

## RECOVERY OF NORBIXIN USING COLLOIDAL GAS APHRONS (CGAS)

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**Abstract.** Annatto dyes are utilized in food worldwide and have their application in cheese, butter, sausages, ice cream, meat, etc. Other applications include: formulation of medicines and cosmetics. Bixin is the main pigment extracted from Annatto seeds. The carotenoid bixin represents more than 80 % of the total carotenoids found in the outer coat of the seeds. Norbixin is the water-soluble form of bixin. The tonality of Annatto dyes varies between yellow and red. Bixin is unstable: it degrades when exposed to certain conditions of temperature (above 80 °C), light and air. Therefore in drying processes, when high temperatures are applied, oxidation and isomerization reactions can occur leading to degradation and a predominantly yellow colour range of products. Basically, annatto dyes can be either extracted using mechanical processes or with alkali solutions, edible oil or organic solvents. In this study an alternative process using CGAs has been developed. CGAs are surfactant-stabilised microbubbles (10-100 µm), which are generated by stirring at high speeds (8000 rpm) a surfactant solution. The main characteristics of these microbubbles are: large interfacial area, relative stability and potential to adsorb molecules oppositely charged when generated from ionic surfactants. In the present work a volume of CGAs generated from a cationic surfactant (CTAB) is mixed with a volume of annatto solution in KOH and when the mixture is allowed to settle it separates into the top aphron phase and the bottom liquid phase. The norbixin in the mixture will interact with the surfactant in the aphron phase, which results in its effective separation.

**Keywords:** Annatto, Norbixin and Surfactant.

### 1. Introduction

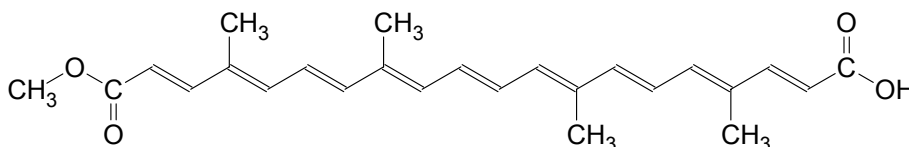
Annatto dyes are obtained from the outer coat of the seeds of the tropical shrub *Bixa orellana L.* They are widely utilized in food and have their application in cheese, butter, sausages, ice cream, meat, etc. Other applications include formulation of medicines and cosmetics (Baliane, 1982). Rare characteristics, such as the possibility to obtain hydrosoluble and liposoluble extracts from the same source and its stability due to its property to bond to certain proteins, make the annatto extracts one of the main natural pigments utilized in food worldwide. Bixin (Figure 1), a monomethylester, is the main pigment extracted from annatto seeds. The carotenoid bixin represents more than 80% of the total carotenoids found in the outer coat of the seeds. Norbixin (Figure 2), a dicarboxylic acid, is the water-soluble form of bixin. The tonality of annatto dyes varies between yellow and red. Bixin is unstable: it degrades when exposed to certain conditions of temperature (above 80 °C), light and air (Alves, 2001). Therefore in drying processes, when high temperatures are applied, oxidation and isomerization reactions can occur, leading to degradation and a predominantly yellow colour range of products. According to Lancaster and Lawrence (Lancaster and Lawrence, 1996), the temperature and duration of the heating process regulates the red/yellow balance. Annatto dyes can be either extracted using mechanical processes such as spouted beds (Guimarães et al., 1989, Barreto et al., 1989, Massarani et al., 1992, Paasos et al., 1997) or with alkali solutions, edible oil or organic solvents (Alves, 2001, Prentice-Hernandez e Rusig,

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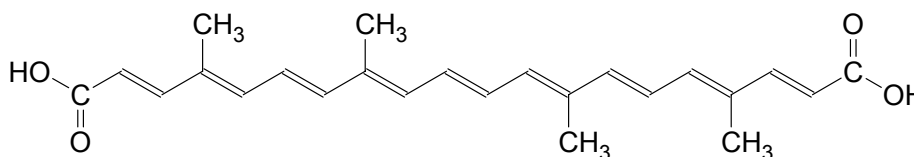
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1992, Simpson et. Al., 1993, Carvalho, 1990, Preston and Rickard, 1980). Usually the separated extracts have low concentration of bixin/norbixin although the process yield can achieve up to 80% average (Alves, 2001). According to Bouvier *et al.* (2003), from an economic point of view, bixin ranks second among natural colour additives used in industry and they suggest that there will be an increasing demand for this compound. Their proposed process involves fermentation, which will have to be followed by an effective separation process. In this study an alternative process using colloidal gas aphrons (CGAs) has been developed. CGAs (Figure 3) are surfactant-stabilised microbubbles (10-100  $\mu\text{m}$ ), which are generated by stirring a surfactant solution at high speeds (8000 rpm). The main characteristics of these microbubbles are: large interfacial area, relative high stability (Noble et al., 1998), have similar flow properties to those of water, separate easily from the bulk liquid phase (Jauregi et al., 2000) and potential to adsorb molecules oppositely charged when generated from ionic surfactants. Several applications have been described for CGAs such as protein recovery (Noble et al., 1998, Jauregi et al., 1997, Jarudilokkul et al., 2003), removal of toxic waste from soil (Roy et al., 1994a,b, Roy et al., 1995), recovery of fine cellulose fibres from paper mill wastewater (Hashim and Gupta, 1998), production of fine divided tin metal powders (Riviello et al., 1994) among other uses. In this work, CGAs are generated from a cationic surfactant (CTAB) and it is expected that the Potassium norbixinate, present in the alkali extract of annatto seeds, will adsorb to CGAs driven mainly by electrostatic interactions. A series of experiments have been carried out to establish the main operational parameters.



**Fig. 1.** Bixin ( $\text{C}_{25} \text{H}_{30} \text{O}_4$ )



**Fig. 2.** Norbixin ( $\text{C}_{24} \text{H}_{28} \text{O}_4$ )

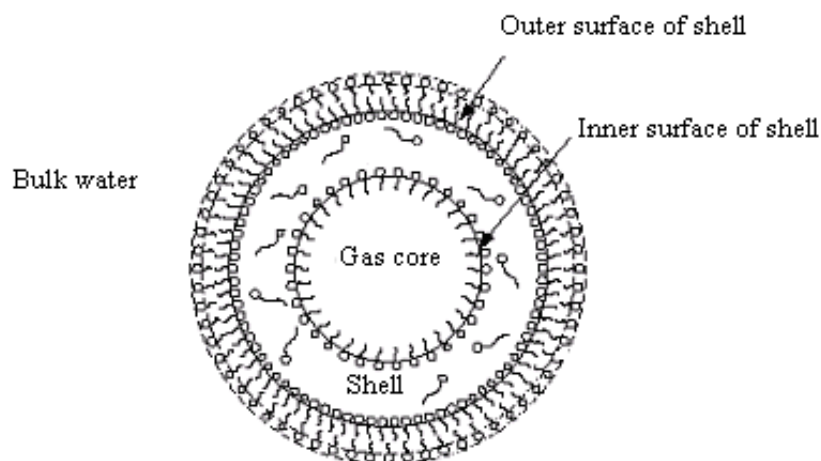


Fig. 3. CGA

## 2. Material and Methods

All chemicals were of analytical grade. CTAB (cetyltrimethylammonium Bromide) and ferric oxide were supplied by Fluka BioChemika. Tris[hydroxymethyl]aminomethane and Chrome azurol S were supplied by Sigma. Hydrochloric acid, acetic acid glacial, potassium hydroxide, chloroform, dibasic sodium phosphate, monobasic sodium phosphate, sodium carbonate and potassium hydroxide were supplied by BDH Laboratory Supplies (UK). Filter paper was supplied by Whatman. Annatto seeds were supplied by Chr. Hansen Indústria e Comércio Ltda (Valinhos/SP, Brazil) and bixin standard was supplied by Chr Hansen A/S (Hørsholm, Denmark). The laboratory mixer (SL2T) fitted with a four-bladed impeller ( $D=30\text{mm}$ ) surrounded by a high shear screen and with a digital readout impeller speed was supplied by Silverson Ltd. (Waterside, Bucks, UK). The spectrophotometers utilised were a Ultrospec 1100 pro supplied by Amersham Pharmacia Biotech (Biochrom Ltd., Cambridge, UK) and a Perkin Elmer Lambda2 UV/Vis Spectrometer coupled to a microcomputer.

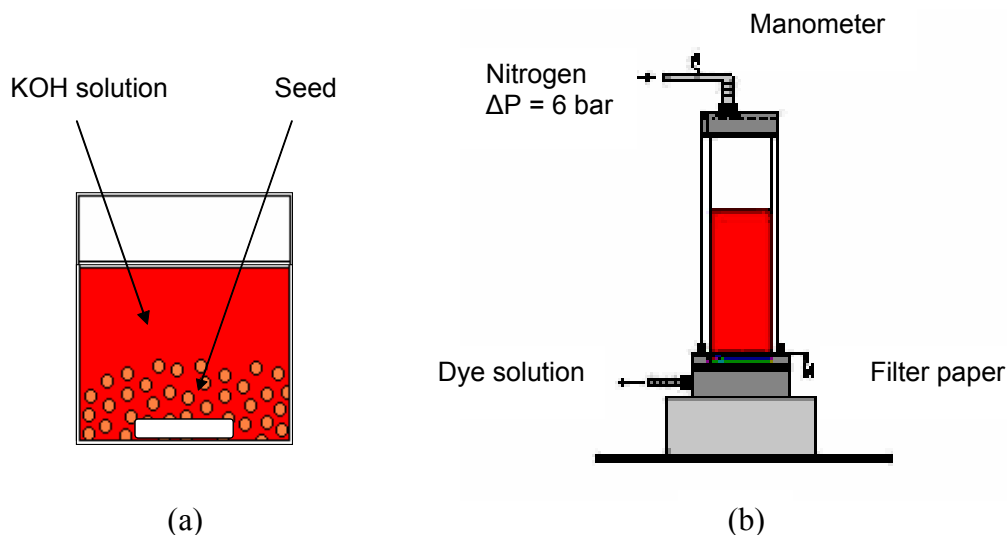
### 2.1. Seeds Analysis

Annatto seeds were analysed using a methodology described by Yabiku and Takahashi (1992) where the pigment of the seeds were extracted with a boiling solution of KOH 5% and the norbixin concentration was measured by UV/Vis spectrophotometer wavelength at 453 in a KOH 0.5% solution.

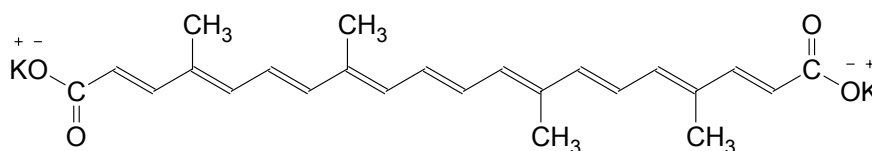
### 2.2. Extraction of Norbixin

A given amount of annatto seeds (10.32 g) was mixed with a solution of KOH 0.5% (200 mL) and magnetically stirred for 30 minutes. The solution was then filtered using a filtration cell with a filter paper of

medium filtration speed under nitrogen ( $\Delta P = 6$  bar) to avoid degradation of the pigment as described in Figure 4. The filtered solution contains the potassium salt of norbixin, ie: potassium norbixinate (Figure 5). The content of norbixin was determined by spectrophotometric analysis.



**Fig. 4.** Norbixin extraction. (a) Extraction of norbixin from the seeds by KOH solution. (b) Filtration of norbixin solution using a glass filtration cell under nitrogen  $\Delta P = 6$  bar



**Fig. 5.** Potassium norbixinate

### 2.3 - Generation of CGAs

The CTAB solutions were prepared in a Tris[hydroxymethyl]aminomethane – HCl buffer pH 8 at concentrations of 1 mmol/L, 2 mmol/L and 4 mmol/L . Also a 2 mmol/L CTAB solution was prepared in distilled water (pH 6). CGAs were generated by stirring 400 mL of CTAB solution, at 8000 rpm for 5 minutes at room temperature using a high-speed impeller.

## 2.4 - Norbixin recovery using CGAs

For all experiments, 1 mL of the dye-filtered solution was mixed with a known volume of CGAs at room temperature and magnetically stirred for 5 minutes. After that the solution was allowed to settle for 5 minutes and separated into two phases: CGAs (top) phase and the liquid (bottom) phase (Figure 6). The bottom phase was removed by pipet and the top phase was dried overnight in an oven at 30 °C to obtain a fine red powder; The dried top phase was washed with distilled water to remove any free surfactant then it was dried again overnight in an oven at 30 °C. Norbixin recovery was calculated by the difference between the initial content of norbixin in the sample and the remaining norbixin content in the liquid phase which were determined by spectrophotometric analysis. A calibration curve was done in KOH 0.5% at 453 nm where a known mass of known concentration of bixin standard was completely converted to norbixin in a KOH 0.5% solution. The norbixin concentration of the sample taken from the remaining liquid phase was then calculated from the calibration curve after reading the absorbance of the sample at 453 nm

The volume of CGAs to volume of the norbixin solution ratio was varied to study the effect of variations in the surface available for norbixin adsorption. Experiments were carried out at constant norbixin concentration (1.47 g/L) and solutions of varying surfactant concentration (1, 2 and 4 mmol/L). One millilitre of the norbixin solution was mixed with different volumes of CGAs (5, 10, 15, 20 and 30 mL), consequently the norbixin to surfactant molar ratio in the mixture was modified.

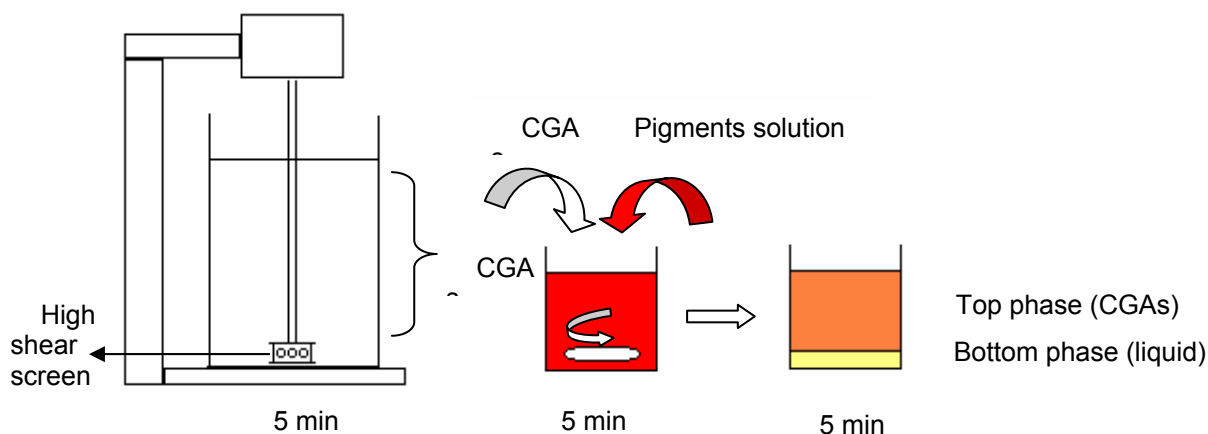


Fig. 6. Norbixin recovery using CGAs

## 2.5 - Surfactant analysis

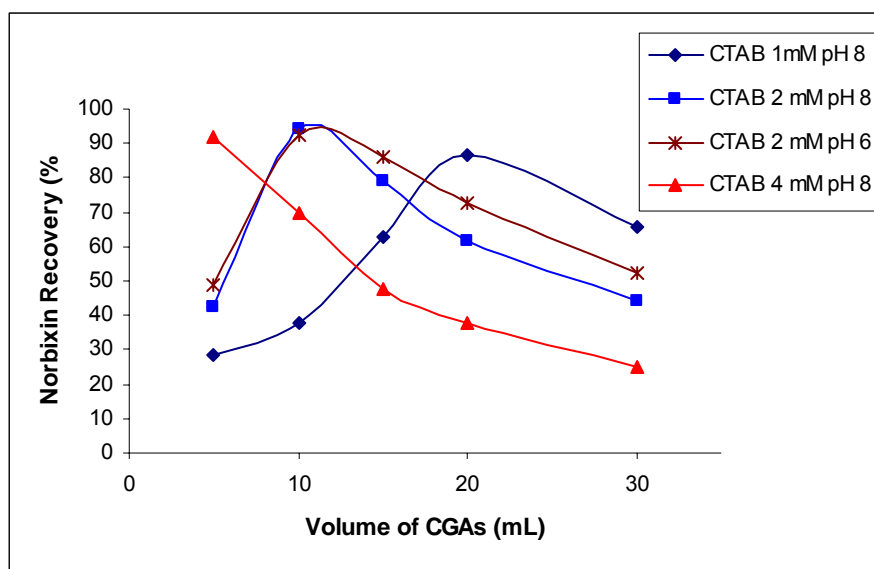
The content of surfactant in the bottom phase was analysed using a methodology adapted from Song and Liang (1996), as described below. The following reactant solutions were prepared: a 2 mmol/L chrome azurol S (CAS) solution, a  $1 \times 10^{-3}$  mol/L of Fe (III) solution, a phosphate buffer solution (pH 5.8, 0.2 mol/L) and a 0.05 mol/L sodium carbonate solution. The following solutions were added in sequence to a test tube: 1 mL of CAS solution, 1 mL of Fe (III) solution, 3 mL of phosphate buffer solution, 0.3 mL of sodium carbonate solution,

then vortexed for 20 seconds. Then 50 to 500  $\mu\text{L}$  of sample was added and diluted with buffer to complete 10 mL of total solution. The final solution was then vortexed for another 20 seconds and analysed using a spectrophotometer. The surfactant concentration in the top phase was obtained from a mass balance.

### 3 - Results and Discussion

The norbixin content in the seeds was 3.26% and the extraction yield was 81%.

Results in Figure 7 show maximum norbixin recovery (94%) when using 10 mL of CGAs generated from a 2 mmol/L CTAB solution. Similar recoveries were obtained for experiments carried out at different surfactant concentration and volume of aphrons but equivalent surfactant to norbixin molar ratio (Figure 8). Maximum norbixin recovery (94%) was achieved at ratio = 3.3. The recovery decreased when the surfactant to norbixin molar ratio increased above this ratio. It could be due to a difficult mixing of the dye solution with the CGAs therefore reducing the surface available for adsorption. In experiments carried out at high ratios of volume of CGA to volume of norbixin extract it was observed that mixing between the two phases was not very homogeneous which may hinder effective adsorption of norbixin hence lower recovery is achieved.



**Fig. 7.** Norbixin recovery in CGA phase at varying volume of CGA for four different solutions of CTAB.

For all volumes of CGAs utilised (5, 10, 15, 20 and 30 mL), the pH of norbixin-CGAs mixture was 9.3, 8.3, 8.0, 8.0 and 8.0, respectively, when using the CTAB buffered solutions. For a 2 mmol/L CTAB solution prepared in distilled water, the pH of the mixture was 11.6, 11.4, 11.2, 11.1 and 11.0 for the volumes of CGAS 5, 10, 15, 20 and 30 mL, respectively. However as shown in Figures 7 and 8 similar results were obtained for experiments carried out with 2 mmol/L CTAB in buffered solutions and 2 mmol/L CTAB solutions in distilled water which proves that an increased in pH of the mixture did not have an effect on recovery.

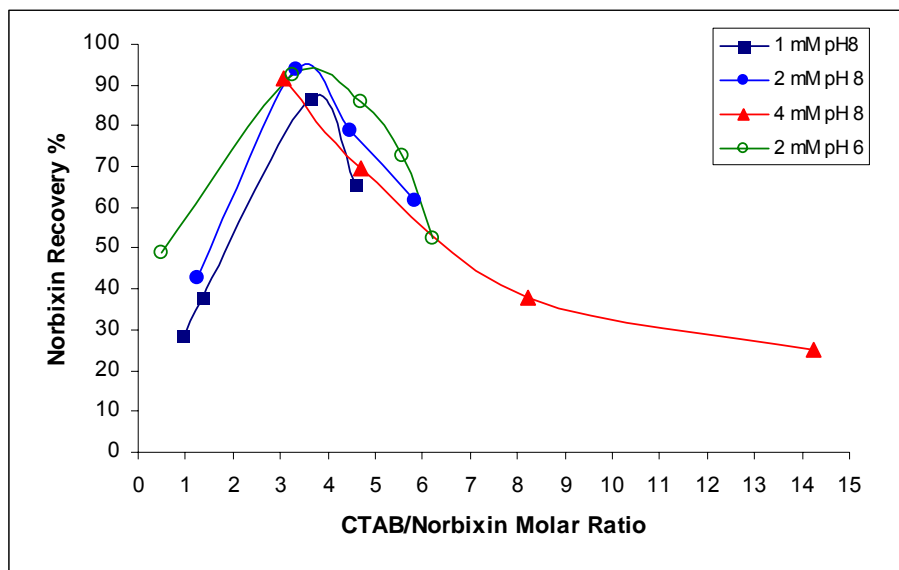


Fig. 8. Surfactant/norbixin molar ratio

It is postulated here that norbixin adsorbs to the CGA mainly driven by electrostatic interactions. Therefore higher yields should be achieved at conditions at which attractive electrostatic interactions between norbixin and surfactant molecules occur and on the contrary, lower yields should be expected at conditions at which repulsive interactions occur. Furthermore in studies carried out in our laboratory we found that the separation of protein molecules with CGA generated with a CTAB is governed by electrostatic interactions between oppositely charged proteins and CGA (Fuda et al., 2004).

It is also postulated here that the interaction between potassium norbixinate and oppositely charged CTAB molecules leads to the formation of a norbixin-CTAB complex which is stabilised by ionic interactions between ionised groups in norbixin and CTAB molecules. In addition, results on CTAB to norbixin mass ratio measurements in the CGA phase, where the best ratio = 3.3, an excess of surfactant is observed which can be due to free surfactant that was not completely removed after the dried material was washed. This mechanism is also supported by Khandurina et al. (1995), where they studied the structure of sodium polyacrylate and CTAB complexes.

Complementary experiments were carried out aiming to remove the surfactant from the formed complex (norbixin-surfactant), where the following solutions were added separately to the top phase: KOH 0.5%, KOH 5%, HCl 0.2 M, CHCl<sub>3</sub> and distilled water. In the experiments using the KOH solutions and water, same behaviour for each solution was observed: the formation of a pale yellow solution and not complete solubilization. With CHCl<sub>3</sub> added to the top phase, a red solution was observed with some particles in suspension. Using a solution of HCl 0.2 M it was observed the formation of a red solution with no particles in suspension and with formation of foam after shaking showing the separation of surfactant. This solution was then centrifuged at 4000 rpm for 30 minutes and a red precipitate was obtained, which was not observed in the

experiments with KOH solutions and distilled water. This precipitate is probably the protonated form of norbixin, which becomes insoluble in water upon acidification.

#### 4 - Conclusions

Results have shown a viable process for recovery of norbixin from an annatto solution using CGAs generated from a cationic surfactant with a good recovery of the pigment.

The best norbixin recovery was achieved with surfactant to norbixin molar ratio of 3.3 giving 94% recovery using CTAB. The main operating parameter to achieve high recovery is the CTAB to norbixin molar ratio whilst pH of the norbixin-surfactant mixture did not have an effect on the recovery.

Finally removal of the surfactant from the formed complex showed to be possible by decreasing the pH to 2 with a solution of HCl 0.2 M which resulted in the precipitation of the protonated form of norbixin with the surfactant remaining in solution, however further research is needed to establish the best parameters for the removal of the surfactant as well as the stability that might be conferred to norbixin while in a complexed form with the surfactant.

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