 4.32 and 3.9% were found significant differences with a 95% confidence level. This means that 4.32% was the maximum concentration where perceptible changes in color was observed, it was defined as the tolerance threshold for the formulation in study. Therefore, dye samples that reached a remaining norbixin concentration less than 4.32% had to be rejected because a change of color with respect to the initial formulation could be observed visually.

Due to the light was not a significant factor in degradation in formulation at 5.58%, the estimation of shelf life was performed under the influence of temperature. To define the storage isothermal conditions to conduct the long term stability study, it was revised the proposed conditions by Grimm

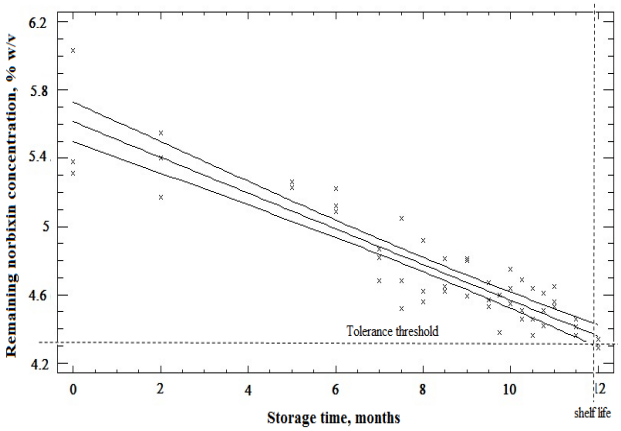

Figure 2. Degradation of norbixin formulation (5.58%) at 30°± 2°C and 75%±5% H.r. packed in containers of opaque white polyethylene

(1998), who proposed four climatic zones for long term stability studies, through employing meteorological and kinetic data, and calculating the kinetic mean temperature. These zones have been adopted by the ICH, FDA and subsequently by WHO with certain modifications. Due to the product will be distributed in the tropical region that corresponds to zone IVB (WHO, 2009), this study was carried out by exposing the samples at 30°C and 75% rh. This means that an isothermal storage at 30ºC will simulate effect that would suffer a product stores at ambient conditions in zone IVB.

Figure 2 shows the behavior of the remaining norbixin concentration during storage time. The degradation of norbixinin under natural storage conditions could be fitted to a linear regression equation. The analysis of variance indicated a significant relationship ($P<0.05$) between the norbixin content and storage time. The equation fitted to the following model:

$$Concentration = 5.624 - 0.107 * time, \quad r^2 = 0.83$$

This model showed that 83% of the variability in the norbixin content was due to the storage time. The result obtained in this study could not be directly compared with those reported by Parvin *et al.* (2011) and Ferreira *et al.* (1999), due to differences in concentrations and temperature range studied. Moreover, it should be noted that the results obtained here did not conclusively determine the reaction order of the kinetics, since the level at which the reaction advances did not exceed 50%.

Table 2 reports the remaining concentrations of norbixin for an observation time of 12 months and their 95% confidence limit. It can be seen that the tolerance threshold was reached at 12 months, and therefore this time was established as the shelf life of the studied formulation. It is expected that during

this period the product will maintains its sensory properties of color as long as it is kept stored in conditions zone IVB (WHO, 2009) and within its commercial packaging. The shelf life determined for the formulation in this study is suitable for the commercialization and industrial use of norbixin dye.

Prabhakara Rao (2002) reported that solutions of norbixin (0.224 g/L) and formulations (30.6 mg/L), exposed at ambient conditions during 90 days, within amber containers, suffered a degradation close to 80% and 10%, respectively. Since the study was performed in Hyderabad-India, it is expected the ambient conditions correspond to the climatic zone IV. In the present study where formulations (5.58%) were stored at 30º±2ºC, a degradation of about 5.3% was found for the same period of 90 days. Assuming that the conditions between these two studies are comparative, the divergence of results suggests that other properties of the matrix as viscosity, pH, aqueous activity, additives affect the stability of norbixin. The aqueous character of the formulation probably favors its stability, since in previous studies it was found that a high or intermediate water activity promoted the stability of the dye (Gloria *et al.*, 1995).

Conclusion

The present study shows that high concentration of norbixin can be a way for maintaining the quality and extending shelf-life of aqueous solutions of annatto against to degradation promoted by light. Through sensorial analysis was determinated that the formulation lost its functional attribute when the concentration of norbixin decreased below 4.32%, where a change in color was visualized.

It was found that the degradation of norbixin dye in natural storage conditions (specified as climatic zone IVB) can be fitted to a linear model, and the shelf life of this formulation for these conditions is 12 months. This study shows that formulation of norbixin in aqueous medium at concentration 5.58% is appropriate to deliver annatto dye in industrial processes, showing a shelf life enough to cover its own storage, distribution and use.

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