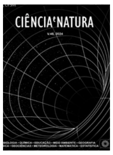

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Chemistry

Annatto seeds: Extraction of essential oils of four cultivars, characterization by LC-MS/MS, and non-destructive NIR Spectrometer

Sementes de urucum: Extração do óleo essencial de quatro cultivares, caracterização por LC-MS/MS e espectrômetro NIR não destrutivo

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ABSTRACT

In annatto seeds, we find a healthy and nutritious pigment in the diet. Annatto seeds are grown in South and Central America and have been used for centuries as a natural food coloring. The Bixin pigment can be extracted after extracting the essential oil, adding more value to the seed. Essential oil also has great value in the industry. The identification of four cultivars of annatto seeds in the trade is important, and quick evaluation with preserving the seeds as near-infrared techniques can be a good alternative. The current work investigated near-infrared spectroscopy (NIRS) for annatto seeds and essential oil. The essential oil was extracted with the Clevenger apparatus and analyzed by gas chromatography coupled with mass spectroscopy. NIR spectra were obtained from fresh seeds and after the essential oil extraction. The best pre-treatment was the second derivative for fresh seeds, and after oil extraction both the first and second derivatives are adequate. NIR spectroscopy showed the potential for discrimination of annatto seed cultivars non-destructively.

Keywords: Near-infrared spectroscopy; α-Pinene; Geranylgeraniol; *Bixa orellana* seeds

RESUMO

Nas sementes de urucum encontramos um pigmento saudável e nutritivo para a dieta. As sementes de urucum são cultivadas na América do Sul e Central e são usadas há séculos como corante alimentar natural. O pigmento Bixina pode ser extraído após a extração do óleo essencial, agregando mais valor à semente. O óleo essencial também tem grande valor na indústria. A identificação de quatro cultivares de sementes de urucum no comércio é importante, e uma avaliação rápida com técnicas de preservação das sementes no infravermelho próximo pode ser uma boa alternativa. No presente trabalho, a espectroscopia de infravermelho próximo (NIRS) foi investigada para as sementes de urucum e o óleo essencial. O óleo essencial extraído com aparelho de Clevenger e analisado por cromatografia gasosa acoplada à espectroscopia de massas. Os espectros NIR foram obtidos de sementes frescas e após a extração do óleo essencial. O melhor pré-tratamento foi o segundo derivado para sementes frescas, e após a extração do óleo tanto o primeiro quanto o segundo derivados são adequados. A espectroscopia NIR mostrou o potencial de discriminação de cultivares de sementes de urucum de forma não destrutiva.convergirem para algumas indicações populares, ainda são necessários outros estudos para corroborar a efetividade terapêutica das plantas.

Palavras-chave: Espectroscopia de infravermelho próximo; α-Pineno; Geranilgeraniol; Sementes de *Bixa orellana*

1 INTRODUCTION

Annatto (*Bixa orellana* L.) seeds are a rich source of compounds with interest in several areas. The seeds contain 2–3% oil mainly composed of δ-tocotrienol, γ-tocotrienol, tocopherols, and geranylgeraniol The species annatto from Bixaceae family, is cultivated in the tropics all over the world Kang et al. (2010) e Zabot et al. (2019). In Brazil, it was identified as a plant native to the northern and northeastern states, mainly Amazonas, Pará, Paraíba, Piauí, Maranhão, Ceará, Bahia and is cultivated in other regions Calvo-Irabien (2018), Pacheco et al. (2019) e Stringheta et al. (2018).

Few studies identifying the essential oil of annatto were made, and among different cultivars. The volatile compounds identified in the seed oil of *B. orellana* was dominated by (Z, E) - farnesyl acetate (11.6%), occidentalol acetate (9.7%), spathulenol (9.6%) and ishwarane (9.1%) Pino; Correa (2003). The compounds detected by gas chromatography-mass spectrometry analysis, the ishwarane (18.6%) and geranylgeraniol (9.1%) were the major components Monzote et al. (2014)

The presence of several volatile compounds, make this plant important for commercialization Bitencourt et al. (2018) e Pino; Correa (2003) e Tabari et al. (2017).

This study aimed to identify the composition of essential oils of four cultivars the brown (BOB), green (BOG), brown small (BOBS) and red (BOR) of annatto from southern Brazil and evaluate the potential of Near Infrared Spectroscopy to application in cultivars discrimination.

2 MATERIAL AND METHODS

The plant samples of annatto were collected in August 2017, from the Environmental Institute of Parana (IAP), in Morretes city, state of Paraná, southern Brazil (Coordinates: COD 02548038/25°30'S, 48°49' W/ Alt 59 m). The identified plant material in the Botanical Museum of Curitiba - PR, Brazil, and a voucher specimen deposited. (#379.394); IBAMA (02001.001165/2013-47) and is registered on SISGEN with number A0EB51A.

The seeds from four annatto cultivars were collected: the brown (BOB), green (BOG), brown small (BOBS) and red (BOR).

2.1 Essential oil extraction

The collected seeds were stored in plastic bags in a freezer at 16 °C until the extraction of the essential oil by hydrodistillation in the Clevenger apparatus, according to the methodology recommended by the Brazilian Pharmacopoeia for volatile oils BrasilBrazilian Pharmacopoeia (2019).

The annatto seeds (100 g ± 1) were used without grinding (whole seeds), was combined with 1000 cm^3 of water (hydromodulus plant material:water ratio of 1:10 m/v). Essential oil was extract by hydrodistillation method for 4 hours using a Clevenger hydrodistillation. In the end, the essential oil was collected and stored at -16°C for chemical analysis. The yield was expressed in g of oil per 100 g of the plant material (g/100 g p.m.). BrasilBrazilian Pharmacopoeia (2019). After the oil extraction the seeds were again dried in an oven at 50 °C, and after stabilization was analyzed in Fourier transform near-infrared (FT-NIR) spectroscopy.

2.2 FT-NIR spectroscopy measurements

The four cultivars of annatto were analyzed by FT-NIR. The fresh seeds have been placed directly on the equipment, without any sample preparation. For each cultivar, a total of 25 spectra was collected. After oil extraction, the procedure was repeated.

The spectra were collected in a Spectrometer Tensor 37 (Bruker) equipped with an integrating sphere and operating in diffuse reflectance mode. The readings were performed with 64 scans and 4 cm^{-1} resolutions, in the range of 10,000- 4,000 cm^{-1}.

The data were analyzed in the program Unscrambler X (version 10.1, of CAMO Software AS). Exploratory models were obtained by analyzing the scores and loadings graphs obtained through principal component analysis (PCA) using the NIPALS algorithm and cross-validation method random with 20 segments. Data were processed in raw form and after pretreatment of the first derivative (polynomial order = 2, smoothing points = 11) and second derivative (polynomial order = 2, smoothing points = 21) of Savitzky-Golay to eliminate noise from surface irregularity. A linear discriminant analysis (LDA) applying Mahalanobis method assuming equal prior probabilities was performed with PCA score data to verify de distinction of material and confusion matrix was evaluated. Data collection and analysis followed ASTM E1655-05Xu et al. (2013).

2.3 Chromatographic profile of the essential oils

The essential oil from annatto was analyzed by gas chromatography-mass spectrometry using a Shimadzu GC-MS-QP 2010 Plus analyzer, equipped with a capillary column Rtx -5MS (30 m × 0.25 mm × 0.25 μm.) with a splitless injector mode at 250°C, and an ion source and interface at 250°C. The mass window was adjusted for analyzes in the range of 40-350 m/z, using Helium as entrainment gas, with a column flow of 1.0 mL/min. Injection ramp for analysis with injector temperature at 250 °C, column pressure of 59 KPa, starting with a temperature of 60 °C rising to 250 °C at a rate of 3 °C /min. After the analysis, the characterization of the chemical constituents was performed by comparing the Kovats retention indexes obtained in the spectra, with those documented in the literature Melo et al. (2017).

3. RESULTS AND DISCUSSION

3.1. Volatile compounds of four cultivars of annatto

The GC–MS analysis of the essential oil led to the identification of different compounds, representing 0.61% of the total oil from the seeds of BOB, 0.45% of BOG, 0.77% from BOBS and 0.96% from BOR. The identified compounds listed in Table 1, according to their elution order on the capillary column.

There are differences in the volatile profile between soluble extracts in water and oil and annatto extracts contain odors with the potential to influence the aroma of foods Bitencourt et al. (2018 e Satyanarayana et al. (2006).

In the cultivar, Brown (BOB) the major compounds were α-pinene (29.7%). The α -pinene is an alkene and found in the oils of many species of coniferous trees Pavithra et al. (2019). This component has diverse applications as inducing apoptosis evidenced by early mitochondrial potential rupture, reactive oxygen species production, increased caspase-3 activity, heterochromatin aggregation, DNA fragmentation, and phosphatidylserine exposure on the cell surface. Also α -pinene was effective in treating experimental metastatic melanoma, reducing the number of nodules of lung tumors Matsuo et al. (2011). Studies in other plants report that two monoterpenes found in *Salvia lavandulaefolia* (Spanish sage) essential oil, α-Pinene, and geraniol, inhibited the production of thromboxane B2 and leukotriene B4, respectively, in rat leucocytes Perry et al. (2003).

In the small brown cultivar (BOBS) the majority volatile compounds were Isomer – Geranylgeraniol (42.80%). Geranylgeraniol already identified in annatto seeds, and other plants Culioli et al. (1999) e Mercadante et al. (1999) e Spindola et al. (2011). Geranylgeraniol has different properties as antinociceptive Spindola et al. (2011), anticancer Yoshikawa et al. (2009), contributes to the treatment of hypercholesterolemia where simvastatin is used Ferri et al. (2017), inhibited osteoclast formation Hiruma et al. (2004), bisphosphonate-related osteonecrosis of the jaws (BRONJ) Koneski et al. (2018)divided into three groups: two experimental groups (EG1 and EG2.

Table 1 – Percentages (%) of essential oil content of *Bixa orellana* cultivars

	Compounds	**RI**	**BOB**	**BOG**	**BOBS**	**BOR**
1	α-Pinene	931	29.7	9.3	nd	nd
2	β-Pinene	970	23.8	7.4	nd	nd
3	Mircene	981	3.1	nd	nd	nd
4	(Z)-β-Ocimene	1026	2.7	nd	nd	nd
5	δ-Elemene	1330	3.7	19.0	9.4	14.8
6	α-Copaene	1368	nd	nd	nd	2.9
7	β-Elemene	1382	nd	2.4	nd	1.6
8	(E)-Cariofilene	1407	nd	nd	nd	1.8
9	6,9-Guaiadiene	1439	nd	1.3	nd	nd
10	Ishwarane	1450	nd	nd	nd	4.3
11	Germacrene D	1467	3.1	nd	2.8	nd
12	γ-Muurolene	1469	nd	14.3	2.1	nd
13	δ-Selinene	1478	nd	3.0	nd	1.5
14	Germacrene B	1480	nd	nd	nd	13.9
15	Bicyclogermacrene	1483	7.5	5.3	nd	9.2
16	δ-Cadinene	1508	1.6	nd	nd	nd
17	δ-Amorfene	1509	nd	2.3	1.9	3.2
18	Spathunelol	1553	nd	nd	nd	6.5
19	NI ($C_{15}H_{26}O$)	1565	nd	2.4	nd	nd
20	NI ($C_{15}H_{26}O$)	1571	1.9	nd	nd	nd
21	β-Copaen-4-α-ol	1590	nd	nd	nd	6.4
22	NI ($C_{15}H_{26}O$)	1602	nd	6.6	5.6	nd
23	β-Atlantol	1603	nd	nd	10.2	nd
24	NI ($C_{15}H_{24}O$)	1616	nd	nd	nd	1.4
25	α-Muurolool	1618	nd	1.4	1.6	nd
26	β-Springene	2164	nd	3.2	nd	nd
27	Geranyl linalool	2165	8.4	nd	nd	12.3
28	Isomero – Geranyl geraniol	2166	nd	nd	42.8	nd
29	Isomero – Geranyl geraniol	2182	nd	nd	2.3	nd
30	α-springene	2272	nd	nd	1.9	nd
Total identified			83.9	80.3	80.7	80.3
Hydrogenated Monoterpene			59,3	16,8	-	-
Hydrogenated sesquiterpene			16,1	47,8	16,2	49,2
Oxygenated sesquiterpene			1,9	10,5	17,5	18,8
Others			8,4	3,2	45,1	12,3

ND: not detected; RI: retention indices relative to C6 – C20 n-alkanes on RTX-5MS column, Bixa orellana cultivars (BOB: brown, BOG: green, BOBS: brown small, BOR: red)

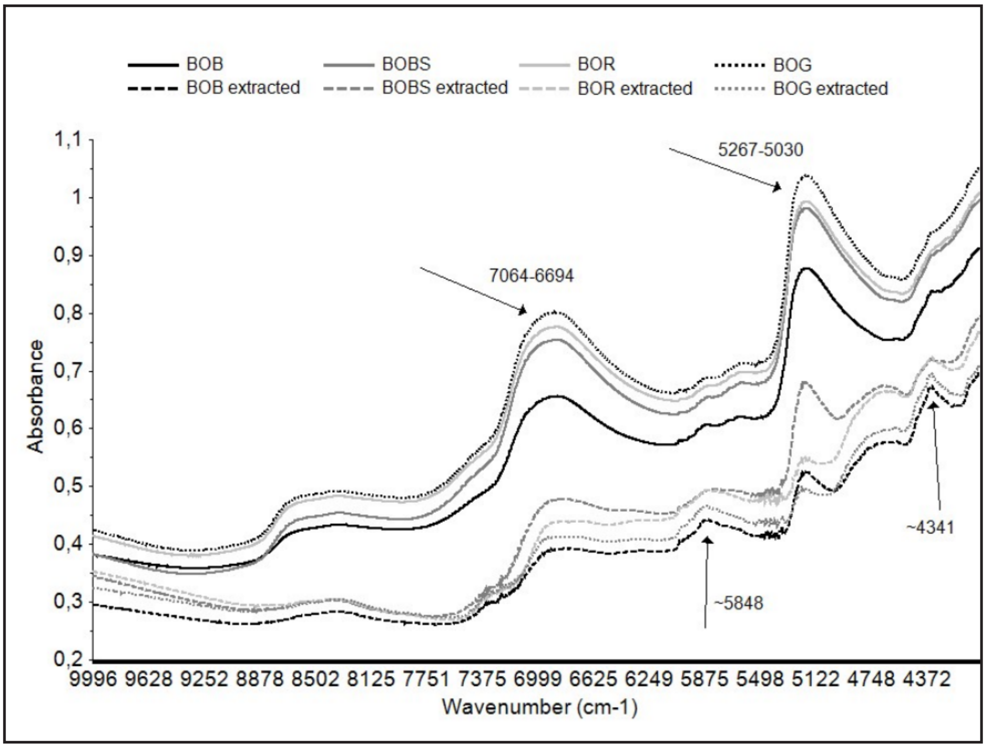
Linalool and β-pinene are two volatile monoterpenes that possess antidepressant-like activity. These are components of many aromatic plants used in folk medicine around the world to relieve anxiety and depression Guzmán-Gutiérrez et al. (2015). The essential oil main component obtained from the leaves of *Baccharis punctulata* (Asteraceae), δ-elemene, presented antioxidant and anti-inflammatory properties Ascari et al. (2019).

The contents of bicyclogermacre and γ-muurolene from Piper species were positively associated with *E. coli* inhibition, whereas, levels of germacrene-D and trans-caryophyllene were associated to the inhibitory activity against all tested bacteria Perigo et al. (2016).

3.2. It is possible to predict seed identity using FT-NIR Spectroscopy?

Mean NIR spectra of four cultivars before and after essential oil extraction shows similar behavior, with the difference in baseline (Figure 1). Absorbance in seeds from brown cultivar (BOB) is slower than others are and in green cultivar is higher. After essential oil extraction, all samples presented slower absorption in near-infrared than fresh material. Also, it is possible to observe an incidence of peaks at ~5848 cm^{-1} and ~4341 cm^{-1}, that are related to CHν+ CHν and CHν+ CHδ Workman Jr.; Weyer (2008).

Figure 1 – NIR mean spectra by cultivar in fresh seeds and after extraction. (BOB: brown, BOBS: brown small, BOR: red, BOG: green)



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An exploratory analysis by Principal Component method (PCA) of the seeds of B. orellana L. was done in three stages: i) fresh material, without extracting the essential oil; ii) after essential oil extraction; iii) with mixed spectra, i.e., fresh seeds and extracted in the same analysis, to observe if there would be a difference between cultivars or some confusion. To eliminate noise caused by surface irregularities first (Figure 2) and the second derivative were evaluated (Figure 3).

The first derivative of spectra from fresh samples shows a clear distinction of brown cultivar and some confusion with others (Figure 2A), and the first PC represents 74% of the variation between samples. When the analysis is done with samples after essential oil extraction (Figure 2B), all cultivars can be observed individually, result in individual intrinsic characteristics. In this group, PC1 represents 79% of spectral characteristics variation. In fresh seeds, some water content is present and was no controlled in the function of natural characteristics of the material, what maybe can interfere in cultivars' discrimination. When the results are from extracted material, some homogenization of external characteristics occur, and seeds were dried, remaining different structure comportment, maybe in the function of genetic parameters that can interfere in spectra.

Evaluating all spectra from fresh material and after extraction together, a clear separation between sample natures is observed (Figure 2C). In fresh samples it is not possible to discriminate cultivars, and in extracted samples, two groups were observed: 1) brown and brown small cultivars; 2) green and red cultivars. In this case, PC1 is related to 91% of the variation between seeds. The separation between fresh seeds and extracted seeds was expected in the function of different water content, also because some H-O bonds can be attached to some oil compounds. Comparing to data from Table 1, the percentage of hydrogenated sesquiterpene can influence grouping brown and brown and brown small cultivars; green and red cultivars.

Figure 2 – Score graphic of PCA with first derivative spectra of fresh seeds (A), after essential oil extraction (B) and with both together (C). (BOB: brown, BOBS: brown small, BOR: red, BOG: green)

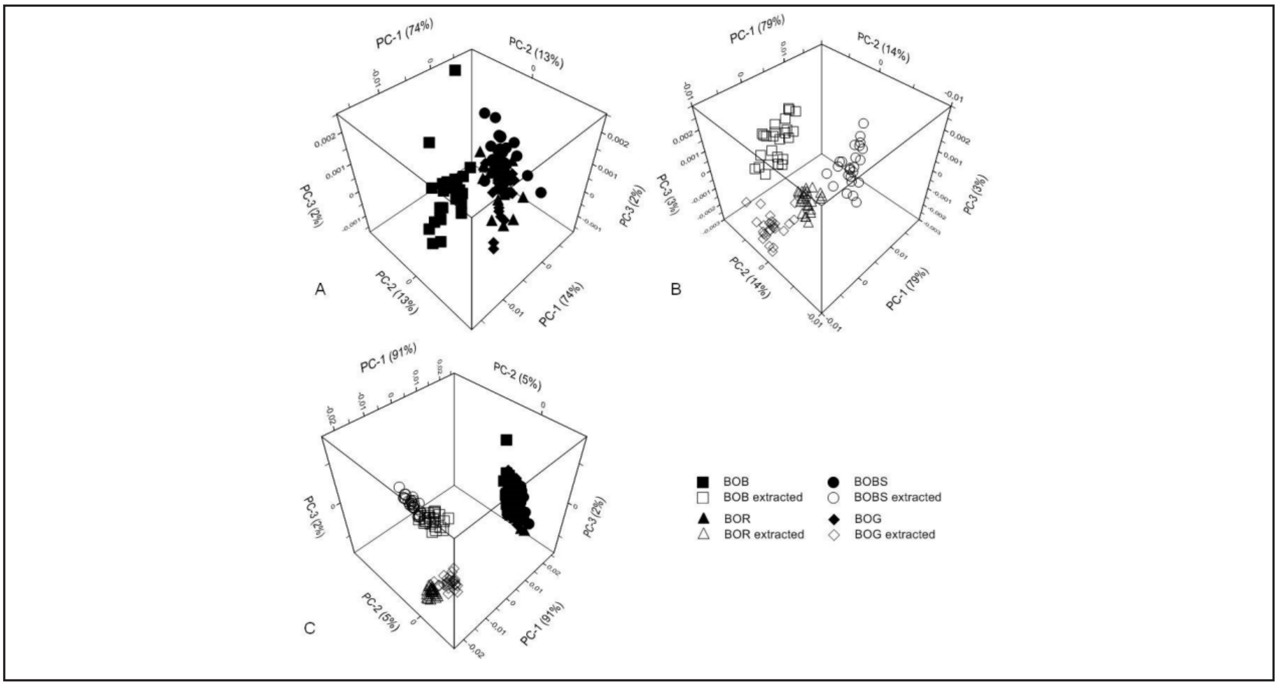

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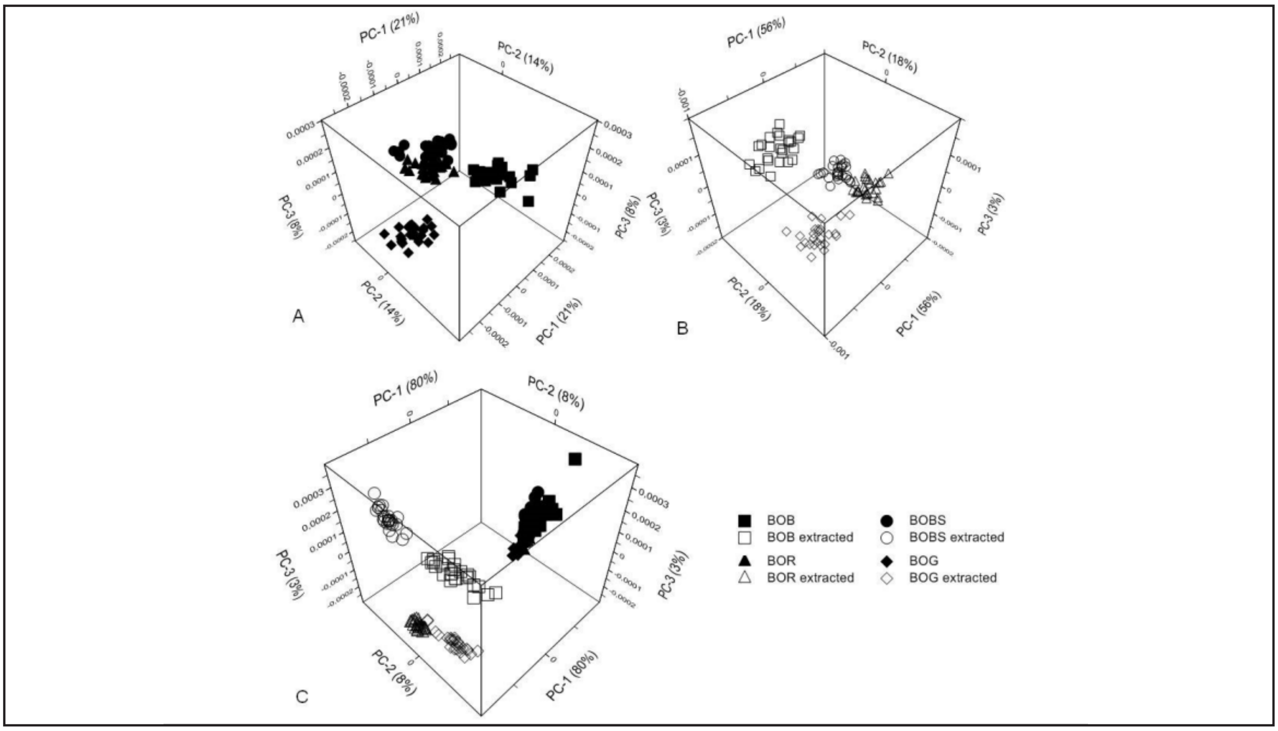
Figure 3 – Score graphic of PCA with second derivative spectra of fresh seeds (A), after essential oil extraction (B) and with both together (C). (BOB: brown, BOBS: brown small, BOR: red, BOG: green)



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When the analysis of PCA is done with the second derivative of NIR spectra from fresh samples (Figure 3A), a clear distinction of brown and green cultivars is observed, and a group of brown small and red cultivars. In this case, PC1 responds only to 21% of the variation. For samples after essential oil extraction (Figure 3B), all cultivars can be observed individually, and the proximity of brown small and red cultivars; and PC1 represents 56% of differences between cultivars. In addition, some influence of water content can be present and justify why in fresh samples there is no total distinction of cultivars. When the analysis is done with spectra from fresh material and after extraction together, a clear separation between sample natures is observed (Figure 3C), and PC1 represents 80% of differences. In fresh samples, it is not possible to discriminate cultivars, and in extracted samples, two groups were observed: 1) brown and brown small cultivars; 2) green and red cultivars.

Table 2 – Confusion matrix from LDA based on PCA scores of fresh seeds and after extraction (A) and confusion matrix from PCA scores with all samples (B)

(A) **Fresh seeds**

	BOB		**BOBS**		**BOR**		**BOG**	
derivative	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**
BOB	25	25	0	0	0	0	0	0
BOBS	0	0	21	24	5	1	0	0
BOR	0	0	4	1	19	24	1	0
BOG	0	0	0	0	0	0	24	25

(A) **After oil extraction**

	BOB		**BOBS**		**BOR**		**BOG**	
	1st	**2nd**	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**
BOB	25	25	0	0	0	0	0	0
BOBS	0	0	25	25	0	0	0	0
BOR	0	0	0	0	25	25	0	0
BOG	0	0	0	0	0	0	25	25

BOB: brown, BOBS: brown small, BOR: red, BOG: green

(B) **First derivative**

	BOB	**BOBS**	**BOR**	**BOG**	**BOBe***	**BOBSe**	**BORe**	**BOGe**
BOB	25	0	0	0	0	0	0	0
BOBS	0	24	2	0	0	0	0	0
BOR	0	1	18	1	0	0	0	0
BOG	0	0	5	24	0	0	0	0
BOBe	0	0	0	0	25	0	0	0
BOBSe	0	0	0	0	0	25	0	0
BORe	0	0	0	0	0	0	25	0
BOGe	0	0	0	0	0	0	0	25

(B) **Second derivative**

	BOB	**BOBS**	**BOR**	**BOG**	**BOBe**	**BOBSe**	**BORe**	**BOGe**
BOB	25	0	0	2	0	0	0	0
BOBS	0	19	9	1	0	0	0	0
BOR	0	1	11	3	0	0	0	0
BOG	0	5	5	19	0	0	0	0
BOBe	0	0	0	0	25	0	0	0
BOBSe	0	0	0	0	0	25	0	0
BORe	0	0	0	0	0	0	25	0
BOGe	0	0	0	0	0	0	0	25

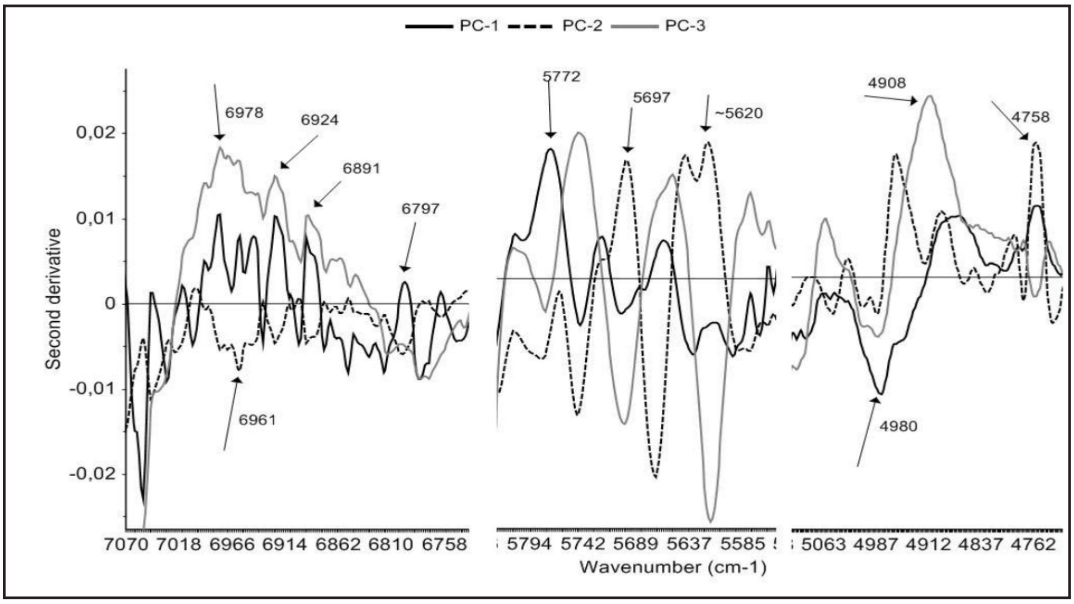
BOB: brown, BOBS: brown small, BOR: red, BOG: green; e = extracted cultivars

To verify which pretreatment is better for cultivars distinctions, a confusion matrix from a linear discriminant analysis based on the first and second derivative of NIR spectra from fresh seeds and after extraction (Table 2 A) and with all samples (Table 2 B) was evaluated and indicate the potential of distinction of seeds cultivar. For fresh seeds, the best pretreatment was the second derivative, and after extraction, both first and second derivative had 100% correct classification.

When the analysis done with all samples together, a distinction of fresh material and after oil extraction is evident, and in extract samples, all cultivars identified. Some confusion between cultivars verified in fresh material and the first derivative is the pretreatment more indicate to its differentiation.

The analysis of the loading graph (Figure 4) shows the peaks with more influence in sample discrimination. Wavenumber at 6978 cm^{-1}, 6961 cm^{-1}, 6924 cm^{-1}, 6891 cm^{-1}, 6797 cm^{-1}, 5772 cm^{-1}, 5697 cm^{-1}, 5620 cm^{-1}, 4980 cm^{-1}, 4908 cm^{-1}, 4758 cm^{-1} are related to alkene functional group: vinyl (hexene) CH2 = CH-, benzene band assignments CHν + CCν and CHν + CHν compounds Workman Jr.; Weyer (2008). Wavenumbers at regions from 6100–7000 cm^{-1} might be the overlapping of the first overtones of O–H (H2O) stretching and N–H stretching, reflecting the variations of water contents in different seeds. The peaks around 5600-5700 cm^{-1} attributed to the first overtones of C–H stretching in various groups. The absorbance in 4600–4800 cm-1 is the combination of O–H bending and C–O stretching and carries much chemical informationXu et al. (2013).

Figure 4 – Loading graph of PCs based on PCA with second derivative with all seeds, fresh and after oil extraction



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Roque et al., (2017) tested the potential of NIR spectra obtained directly from *Jatropha curcas* in the prediction of phorbol ester percentage in essential oil composition. The results indicate that the technique had limited sensitivity and was not able to make accurate predictions of the component directly, but the model tested showed potential for classification purposes and used in genetic breeding programs. Teixeira & Sousa, (2019) presented a revision from applications of near-infrared in nuts characterization, describing qualitative and quantitative results, between others, and highlight some limitations of the methodology, as necessity of chemometric tools to extract more important spectral information's, water influence, and the lack of validated spectral Intra and interlaboratory databases. Ishikawa et al., (2017) related that NIR measurements for single seeds could be useful to determine the uniformity of grain quality, or for rapid screening of the nitrogen content in the seeds of cowpea genotypes.

Xu et al. (2013) applied NIR spectroscopy to the determinate geographic origin of *Torreya grandis* seeds and concluded that second derivative NIR spectra was the most effective pretreatment and resulted in the adequate prediction of age and geographic origin discrimination.

4 CONCLUSION

The essential oils obtained from the four cultivars differed concerning their components. This result is important for future seed studies and the natural products industry.

NIR spectroscopy showed the potential for discrimination of annatto cultivars. The best pre-treatment was the second derivative for fresh seeds, and after oil extraction both first and second derivative are adequate. For a quick analysis for quality control of the seeds in industry, the spectra collected directly from the fresh seeds can be applied with adequate results.

NIRS technology proved to be effective for discriminating species of the Bixaceae family and should be extended to a larger number of family varieties and cultivars before is suitable for practical applications non-destructively.

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Conflict of interest statements

The authors have no competing interests.

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