



The Annatto Carotenoids and the Norbixin Absorption Coefficient

Carotenoides de Urucum e o Coeficiente de Absorção da Norbixina

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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 $85 \text{ g } 100 \text{ mL}^{-1}$, respectively.

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

RESUMO

O principal carotenoide 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 absorvâncias são obtidas nos dois picos espectrais de maior intensidade, mas discrepâncias no valor de absorvabilidade 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 $85 \text{ g } 100 \text{ mL}^{-1}$, respectivamente.

Palavras-chave. *Bixa orellana*, pigmentos, solventes, quantificação, estimação da incerteza.

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 $\pi \rightarrow \pi^*$ with excited state of comparatively low energy, which corresponds to light in the visible wavelength region. The solvent effect on the $\pi \rightarrow \pi^*$ transition energy of pigment in different solvents which allows to obtain the absorption coefficient with the Beer-Lambert law^{1,2,3}.

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*-configuration¹.

Annatto seeds (*Bixa orellana* L.) present *cis*-bixin as the major carotenoid, representing more than 80% of the carotenoids present⁴. *Cis*-bixin is designated as the monomethyl ester of *cis*-norbixin dicarboxylic acid (9'-*cis*-norbixin: 6,6'-diaprocarotenedioic acid) (). 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 process⁵.

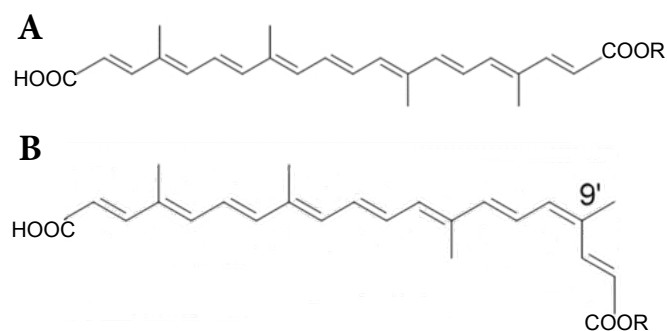


Figure 1. Chemical structure of (A) *trans*- and (B) *cis*-bixin (for R=CH₃) or norbixin (for R=H) (Dias et al)⁶

Seeds with the highest pigment contents are looked for in order to produce the dye, and hence the value of the seeds is directly related to the carotenoid concentration, making the exactness of the quantification method an extremely important factor.

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 () for the quantification of these pigments has been widely divulged^{3,6,7,8}. 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 path⁹.

The values divulged in the literature for the norbixin absorption coefficient have undergone changes with time, without changing the wavelength or solvent. Reith and Gielen¹⁰ and FAO/WHO¹¹ mentioned values for () of 2850±40 for spectrophotometric readings of annatto pigments in 0.1N sodium hydroxide at 453 nm, but in 1982 FAO/WHO¹² changed the value to 3473. In 2006, FAO/WHO¹³ 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 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 Mercadante¹⁴ and Rios and Mercadante¹⁵. 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 °C, 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 Scotter¹⁶. 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^{1,3,7,17,18,19,20}.

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 (λ max)^{5,10,15,21}.

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 procedures²². According to this definition, the uncertainty estimation associated with the absorption coefficient () in g 100g⁻¹ obtained by spectrophotometry, was calculated from the graph equation (), and the uncertainty sources listed on the cause - effect graph (). Thus the combined uncertainty () 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.

(Equation 1)

(Equation 2)

where: () = the absorption coefficient; C_{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; $E_{\text{spect}1,2}$ = absorption coefficient spectrophotometer 1,2.

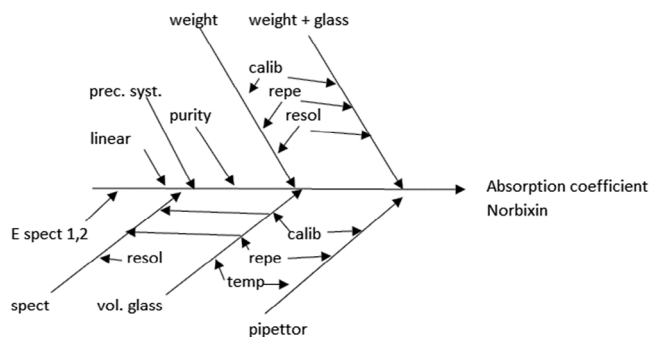


Figure 2. Cause – effect graph showing the uncertainty causes used in the absorption coefficient calculation. Where calib = calibration; spect = spectrophotometer; linear = analytical curve linearity; repe = repeatability; resol = resolution; prec. syst = analytical system precision; and temp = temperature;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

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) () 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 al²³, the *cis* isomer shows an increase in absorption intensity close to 355 nm. The absorption spectrum obtained () 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% (). Scotter et al²³ and Rios and Mercadante¹⁵ 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⁻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. shows the data obtained.

According to Pretsch et al²⁴, the Woodward-Fieser rule can be used to calculate the absorption chromophore value at the maximum absorption wavelength (λ max in nm). The rule estimates that carboxylic acids with α , β -unsaturation present maximum absorption at a wavelength of 195 nm, substitutions at the γ and δ positions contribute 18 nm, each additional conjugated double bond contributes 30 nm, and each alkyl substituent 5 nm. Norbixin presents 7 additional conjugated double

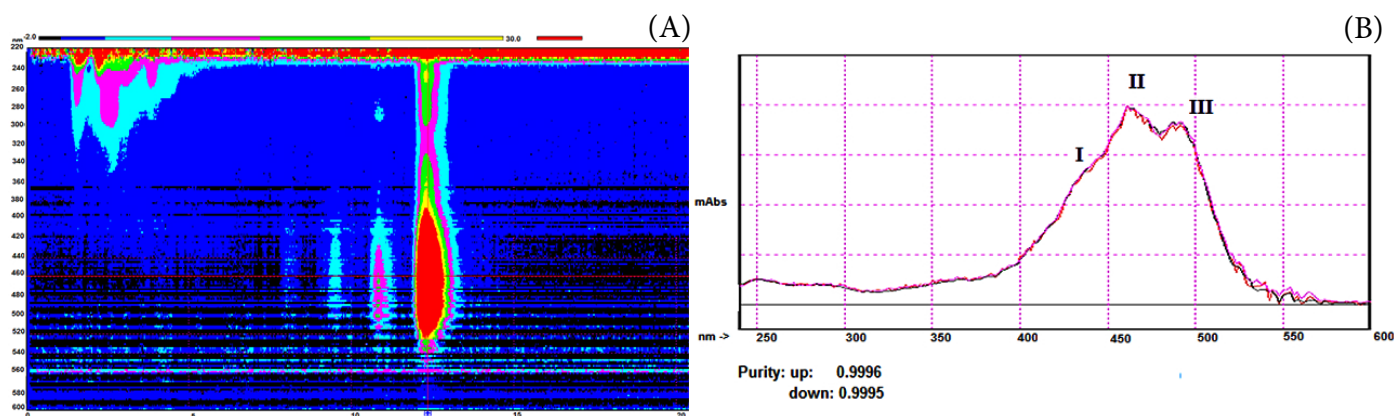


Figure 3. Thin layer chromatogram (A) absorption spectra with the identification of the absorption peaks I, II and III and the spectral purity of the isolated 9-*cis*-norbixin. (B). Analytical conditions described in the text

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_{hydr}) and the relative hydration velocity being influenced by the effects of the steric and electronic structure²⁵. 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. Dilution solvents, wavelengths, spectrophotometers, regression equations of the analytical curves, determination coefficients and absorption coefficients found for *cis*-norbixin, expanded uncertainty

Solvent	λ (nm)	Spect	Analytical curve	r^2			U (K = 2)	% U
					Mean	s		
Methanol	452	1	Abs=2.9122c - 0.0037	1.0000	2912	2896±23	58	2
		2	Abs=2.88c + 0.0009	1.0000	2880			
	480	1	Abs=2.5912c - 0.0011	1.000	2591	2583±11	51	2
		2	Abs=2.5755c + 0.0018	1.0000	2575			
Acetone	457	1	Abs=2.5931c - 0.0514	0.9923	2593	2553±57	98	4
		2	Abs=2.5122c - 0.0453	0.9914	2512			
	486	1	Abs=2.2689c - 0.0488	0.9915	2269	2237±45	83	4
		2	Abs=2.2059c - 0.0399	0.9917	2206			
Chloroform: acetic acid (99:01, v/v)	475	1	Abs=2.7659c - 0.0025	0.9998	2766	2757±12	52	2
		2	Abs=2.7484c - 0.022	0.9999	2748			
	507	1	Abs=2.4245c - 0.0027	0.9998	2424	2417±10	51	2
		2	Abs=2.4098c - 0.0024	0.9999	2410			
0.5% Potassium hydroxide	454	1	Abs=2.9235c + 0.0063	0.9994	2923	2887±51	86	3
		2	Abs=2.8514c + 0.0056	0.9997	2851			
	483	1	Abs=2.614c + 0.0000	0.9995	2581	2546±50	85	3
		2	Abs=2.5109c + 0.0049	0.9997	2511			
0.5% Sodium hydroxide	454	1	Abs=2.8461c - 0.0060	0.9991	2846	2854±11	51	2
		2	Abs=2.8617c - 0.0018	0.9994	2862			
	483	1	Abs=2.5213c - 0.0053	0.9992	2521	2523±2	49	2
		2	Abs=2.5243c - 0.0015	0.9995	2524			

λ = wavelength; Spect = spectrophotometer; r^2 = correlation coefficient; = 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 were 2854 at 454 nm and 2523 at 483 nm, and Scotter²³ and Reith and Gielen¹⁰ 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)¹² and 2870 at 482 nm (2003²⁶ and 2006¹³) 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 equal to 2757 at 475 nm and 2417 at 507 nm were found in the present study, and Scotter et al⁷ and Reith and Gielen¹⁰ 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 (), and 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 2²⁷.

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

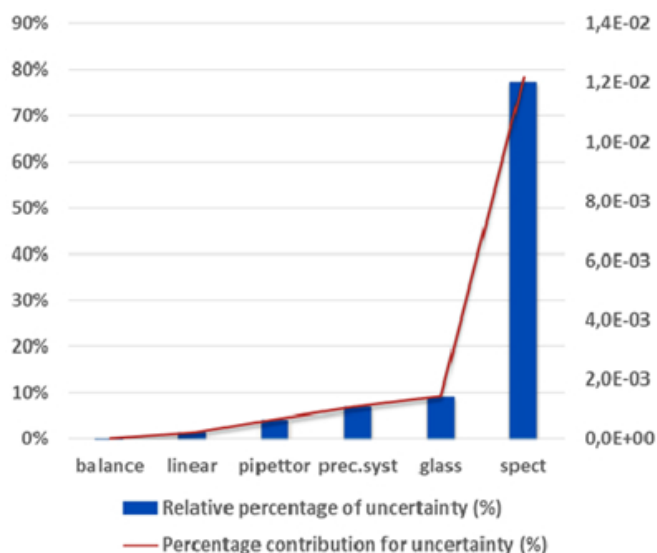


Figure 4. Relative percentage of uncertainty and percentage contribution for uncertainty considered in the estimates

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

1. Bareth A, Strohmar W, Kitzelmann E. HPLC and spectrophotometric determination of annatto in cheese. *Eur Food Res. Technol.* 2002;215:359-64. <http://dx.doi.org/10.1007/s00217-002-0541-y>
2. Rodriguez-Amaya DB. *Food carotenoids: chemistry, biology and technology*, Chichester (UK):John Wiley & Sons, Ltd; 2016.
3. Tupuna DS, Paese K, Guterres SS, Jablonski A, Flôres SH, Rios AO. Encapsulation efficiency and thermal stability of norbixin microencapsulated by spray-drying using different combinations of wall materials. *Ind Crops Prod.* 2018;111:846-55. <http://dx.doi.org/10.1016/j.indcrop.2017.12.001>
4. Preston HD, Rickard MD. Extration and chemistry on annatto. *Food Chem.* 1980;5:47-56. [http://dx.doi.org/10.1016/0308-8146\(80\)90063-1](http://dx.doi.org/10.1016/0308-8146(80)90063-1)
5. Costa CLS, Chaves MH. Extração de pigmentos das sementes de *Bixa orellana* L.: uma alternativa para disciplinas experimentais de química orgânica. *Quím Nova.* 2005;28 (1):149-52. Disponível em: <http://www.scielo.br/pdf/qn/v28n1/23053.pdf>
6. Dias VM, Pilla V, Alves LP, Oliveira HPM, Munin E. Optical characterization in annatto and comercial colorific. *J Fluoresc.* 2011;21:415-21. <http://dx.doi.org/10.1007/s10895-010-0730-1>
7. Scotter MJ, Wilson LA, Appleton GP, Castle L. Analysis of annatto (*Bixa orellana* L.) food coloring formulations. 1. Determination of coloring components and colored thermal degradation products by high-performance liquid chromatography with photodiode array detection. *J Agric Food Chem* 1998;46(3):1031-8. <http://dx.doi.org/10.1021/jf970063+>
8. Scotter, MJ. Methods for the determination of European Union-permitted added natural colours in foods: a review. *Food Addit Contam. A* 2011;28(5):527-96. <http://dx.doi.org/10.1080/19440049.2011.555844>
9. Dean JA. *Lange's handbook of chemistry*. 5.ed. St. Louis (NY):McGraw-Hill Inc;1999.
10. Reith JE, Gielen JW. Properties of bixin and norbixin and the composition of annatto extracts. *J Food Sci.* 1971;36(6):861-4. <https://doi.org/10.1111/j.1365-2621.1971.tb15545.x>
11. FAO/WHO. Specifications for the Identify and Purity of Some Food Colors, Flavours Enhancer, Thickening Agents and Certain other Food Additives. FAO Nutricion Meeting Report Series, 54B: Rome; 1975. WHO Food Additives Series, nº. 7, 1975.
12. FAO/WHO. Evaluation of certain food additives and contaminants. WHO Technical Report Series, nº 683 / Joint FAO/WHO Expert Committee on Food Additives (JECFA). Geneva, Italy; 1982. Disponível em: http://apps.who.int/iris/bitstream/handle/10665/41546/WHO_TRS_683.pdf?sequence=1
13. FAO/WHO. Compendium of Food Additive Specifications. Monographs 3 / Joint FAO/WHO Expert Committee on Food Additives (JECFA), 67th meeting, Food and Agriculture Organization of the United Nations: Rome; 2006. Disponível em: <http://www.fao.org/3/a-a0675e.pdf>
14. Tocchini L, Mercadante AZ. Extração e determinação, por CLAE, de bixina e norbixina em coloríficos. *Ciênc Tecnol Aliment.* 2001;21(3):310-3. <http://dx.doi.org/10.1590/S0101-20612001000300010>
15. Rios AO, Mercadante AZ. Otimização para obtenção de padrão de bixina e das etapas de extração e saponificação para quantificação de bixina em snacks extrusados por CLAE. *Alim Nutr.* 2004;15(3):203-13.
16. Scotter M. The chemistry and analysis of annatto colouring: a review. *Food Addit Contam. A* 2009;26(8):1123-45. <http://dx.doi.org/10.1080/02652030902942873>
17. Carvalho PRN, da Silva MG, Fabri EG, Tavares PER, Martins ALM, Spatti LR. Concentração de bixina e lipídios em sementes de urucum da coleção do Instituto Agrônômico (IAC). *Bragantia.* 2010;69(3):519-24. <http://dx.doi.org/10.1590/S0006-87052010000300002>

18. Cardeñosa V, Lunar ML, Rubio S. Generalized and rapid supramolecular solvent-based sample treatment for the determination of annatto in food. *J Chromatogr A*. 2011; 1218(50):8996-9002. <http://dx.doi.org/10.1016/j.chroma.2011.10.041>
19. Gallardo-Cabrera C, Rojas-Barahona A. Stability study of an aqueous formulation of the annatto dyes. *Int Food Res J*. 2015;22(5):2149-54. Disponível em: [http://www.ifrj.upm.edu.my/22%20\(05\)%202015/\(56\).pdf](http://www.ifrj.upm.edu.my/22%20(05)%202015/(56).pdf)
20. Raddatz-Mota D, Pérez-Flores LJ, Carrari F, Insani M, Asis A, Mendoza-Espinoza JA, León-Sánchez FD, Rivera-Cabrera F. Chemical characterization and quantification of the pigment extraction yield of seven mexican accessions of *Bixa orellana*. *Rev Mex Ing Quím*. 2016;15(3):727-40.
21. Rodriguez-Amaya DB, Kimura M. HarvestPlus Handbook for carotenoid analysis. HarvestPlus Technical Monograph 2. Washington, DC and Cali: International Food Policy Research Institute (IFPRI) and International Center for Tropical Agriculture (CIAT);2004.
22. Magnusson B, Örnemark U. Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, 2. ed.;2014. [acesso 2015 Jun 03]. Disponível em: https://www.eurachem.org/images/stories/Guides/pdf/MV_guide_2nd_ed_EN.pdf
23. Scotter MJ, Thorpe SA, Reynolds SL, Wilson LA, Strutt PR. Characterization of the principal colouring components of annatto using high performance liquid chromatography with photodiode-array detection. *Food Addit Contam*. 1994;11(3):301-315. <http://dx.doi.org/10.1080/02652039409374229>
24. Pretsch E, Bühlmann P, Badertscher M. Structure Determination of Organic Compounds – Table of Spectral Data. 4.ed. Verlag Berlin Heidelberg: Springer; 2009.
25. Carey FA. Química Orgânica. 7.ed., vol. 2. Porto Alegre: AMGH Editora Ltda; 2011.
26. FAO/WHO. Compendium of Food Additive Specifications. Addendum 11 / Joint FAO/WHO Expert Committee on Food Additives (JECFA), 61th meeting, Food and Agriculture Organization of the United Nations: Rome; 2003.
27. Ellison SLR., Williams A. Eurachem/CITAC guide: Quantifying Uncertainty in Analytical Measurement. 3.ed; 2012. [acesso 2015 Jun 03]. Disponível em: https://www.eurachem.org/images/stories/Guides/pdf/QUAM2012_P1.pdf