

CONFIRMATION, THROUGH STRUCTURAL MODIFICATIONS, OF TRI-ACYL-GLYCERIDE (TAG) OBTAINED BY EXTRACTION FROM THE SEED OF JOANESIA PRINCEPS (CUTIEIRA A EUPHORBIACEAE)

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Abstract: In the present work, we report the characterization, after isolation, of a triglyceride (TAG) from seeds of *Joannesia princeps* Vell., a Euphorbiaceae, through extraction in a Soxhlet apparatus, using hexane as an extracting solvent. The proposed structure of TAG was determined by spectrometric analyses: high-resolution GC/MS; I.V.; R.M.N. ^1H and ^{13}C . Spectra of RMN (^1H ; ^{13}C ; $\text{H} \times \text{H}$ COSY e DEPT-45) indicate that all signals observed in the spectra belong to a single compound, a TAG containing two identical units and one different. A structure is proposed that, through hydrolysis in methanol/sodium methoxide, leads to two units of methyl linoleate ester and one of methyl oleate ester. Therefore, a molecular weight of $880\text{g}\cdot\text{mol}^{-1}$ and formula $\text{C}_{57}\text{H}_{100}\text{O}_6$ can be proposed. Confirmation of the structure is also done by comparing the proposed structure with another TAG described in the literature. Reaction of the mixture produced by basic hydrolysis with dimethyl-disulfide ($\text{CH}_3\text{-S-S-CH}_3$) confirms the positions of the unsaturations of the oleic and linoleic units; in addition to demonstrating the obtaining of a product originating from a thermal mechanism, under mass spectroscopy analysis. Therefore, in general, this TAG with a mild and slightly yellowish aroma is a mix of omega-9 and omega-6; which corresponds to approximately 40% of the seed mass, in addition to being probably one of the main responsible for physiological activities described in the literature. Another predominant TAG with a molecular mass of $878\text{g}\cdot\text{mol}^{-1}$, was detected by high-resolution mass spectroscopy, after a period of seed storage in a desiccator.

Keywords: Cutieira, *Joannesia princeps*, triglyceride and extraction.

INTRODUCTION

The species *Joannesia princeps*, studied in the present work, is known as Cutieira, Coco de purga, Purga de paulista, Boleira, andá-assu, among other popular names, and is found in the north, northeast and southeast regions, mainly in the Atlantic forest biome [1]. Due to the quality of the wood produced, the adaptability of the species and the growing conditions, the tree is useful for shading in pastures, but not for street afforestation due to the size and weight of the fruits, in addition to the ease with which the wind breaks its branches. Due to the role it plays in feeding fauna through its fruits, it must not be missing from the composition of forests intended for the repopulation of degraded areas of permanent preservation [2]. The cutieira fruit, illustrated in Figure 1, has soft pulp and a resistant endocarp, generally having 2, rarely 3 seeds, which are popularly used as medicine. They also have a significant amount of lipids.

In folk medicine, this plant is indicated as a purgative, for menstrual disorders, pernicious fever, antimicrobial, syphilis, scrofulosis and swelling. The oil from the root bark is used as a laxative [3-6] and the extract from the seeds exhibits strong anthelmintic activity, being used in folk medicine, due to the induction of intestinal motility [1, 7-11]. The seeds contain 37-55% of a viscous, slightly yellow oil that is useful for medicinal and industrial purposes. [3, 12, 13].



Figure 1. Images of the cutieira fruit and seeds. Collected and photographed by prof. Dr. Lenício Gonçalves.

Through institutional interaction with researchers in the area of Biology – Physiology, it was demonstrated that:

i. *J. princeps* seed oil accelerates the closure of skin wounds, increases angiogenesis, keratinocyte migration and fibroblast activity, while reducing the inflammatory process and oxidative damage [16];

ii. The cytotoxic and genotoxic potential of the aqueous extract of *Joannesia princeps* leaves was investigated and no mutagenic effects were observed [17].

iii. New investigation into the existence of possible mutagenic effects using *Allium cepa* and tests with micronuclei also demonstrated no harmful effects [18];

iv. In more recent studies, it was demonstrated that: *Joannesia princeps* seed oil has antinociceptive and anti-inflammatory action through its topical and systemic administration, promoted by the inhibition of leukocyte recruitment and cytokine production [19].

In this work we define that this oil contained in the seed of *Joannesia princeps*, obtained by soxhlet extraction using hexane as solvent, is composed of a triglyceride mainly present, in addition to showing that simple reactions suggest products originating from interesting thermal rearrangements.

EXPERIMENTAL

ANALYSIS EQUIPMENT AND REAGENTS USED

The solvent used was hexane PA from VETEC and used as received. NMR, IR and GC/MS analyzes were obtained using, respectively, the following devices: AC-500 from BRUCKER; SHIMADZU GC2010 and GCMS-Q2010 Plus. The following spectra were also obtained by NMR: H x H COZY,

COLLECTION AND BOTANICAL IDENTIFICATION

The plant material was always collected between January and February from 2010, in the Lagos Region, Jardim Peró, Cabo Frio – RJ. After botanical identification by Professor Dr. Pedro Germano Filho from the Department of Botany, Institute of Biology and Health Sciences - ``Universidade Federal Rural do Rio de Janeiro`` (IBSC-UFRRJ), the samples in the floriferous phase were deposited in the Department's RBR Herbarium collection. of Botany at UFRRJ, under registration number 34.630.

EXTRACTION IN SOXHLET APPARATUS AND TREATMENT

After natural drying of the fruit, the exocarp and endocarp were carefully broken. Two white seeds measuring approximately 2.0 cm in diameter were obtained per fruit. The average weight of shelled and shelled seeds are, respectively, 9.23 and 6.89g. This material was then crushed; part was stored in suitable bottles. After weighing, the dried and crushed plant material was separated into a smaller quantity, weighing 11.47g, which was placed in a small filter paper cup and placed in a soxhlet, with 200mL of hexane as the extracting solvent. The hexane extract of the species was concentrated in rotary steam at reduced pressure, obtaining 4.53g of oil. As there was a possibility that not all hexane had been evaporated, around 40 mL of dichloromethane were added to this oil and further evaporation was carried out in the rotavapor. After exhaustive evaporation of the solvent, 3.65g of a slightly yellow, mild-smelling oil was obtained (31.82% of the mass contained in the soxhlet). This material did not undergo any other treatment and was analyzed by: ^1H and ^{13}C NMR, IV, GC-MS,

among other analysis methods.

RESULTS

The spectra of I. V. and RMN ^1H - ^{13}C (Figures 3, 4 and 5) are excellent for spectroscopic studies as they clearly present well-differentiated signals. Making an analysis and comparison between them:

ANALYSIS BY I.V

(i) In the infrared spectrum (Figure 2), signals are observed referring to carbonyls (1745 cm^{-1}), C=C double bonds (1600 cm^{-1}), referring to vinyl hydrogen and in CIS configuration (1640 and 760 cm^{-1}), C-O stretching and signals referring to alkyl hydrogens (1160, 2840 and 2920 cm^{-1} , respectively).

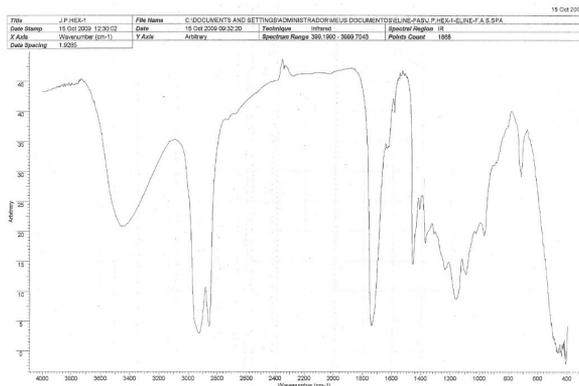


Figure 2: Infrared spectrum of oil extracted from *Joannesia princeps*.

Analysis by R.M.N.:

(i) The analysis of the spectrum referring to the NMR. ^1H and ^{13}C (Figures 3 and 4, respectively) confirm the presence of vinyl hydrogens (CH=CH) at $\text{H} = 5.34\text{ppm}$ (multiplet – 10H) and with equivalent signs, as demonstrated by the carbon spectra: C= 130.06 and 127.8ppm.

(ii) Two doublets are observed, referring to CHaHa and CHbHb signals at $\text{H} = 4.29$ and 4.12 ppm, respectively, region of

CH₂ linked to oxygen, in addition to the signal at H = 5.25 ppm, almost covered by the vinyl H and which refers to CH also linked to ester oxygen, and to the carbon at the center of the chain, hydrogen, located in the triglyceride [5Htotals: (-OC-O-CH₂)₂-CH-O-CO-]. These signals are equivalent to those observed at C = 69.02 and 61.9ppm (Figure 4), referring to CH-O and CH₂-O, respectively, with the signal intensities indicating the 1:2 relationship between these carbons. In the spectrum of 1H x 1H COSY (Figure 5), the interaction between these five hydrogens is evident. Furthermore, the COSY spectrum demonstrates a correlation between all H belonging to the molecule.

(iii) Other signals that are also important in defining the TAG structure are: doubly allylic CH₂ at 2.75ppm (t- 6.27Hz – 4H); CH₂ alpha to carbonyl at 2.3ppm (t- 7.53Hz – 6H); allylic CH₂ at 2.03ppm (m – 12H); CH₂ beta to carbonyl at 1.60ppm (m – 6H); CH₂ distributed along the chain at 1.29ppm (m – 48H); Finally, the signal at 0.87ppm indicates CH₃ at the end of the chain (t – 6.78Hz – 9H).

(iv) Indicative signals in the ¹³C spectrum are: three carbonyls (C=O) at: 173.1; 173.0 and 172.7ppm, CH₂ between 22.6 and 34.1ppm and CH₃ at the end of the carbon chain at 14.0 and 14.1ppm; the first being twice as large.

(v) When differentiating between total ¹³C spectra with DEPT-45, signals referring to CH and CH₃ below the spectral line, CH₂ above and absence of C=O, lead to proposing the molecular structure and the correlation between units 2 :1 suggested.

(vi) The integration of the signals observed in Figure 3 clearly indicates a total of one

hundred (100) hydrogens, considering all belonging to the same molecule. Therefore, the allylic and doubly allylic hydrogens (12 and 4 H, respectively) inform us that we have two double bonds that are repeated in two TAG units (CH₂-CH=CH-CH₂-CH=CH-CH₂); and the third unit containing a double bond (CH₂-CH=CH-CH₂). The intensity of the signals referring to chain-end methyls (14.0 and 14.1ppm) (Figure 4) is also a possible indication of two similar groups and one different. The 1H x 1H COSY spectrum (Figure 5) demonstrates the correlation between the signals observed in the 1H NMR spectra and confirms that all the signals that appear in the spectra in Figures 3 belong to the same molecule, as indicated by the integration and already evidenced. For example, from Figure 5, interactions between hydrogens are observed: (i) Allylic and double allylic with vinylic; (ii) linked to oxygen (ester); (iii) and to carbonyl; (iv) methyl (chain terminus) with methylenes (CH₂-CH₃).

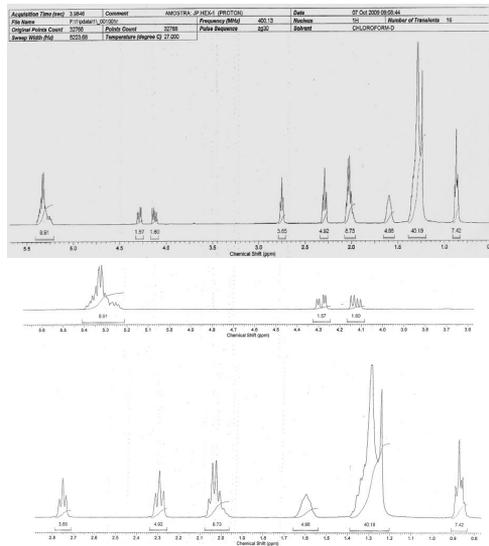


Figure 3: Espectros de RMN de ¹H (500 MHz) of oil extracted from *Joannesia princeps*. Above: chemical shift between 0.5 - 5.8ppm. Below: displacements between two different regions of the spectrum.

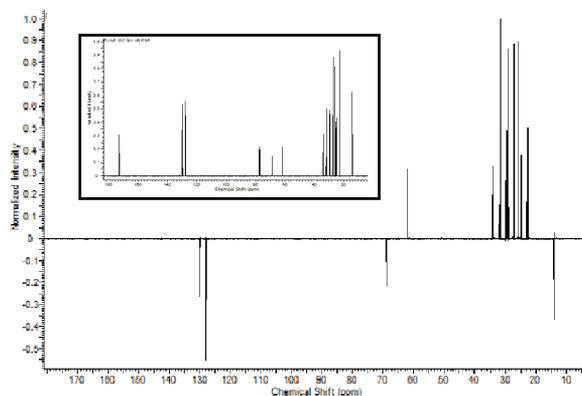


Figure 4: NMR spectra of ^{13}C (500 MHz) of oil extracted from *Joannesia princeps*. Total carbons (smaller figure). Below: DEPT 45.

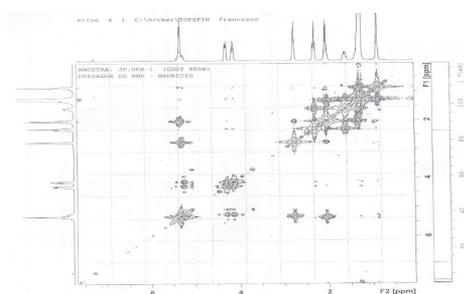


Figure 5: COZY $1\text{H} \times 1\text{H}$ NMR spectrum (500 MHz) of extracted seed oil

BIBLIOGRAPHIC COMPARISON

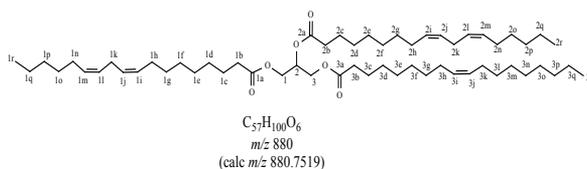
In the only table presented, a correlation is made between the signals referring to the ^1H e ^{13}C , of isolated GAD, comparing with the signs of other GAD described in the literature [14]. It can be seen that the comparison, especially considering the most informative signs, of both carbons and hydrogens are compatible with what has been described: Carbonyls, vinylic, allylic and double allylic H and C; in addition to the terminal methyl groups and H linked to the ester group. Great for discussing in NMR classes.

In evaluating all this spectroscopic information, it is concluded that TAG has the formula $\text{C}_{57}\text{H}_{100}\text{O}_6$ and a molecular weight equal to 880 g.mol^{-1} , that is: it has three alkyl chains, containing eighteen carbons in each

unit (C18); in addition to the three carbons of the main chain. Another important piece of information is that of the three TAG units, two are identical and have homoconjugated double bonds in CIS configuration, while the other unit has only one double bond, also CIS. This spectroscopic observation is confirmed when the acid and basic hydrolysis of TAG is carried out; comparisons of spectroscopic data [15] confirm the presence of the methyl linoleate unit.

The integration of the signal referring to doubly allylic hydrogens (unique for linoleate), compared to all other signals that are common with oleate, confirms the 2:1 ratio between these units.

Concluding the spectroscopic observations, it can be proposed that the signals present at 4.16 and 4.31ppm (Figure 3), and which refer to ester-linked methylenes, (62.04 – Figure 4), are not equivalent because they connect to units other than the TAG. The coupling constant of 11.9Hz (1.2J) is related to the interaction of each of them with the central ester CH, however, the different twin constants, 1.1J of 4.3 and 6.0Hz confirm the initial statement and it can also be proposed that the signal in lower field and with lower J refers to CH_2 bound to the linoleic unit. Therefore, the structure represented in Scheme 1 is proposed for the predominant triglyceride obtained by extraction with hexane from seeds of *Joannesia princeps* Velloso (Euforbiácea – Cutieira).



Scheme 1: Predominant triglyceride present in seeds of *Joannesia princeps* (cutieira).

¹ H (ppm) (m / I)	J (Hz)	¹³ C (ppm)	Duties	¹ H (ppm) (m) ^[14]	¹³ C (ppm) ^[14]
-	-	173,3 / 172,8	C=O	-	173,3 / 173,2
5,39-5,30 (m - 10 H)	-	130,2 / 127,9	-CH=CH-	5,34 (m)	129,9 / 127,9
5,27-5,23 (m - 1 H)	-	68,5	O-CH ₂ -CHO-CH ₂ -O	5,25 (m)	68,9
4,33-4,29 (dd - 2 H)	4,3-11,9	62,04	O-CH _a H _a -CHO-CH ₂ -O	4,29 (dd)	62,1
4,18-4,13 (dd - 2 H)	6,0-11,9	62,04	O-H ₂ C-CHO-CH _b H _b -O	4,15 (dd)	62,1
2,80-2,76 (t - 4 H)	6,8-13,0	25,6	-CH=CH-CH ₂ -CH=CH-	2,78 (dd)	25,5
2,31-2,27 (2t - 6 H)	7,4-17,5 4,5-17,2	34,2 / 34,0 (2:1)	-CH ₂ -CH ₂ -COOR	2,30 (t) 7,0 Hz	34,2
2,06-2,00 (m - 12 H)	-	27,2	-CH=CH-CH ₂ -	2,03 (m)	27,2
1,61-1,59 (m - 6 H)	-	31,9 / 31,5 (3 C)	-CH ₂ -CH ₂ -COOR	1,60 (m)	
1,31-1,25 (m - 48 H)	-	29,7 / 29,1	-CH ₂ -	1,25 (m)	29,7 / 29
0,88-0,85 (2t - 9 H)	7,0-13,3	14,13 / 14,09	-CH ₂ -CH ₃	0,87 (t) 7,0 Hz	14,1

Table: Spectroscopic assignments of the signals observed in the ¹H and ¹³C NMR spectra compared to isolated TAG and described in the literature [14].

Note: m / I: multiplicity / Integration; J: Coupling Constant.

HYDROLYSIS: BASIC AND ACID

i. Sodium shavings were added to 50mL of methanol, left overnight in a sealed flask with a needle to release the gas. The following day, 10mL of CH₃OH containing 1.442g of TAG isolated as described above, coupled to a reflux condenser, were added to the flask. The mixture was heated and refluxed for 6:00 hours, after this period the reaction mixture was transferred to a separation funnel containing 20mL of H₂O and 10mL of CH₃CO₂H (glacial). After stirring, the mixture was then extracted with 3 x 100mL of hexane. The organic phase was treated with anhydrous MgSO₄ and then filtered. After exhaustive evaporation, 1.346g of an oil was obtained with characteristics very similar to the initial material (92.9% yield). Analysis by ¹H - ¹³C NMR and GC/MS indicate the

predominance of two methyl esters with masses 294 - 296g.mol⁻¹ and a third with a relatively low proportion with masses of 298 and 280g.mol⁻¹, probably methyl stearate and linoleic acid (fig. 6 and 7).

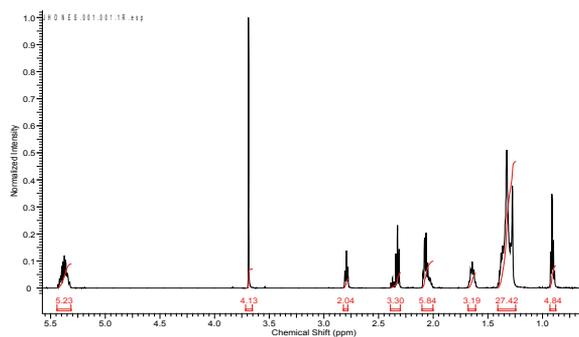


Figure 6: NMR spectrum of ¹H (500 MHz) of basic hydrolysis product oil, after purification.

i. Using 1.0g of oil with 50mL of PA methanol and 1.0mL of glacial acetic acid; the reaction mixture was refluxed for approximately 5:00 hours on a heating

mantle with a relatively low temperature. Next, the reaction mixture was extracted with a hexane - ethyl acetate mixture (2 x 50mL), addition of drying agent and filtration. This material was analyzed only by GC-MS. Chromatograms demonstrated by Figure 6.

REACTION WITH DIMETHYLDISULFIDE

In a 50mL flask adapting: reflux condenser and magnetic stirring, 1.0g of the oil obtained by basic hydrolysis and around 10mL of dimethyl disulfide were mixed. The reaction mixture was gently heated for 12h and analyzed directly by GC/MS. A complex reaction mixture was obtained; however two predominant products have molecular masses (390 and 420g.mol⁻¹) and informative lysis patterns (Figure 8).

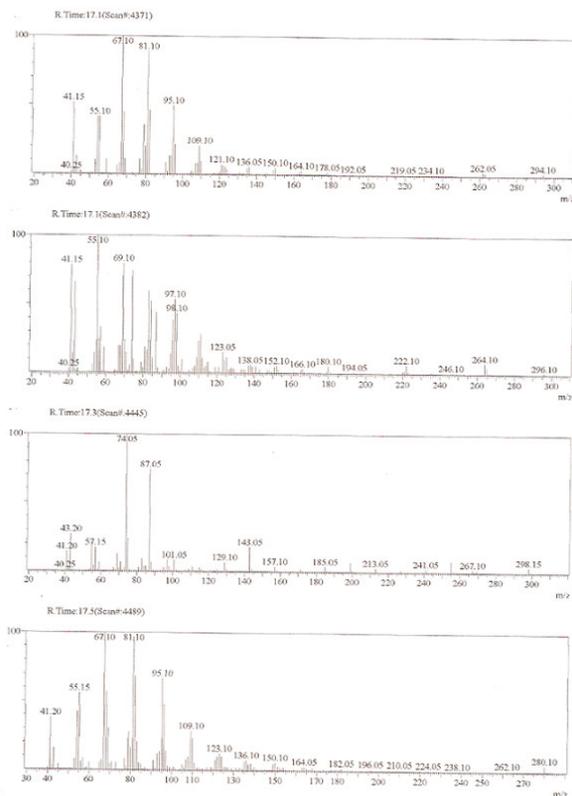


Figure 7: Mixture of Substances obtained by acid hydrolysis of TAG.

Comparing the two forms of transesterification, basic hydrolysis showed better yield and greater efficiency, as demonstrated by the spectra and chromatograms, with acid hydrolysis, the yield of obtaining methyl oleate and linoleate is 37%, while in basic medium it reaches 81% (both, approximately). Boiling point determinations of both the TAG and the mixture obtained by basic hydrolysis indicated the following temperatures: 175oC and 158oC, respectively.

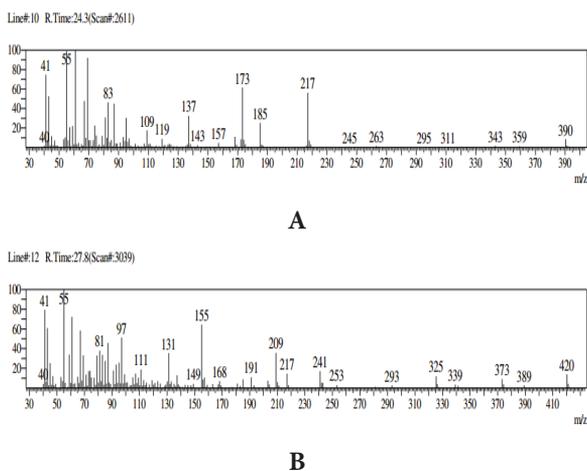


Figure 8: Mass spectrum resulting from the reaction of oil obtained by basic hydrolysis with CH_3SSCH_3 .

HIGH RESOLUTION MASS

The integrations provided by the 1H NMR spectrum (Figure 3) due to the small difference in the hydrogen count, the clear difference between the CH₂ of the tri-glyceric chain; as well as the difference in coupling constants between them, demonstrate the predominance of oil with a mass of 880g.mol⁻¹, in the spectra obtained in very recent extraction periods. However, obtaining a high-resolution mass of oil stored in a desiccator for approximately a few months indicates the presence of other substances, which can be attributed to derivatives. One of these possible derivatives, which has a slightly higher percentage than initially characterized, has a

mass of 878g.mol⁻¹. Which is probably due to the presence of an extra bond. Furthermore, these triglyceride esters predominate in the mass spectrum. Data relating to these two triglycerides, as well as information provided by the library contained in the analysis system media, for the highest percentage peaks are contained in Figure 9.

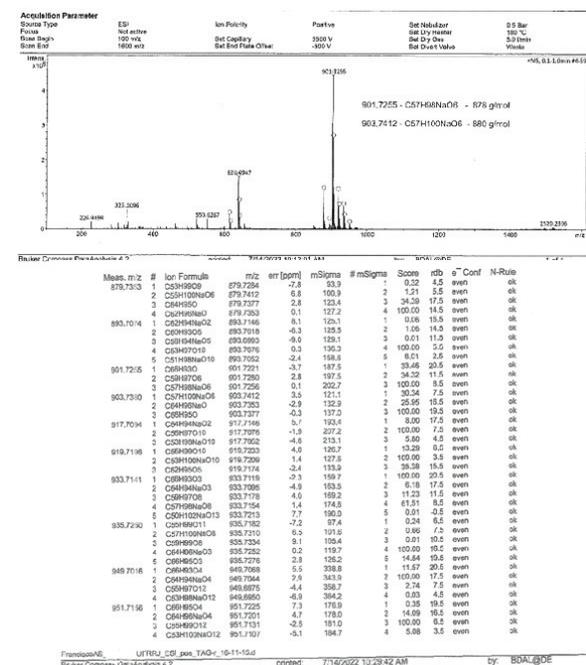


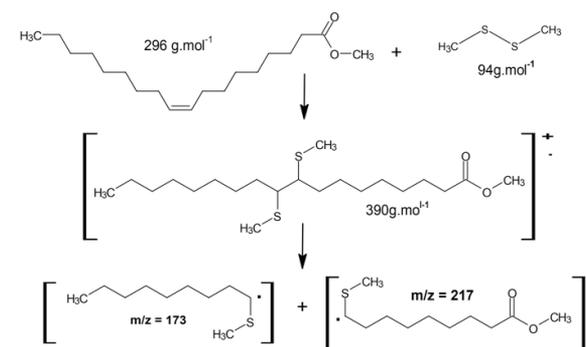
Figure 9. Chromatogram obtained by Mass Spectrometry (High Resolution), of the oil. Below is a table containing a report of molecular formulas suggested by the device's media. The highest intensity peaks stand out.

DISCUSSION

Previous studies demonstrate that Cutieira seed oil is composed of oleic and linoleic units. It was possible to demonstrate, through spectroscopic analysis, that these unsaturated compounds have their origin in a TAG with a molecular weight of 880g.mol⁻¹, containing two linoleic units and another oleic one, and at the ends of the tri-glyceric chain the units are differentiate (Figures 3 and 4). Hydrolysis reactions clearly demonstrate (figures 6 and 7) the obtaining of methyl oleate and linoleate,

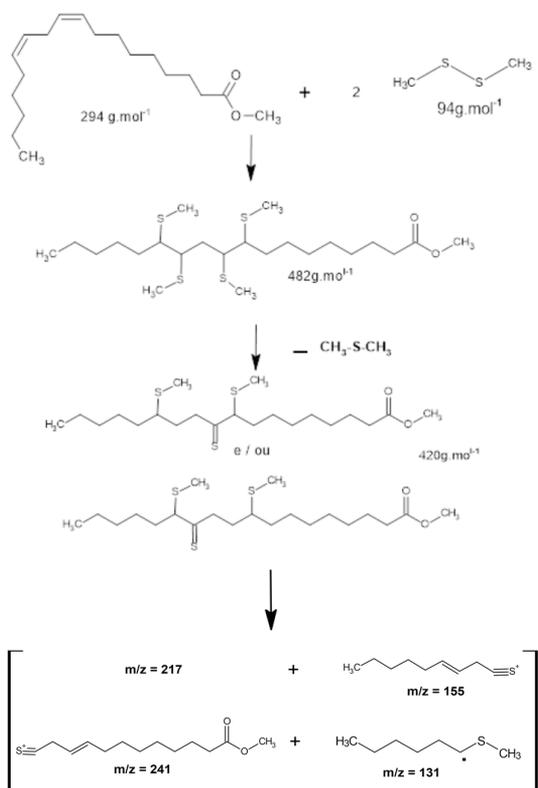
and in acid hydrolysis one of the spectroscopic records also demonstrates a compound with a lysis pattern similar to linoleate (294g.mol⁻¹), it has a molecular weight of 280g.mol⁻¹, most likely being linoleic acid. Different analyzes of the transesterification reaction in an alkaline medium demonstrate greater selectivity and efficiency (Figures 6 and 8).

In order to confirm the position of unsaturations in the detected units, the reaction was carried out with dimethyl disulfide (CH₃SSCH₃ – 94g.mol⁻¹). For this, the mixture of products obtained by alkaline hydrolysis was used. Two new products stand out when analyzed by GC/MS. One containing a molecular weight of 390 g.mol⁻¹ and the other 420 g.mol⁻¹. The compound with the lowest mass is attributed to the reaction starting from oleate (296 + 94 - scheme 2).



Scheme 2: Reaction to define the position of the double bond of methyl oleate and its probable fragments by mass spectroscopy.

However, for the compound with a larger mass, a more complex thermal reaction mechanism is proposed. Four CH₃S radicals would be added to the linoleic units, but there would be extrusion of CH₃SCH₃ (294 + 188 – 62 = 420), generating a C=S bond (scheme 3).



Scheme 3: Reaction defining the position of the double bond of methyl linoleate and its probable fragments by mass spectroscopy.

GC/MS analysis of this reaction mixture shows, from the first compound (scheme 2), peaks with 217 and 173 m/z , indicating that the double bond is located between carbons C9 and C10 of the TAG oleic unit. From the second onwards, peaks at 217 and 131 m/z are observed, in addition to signals referring to 241 and 155 m/z , indicating that the homoconjugated double bonds are located between carbons C6-7 and C9-10 of the linoleic units. of the TAG, considering the omega chain reading. Important information in these compounds is completed by lyses that indicate extrusion of CH_3O and CH_3S , with 31 and 47 g.mol^{-1} , respectively.

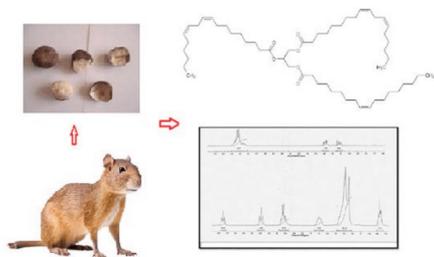
High-resolution mass analysis on material stored in a desiccator for approximately six months, the presence of another TAG with two smaller molecular weight units (878g.mol^{-1}) was observed, in addition to that already

defined by the mentioned techniques (figure 9). These made up a more complex mixture and dominated, in percentage terms, the mixture. This compound is possibly attributed to a third linoleic unit. This deduction is based on the observation of acid and basic hydrolysis products that did not demonstrate unity with 292g.mol^{-1} (linolenic).

CONCLUSION AND CONSIDERATIONS

Results obtained by several researchers, including those developed in our laboratories, demonstrate a similarity with the popular use of *Joannesia princeps (cutieira)*. In the present work, it is evident that this oil is composed of a triglyceride rich in omega-6 and -9. Through the different spectra, thermal reactions with the isolated oil; In addition to comparisons of the data obtained, a structure formed by two linoleic and one oleic unit can be proposed, distributed in such a way that the carbons at the ends of the triglyceride chain do not support the same units. Storage of its fruit indicates that structural changes may still occur.

The agouti, with its eating habits, is a disseminator of this plant. Being rich in unsaturated oils, it can have a wide application for human and fauna needs. The percentage of oil present in the seed (approximately 40%) is a favorable factor for its study and popular use. In tests carried out and published, the absence of mutagenesis was demonstrated, another important observation that agrees with the popular testimonies obtained [20]. Much can still be done, research is always ongoing and enlightening. The figure below is intended to demonstrate the importance of the relationship between agouti and cutieira. The small wild animal is one of the main seed dispersers.



Graphical Summary: agouti; *Joannesia princeps* seed; formula and spectrum RMN ^1H of the oil extracted - $\text{C}_{57}\text{H}_{100}\text{O}_6$.

THANKS

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