The Annatto Carotenoids and the Norbixin Absorption Coefficient

Carotenoides de Urucum e o Coeficiente de Absorção da Norbixin

ABSTRACT

Annatto seeds present cis-bixin as the major carotenoids, but the norbixin salt is the main pigment present in dyes obtained from the alkaline extraction process. For analyzing the norbixin, the absorptions are obtained in the two spectral peaks with higher intensity, but discrepancies in the published extinction values have led to serious doubts. Taking into account the use of absorption coefficient for evaluating the norbixin concentration in annatto seeds and extracts, the present study evaluated the absorption coefficient in various solvents and the total uncertainty associated with this value was determined. The norbixin standard was prepared from annatto seeds, purified by column chromatography and the purity of norbixin was evaluated by HPLC-DAD. The absorption coefficients were determined for norbixin in different solvents and the uncertainty was evaluated. The 0.5% potassium hydroxide, the main solvent used for marketing the annatto seeds and extracts, showed the absorption coefficients of 2887 at 454nm and of 2546 at 483nm, and the estimation of expanded uncertainty (K=2) was ±86 and 85 g 100 mL$^{-1}$, respectively.

Keywords. Bixa orellana, pigments, solvents, quantification, uncertainty estimation.

RESUMO

O principal carotenóide em sementes de urucum é a cis-bixina, mas o sal de norbixina torna-se o principal pigmento presente em corantes obtidos pelo processo de extração alcalino. Para a análise de norbixina as absorbâncias são obtidas nos dois picos espectrais de maior intensidade, mas discrepâncias no valor de absorptividade levam a sérias dúvidas. Levando-se em conta o uso do coeficiente de absorção na avaliação da concentração de norbixina em sementes de urucum e extratos, este trabalho teve como objetivo avaliar o coeficiente de absorção em vários solventes e determinar a incerteza expandida associada a esse valor. Para a execução do estudo, um padrão de norbixina foi preparado a partir de sementes de urucum, purificado em coluna aberta e a pureza da norbixina foi avaliada por HPLC-DAD. O coeficiente de absorção da norbixina foi determinado pela construção de curvas analíticas em diferentes solventes e a incerteza expandida foi avaliada. O hidróxido de potássio a 0,5%, principal solvente utilizado na comercialização de sementes de urucum e extratos, apresentou o coeficiente de absorção determinado em 2887 a 454nm e 2546 a 483nm e a estimação da incerteza expandida (K=2) foi 86 e 85 g 100 mL$^{-1}$, respectivamente.

INTRODUCTION

Carotenoids are yellow, orange, or red pigments with wide distribution in nature. They are formed of isoprenoid units that constitutes the light-absorbing chromophore and are responsible for their color, properties and functions. The π electrons of the conjugated double bond system show the relevant transition π→π* with excited state of comparatively low energy, which corresponds to light in the visible wavelength region. The solvent effect on the π→π* transition energy of pigment in different solvents which allows to obtain the absorption coefficient with the Beer-Lambert law.

The carotenoids exist in nature generally more thermodynamically stable form, all-trans-configuration. However, the bixin is an exception because it occurs naturally in the cis-configuration.

Annatto seeds (Bixa orellana L.) present cis-bixin as the major carotenoid, representing more than 80% of the carotenoids present. Cis-bixin is designated as the monomethyl ester of cis-norbixin dicarboxylic acid (9'-cis-norbixin: 6,6'-diaprocarotenedioic acid) (Figure 1). Norbixin is found in low concentrations in annatto seeds, but the norbixin salt becomes the main pigment present in dyes obtained by the alkaline extraction process.

A spectrophotometer with the wavelength in the visible region can be used for a quick evaluation of the pigments in annatto seeds or of the water soluble or liposoluble dyes obtained using the alkaline extraction process. However, due to the difficulty of obtaining and maintaining an analytical standard of norbixin, the use of the absorption coefficient ($E_{1\text{cm}}^{1%}$) for the quantification of these pigments has been widely divulged. The absorption coefficient is the measure of the decrease in radiation intensity that passes through a layer containing a constant fraction of a determined substance in a one centimeter optical path.

The values divulged in the literature for the norbixin absorption coefficient have undergone changes with time, without changing the wavelength or solvent. Reith and Gielen and FAO/WHO mentioned values for ($E_{1\text{cm}}^{1%}$) of 2850±40 for spectrophotometric readings of annatto pigments in 0.1N sodium hydroxide at 453 nm, but in 1982 FAO/WHO changed the value to 3473. In 2006, FAO/WHO suggested the use of an absorption coefficient of 2870 absorption units for readings obtained at 482 nm for annatto dyes, using 0.5% potassium hydroxide as the solvent.

Due to doubts concerning the values currently used, a study was carried out to determine the $E_{1\text{cm}}^{1%}$ of norbixin and the total uncertainty associated with this value.

MATERIAL AND METHODS

Reagents

In the present study the solvents methanol, ethyl acetate and acetonitrile (JT Baker, Center Valley, USA) were of chromatographic grade, and the following reagents (Synth, São Paulo, Brazil) were of analytical grade: petroleum ether, ethanol, dichloromethane, chloroform, acetone, acetic acid, potassium hydroxide and sodium hydroxide.

Preparation of norbixin

The norbixin standard was separated as described by Tocchini and Mercadante and Rios and Mercadante. A mass of approximately 200 g of annatto seeds was washed with two successive portions of 400 mL of petroleum ether.
to remove the lipids, and the pigments then extracted from the defatted seeds with two portions of 400 mL of ethanol: dichloromethane (1:1, v/v). The dichloromethane was eliminated in a rotary evaporator at 40 °C and 200 mL of a 10% (m/v) ethanolic solution of potassium hydroxide added to the residue. The pigments were saponified overnight at room temperature and the saponified pigments neutralized and precipitated with glacial acetic acid and collected by filtration.

The norbixin was purified by open column chromatography using a glass column with a 10 mm internal diameter and length of 250 mm, filled with the absorbent silica gel 60 with particle diameters from 0.063 mm to 0.200 mm (Merck, Darmstadt, Germany, article 1.07734). The pigment collected by filtration was dissolved in ethyl acetate. Some drops of ethanol and petroleum ether were added to the extract to facilitate dissolution of the pigment, which was then transferred to a chromatographic column previously conditioned with ethyl acetate. A yellow band was eluted with portions of ethyl acetate: ethanol (1:1, v/v) and ethyl acetate: methanol (1:1, v/v). After eluting the yellow band, the norbixin (orange band) was eluted with 100% methanol. The solvents were eliminated in a rotary evaporator at a maximum temperature of 40 ºC, and the norbixin dried in a nitrogen flow and collected.

The purity of the norbixin was evaluated by high performance liquid chromatography with a diode array detector (HPLC-DAD) according to the method described by Scotter. A Shimadzu model CLASS 10 chromatograph (Shimadzu, Tokyo, Japan) was used for this evaluation equipped with a 250 mm long x 4 mm internal diameter C18 reverse phase column (Merck, Darmstadt, Germany). The mobile phase was composed of acetonitrile in a 2% aqueous acetic acid solution (65:35, v/v) with a flow rate of 1 mL min⁻¹ in an isocratic system.

### Determination of the norbixin absorption coefficient

In order to determine the norbixin absorption coefficient, analytical curves with ten points were constructed with norbixin concentrations between 0.035 and 0.355 mg mL⁻¹ in the solvents methanol; acetone; chloroform: acetic acid (99:01, v/v); and aqueous 0.5% (m/v) solutions of potassium hydroxide and sodium hydroxide. The solvents were chosen based on the reagents most commonly used in commercialization of the seeds and in the quality control of annatto dyes. The absorbance readings were obtained using two Varian (Varian, Victoria, Australia) model Cary 50 spectrophotometers, one bought in 2003 (equipment 1) and the other in 2007 (equipment 2). The wavelengths showing maximum absorption, peaks II and III, were obtained by a spectral scan between 300 nm and 600 nm for each solvent used. Peak II is usually considered as the maximum absorption wavelength ($\lambda_{\text{max}}$).

### Uncertainty estimation

By definition, “Uncertainty is an interval associated with a measurement result which expresses the range of values that can reasonably be attributed to the quantity being measured. An uncertainty estimate should take account of all recognized effects operating on the result. The uncertainties associated with each effect are combined according to well-established procedures.” According to this definition, the uncertainty estimation associated with the absorption coefficient ($E_{\text{abs}}$) in g 100 g⁻¹ obtained by spectrophotometry, was calculated from the graph equation (Equation 1), and the uncertainty sources listed on the cause – effect graph (Figure 2). Thus the combined uncertainty (Equation 2) was determined from the sum of the squares of the norbixin concentration uncertainties, the linearity of the analytical curves, the uncertainties associated with the spectrophotometer and the precision of the analytical system.

$$E_{\text{abs}} = C_{\text{norb}} \times \text{intercept} + \text{slope} \quad \text{(Equation 1)}$$

$$\mu_{\text{abs}} = \sqrt{\left(\frac{n_{\text{norb}}}{C_{\text{norb}}}\right)^2 + (\text{linear})^2 + (\text{spect})^2 + (\text{prec.syst})^2 + (E_{\text{spect1,2}})^2} \quad \text{(Equation 2)}$$

where: ($E_{\text{abs}}$) = the absorption coefficient; $C_{\text{norb}}$ = the norbixin concentration; linear = the analytical curve linearity; spect = the uncertainty associated with the spectrophotometer; and prec.syst = the precision of the analytical system; $E_{\text{spect1,2}}$ = absorption coefficient spectrophotometer 1,2.
RESULTS AND DISCUSSION

Analytical curve preparation

The purity of the norbixin isolated and determined by high performance liquid chromatography with a diode array detector (HPLC-DAD) (Figure 3) was calculated as 89% with a spectral purity of 99%.

The trans and cis isomers can be distinguished based on the UV-VIS absorption spectra between 250 and 600 nm. According to Scotter et al.\textsuperscript{23}, the cis isomer shows an increase in absorption intensity close to 355 nm. The absorption spectrum obtained (Figure 3) showed an increase in intensity at 355 nm, indicating that the pigment isolated corresponded to cis-norbixin.

Under the HPLC-DAD analytical conditions used, the maximum absorption for 9'-cis-norbixin occurred at 463 nm and presented a spectral purity of 99% (Figure 3). Scotter et al.\textsuperscript{23} and Rios and Mercadante\textsuperscript{15} reported maximum absorption for cis-norbixin at 461 nm and a chromatographic purity of 92% and 93%, respectively, under the same analytical conditions used in the present study.

As from the norbixin solution in methanol, with the concentration corrected for chromatographic purity (44.44 mg 100 mL\textsuperscript{-1}), 2 mL aliquots were taken and dried in a nitrogen flow before diluting to 100 mL in the solvents mentioned above. An analytical curve with 10 points (not including the origin) was prepared and a spectral scan carried out between 300 nm and 600 nm to verify the absorption maxima of peaks II and III. After the spectral scan and determining the absorption maxima of peaks II and III, absorption reading was taken at each peak and the curve equation determined for each wavelength evaluated in each solvent. Table shows the data obtained.

According to Pretsch et al.\textsuperscript{24}, the Woodward-Fieser rule can be used to calculate the absorption chromophore value at the maximum absorption wavelength (\(\lambda_{\text{max}}\) in nm). The rule estimates that carboxylic acids with \(\alpha, \beta\)-unsaturation present maximum absorption at a wavelength of 195 nm, substitutions at the \(\gamma\) and \(\delta\) positions contribute 18 nm, each additional conjugated double bond contributes 30 nm, and each alkyl substituent 5 nm. Norbixin presents 7 additional conjugated double

Figure 2. Cause – effect graph showing the uncertainty causes used in the absorption coefficient calculation. Where calib = calibration; spect = spectrophotometer; linear = analytical curve linearity; repe = repeatability; resol = resolution; prec. syst = analytical system precision; and temp = temperature; E spect 1,2 = absorption coefficient of spectrophotometer 1,2

Figure 3. Thin layer chromatogram (A) absorption spectra with the identification of the absorption peaks I, II and III and the spectral purity of the isolated 9-cis-norbixin. (B). Analytical conditions described in the text
bonds, one substitution at position γ and another at position δ, and 4 alkyl substituents, and hence, according to the rule, should present maximum absorption at a wavelength of 461 nm, not considering the solvent. The same rule mentions correction for some solvents, such as water, of -8 nm. Considering correction for the solvent, the maximum absorption wavelength calculated for norbixin would change from 461 nm to 453 nm. Maximum absorption of 454 nm was observed for the solutions of 0.5% sodium hydroxide and 0.5% potassium hydroxide.

The spectrophotometers used in the study were located in buildings far from each other, and the absorbance readings were made in an interval of approximately 30 minutes, which allowed for some observations of the solvents of acetone and of the alkalis.

Acetone, which has one carbonyl group, shows a tendency to suffer nucleophilic addition reactions, which occur quicker in acid and basic solutions than in neutral solutions, the hydration equilibrium constant (K\text{hydr}) and the relative hydration velocity being influenced by the effects of the steric and electronic structure.\textsuperscript{25} A contact period was found to be necessary for the solvents of potassium hydroxide and sodium hydroxide in order to establish chemical equilibrium and the formation of the norbixin salts. The readings taken using equipment 2 were made soon after preparation, and could have been taken before the acetone and potassium hydroxide solutions had reached equilibrium. However this was not observed for the 0.5% sodium hydroxide solution, probably because the curves made with the two alkaline solutions were prepared in sequence, and the absorbance readings made first were those prepared in the potassium hydroxide solution (Table).

**Table.** Dilution solvents, wavelengths, spectrophotometers, regression equations of the analytical curves, determination coefficients and absorption coefficients found for cis-norbixin, expanded uncertainty

<table>
<thead>
<tr>
<th>Solvent</th>
<th>λ (nm)</th>
<th>Spect</th>
<th>Analytical curve</th>
<th>r²</th>
<th>E\textsuperscript{1%}\textsubscript{1cm} Mean ± s</th>
<th>U (K = 2)</th>
<th>% U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>452</td>
<td>1</td>
<td>Abs=2.9122c + 0.0037</td>
<td>1.000</td>
<td>2912</td>
<td>2896±23</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>Abs=2.88c + 0.0009</td>
<td>1.000</td>
<td>2880</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>480</td>
<td>1</td>
<td>Abs=2.5912c + 0.011</td>
<td>1.000</td>
<td>2591</td>
<td>2583±11</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>Abs=2.5755c + 0.018</td>
<td>1.000</td>
<td>2575</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>457</td>
<td>1</td>
<td>Abs=2.5931c + 0.0514</td>
<td>0.993</td>
<td>2593</td>
<td>2553±57</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>Abs=2.5122c + 0.0453</td>
<td>0.991</td>
<td>2512</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroform: acetic acid</td>
<td>475</td>
<td>1</td>
<td>Abs=2.2689c + 0.0488</td>
<td>0.991</td>
<td>2269</td>
<td>2237±45</td>
<td>83</td>
</tr>
<tr>
<td>(99:01, v/v)</td>
<td>507</td>
<td>2</td>
<td>Abs=2.2059c + 0.0399</td>
<td>0.991</td>
<td>2206</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5% Potassium hydroxide</td>
<td>454</td>
<td>1</td>
<td>Abs=2.9235c + 0.0063</td>
<td>0.999</td>
<td>2923</td>
<td>2887±51</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>Abs=2.8514c + 0.0056</td>
<td>0.997</td>
<td>2851</td>
<td></td>
<td></td>
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<tr>
<td>0.5% Sodium hydroxide</td>
<td>483</td>
<td>1</td>
<td>Abs=2.614c + 0.0000</td>
<td>0.999</td>
<td>2581</td>
<td>2546±50</td>
<td>85</td>
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<tr>
<td></td>
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<td>Abs=2.5109c + 0.0049</td>
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<tr>
<td></td>
<td>454</td>
<td>1</td>
<td>Abs=2.8461c + 0.0060</td>
<td>0.999</td>
<td>2846</td>
<td>2854±11</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>Abs=2.8617c + 0.0018</td>
<td>0.999</td>
<td>2862</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>483</td>
<td>1</td>
<td>Abs=2.5213c + 0.0053</td>
<td>0.999</td>
<td>2521</td>
<td>2523±2</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>Abs=2.5243c + 0.0015</td>
<td>0.999</td>
<td>2524</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

λ = wavelength; Spect = spectrophotometer; r² = correlation coefficient; E\textsuperscript{1%}\textsubscript{1cm} = calculated absorption coefficient; s = standard deviation estimate; U = expanded uncertainty; %U = percentage contribution of uncertainty for absorption coefficient
Of the solvents used to determine the absorption coefficient of the 9'-cis-norbixin isolated with a purity of 89%, 0.5% sodium hydroxide was the one that presented an absorption coefficient closest to the values reported in the publications consulted. The mean values determined for $E_{1\%}^{1\%}$ were 2854 at 454 nm and 2523 at 483 nm, and Scotter\textsuperscript{23} and Reith and Gielen\textsuperscript{10} found values of 2818 and 2850 at 453 nm, and 2503 and 2550 at 482 nm, respectively, although the sodium hydroxide concentration used was 0.1N.

The values calculated for the solvent 0.5% potassium hydroxide were 2887 at 454 nm and 2546 at 483 nm, which differed from the values found in publications by FAO/WHO, where values of 3473 at 453 nm (1982)\textsuperscript{12} and 2870 at 482 nm (2003\textsuperscript{26} and 2006\textsuperscript{13}) were presented.

Although the concentration of acetic acid used in the chloroform (99:01, v/v) was not the same as the concentration used in other publications (99.5:0.5, v/v), the values encountered presented less variation. Values for $E_{1\%}^{1\%}$ equal to 2757 at 475 nm and 2417 at 507 nm were found in the present study, and Scotter et al\textsuperscript{7} and Reith and Gielen\textsuperscript{10} established values of 2620 at 473 nm and 2290 at 503 nm.

**Estimate of the measurement uncertainty**

In order to estimate the measurement uncertainty, the uncertainties associated with each analytical step were considered according to the cause and effect graph (Figure 2), and Table shows the results obtained. The expanded uncertainty was calculated at a confidence level of 95.45% using the fact that the coverage factor K is equal to 2\textsuperscript{27}.

The greatest source of uncertainty observed was the spectrophotometer (Figure 4) contributing about 80% of the associated uncertainties.

**CONCLUSION**

The results obtained in this study indicate the need for a better discussion concerning the values for the absorption coefficient for norbixin found in the literature. These values, used in various scientific studies, quality control analyses and in the commerce of annatto dyes and seeds, should always be accompanied by the indication of their uncertainty.

Although this study did not use analytical techniques such as nuclear magnetic resonance or mass detector in the identification of norbixin, demonstrated that the absorption coefficient must be adequate for the intended use, be supported by evidence and have adequate uncertainty.
REFERENCES


