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Development of an eco-fractionation process for *Ricinodendron heudelotii* oil to obtain α -eleostearic acid and β -eleostearic acid

Éric Lacroux¹ · Diakaridja Nikiema^{1,2} · Muriel Cerny¹ · Jean-François Fabre¹ · Zéphirin Mouloungui¹ · Koua Oi Koua² · Christine Raynaud¹ · Ané Adjou² · Othmane Merah^{1,3} · Romain Valentin¹

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Abstract

The present article studies the transformations and fractionation of the reserve lipids of *Ricinodendron heudelotii*. The native triglycerides of this oil are composed mainly of conjugated polyunsaturated fatty acids, in particular α -eleostearic acid C18:3 n-5 (9c, 11t, 13t) at 60%. This particular fatty acid of CLnA family exerts many activities potentially beneficial for health: anti-inflammatory, anti-leukemic, anti-microbial, anti-tumor, anti-ulcer and anti-diabetic. A process for transforming this acid into its isomers β -eleostearic acid and catalpic acid was explored with the aim of obtaining fractions enriched in β -eleostearic acid C18:3 n-5 (9t, 11t, 13t). The β -eleostearic acid is a fatty acid of great interest because it is even more reactive and more efficient as antioxidant than α -eleostearic acid due to its higher *trans* content. Isomerization reaction of α -eleostearic acid in oil was carried out using artificial solar radiation treatment. The enrichment of concentrates in β -eleostearic acid was therefore tested using an eco-fractionation process. This process was carried out in two stages. The first step was the *Candida rugosa* lipase-catalyzed hydrolysis of triglycerides from native or isomerized *Ricinodendron heudelotii* oil. The second step was fractionation of the reaction medium obtained after hydrolysis. Triglyceride hydrolysis was complete, with a yield of free fatty acids of over 95% after 2 h of reaction. Treatment of the reaction media yielded 3 lipid concentrates with new chemical compositions of polyunsaturated fatty acids: a first concentrate, derived from hydrolysis of the native oil, composed of 60% α -eleostearic acid and 22% linoleic acid, and two other concentrates derived from fractionation of the hydrolysate of the oil isomerized by radiation. One was composed of 34% linoleic acid, 21% β -eleostearic acid, 11% α -eleostearic acid and 6% catalpic acid, and the last concentrate was composed of over 80% β -eleostearic acid. In addition to offering nutritional benefits similar to those of α -eleostearic acid, β -eleostearic acid also offers an interesting physical property: a very high melting point of 72 °C. Such a polyunsaturated fatty acid, which is solid at room temperature, could prove to be a raw material of choice, particularly in the world of food formulation, where manufacturers are looking for replacements with physical properties close to those of palm oil (the melting point of palmitic acid is 62 °C). *Ricinodendron heudelotii* oil has thus demonstrated its major role as a source of the triptych of α -eleostearic, β -eleostearic and linoleic fatty acids.

Keywords *Ricinodendron heudelotii* · Eco-fractionation · CLnA · β -eleostearic acid · α -eleostearic acid · Linoleic acid

Introduction

The human population is growing at a galloping pace, with an average of one billion additional people every 12 years. This requires the production and diversification of foodstuffs not only to meet their nutritional needs, but also to ensure their health [1]. The main source of lipids for human consumption remains vegetable oils. The diversification of lipid requirements due to the multiplication of industrial outlets has become important and demanding. To meet these needs, the first solution was to genetically transform (classically or

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by genetic engineering) already cultivated oilseeds to produce specific fatty acids. A second approach is to prospect for new plant sources [2–6]. Another approach is to develop oil and fatty acid fractionation processes to produce the desired free fatty acids [7, 8]. This is all the more true and important given that the resources exist, and that no current solution allows them to be used to meet these specific needs.

As a result, oils with a high content of CLnA-type (Conjugated Linolenic Acid, with eighteen carbon atoms and three conjugated double bonds) polyunsaturated fatty acids are receiving particular attention from researchers and industry alike. Due to their specific physico-chemical, physical and biological properties, CLnA fatty acids are increasingly used in the pharmaceutical, chemical and cosmetics industries [9–11]. Studies have shown the plant origin of these lipids, composed of conjugated polyunsaturated carboxylic acids with eighteen carbon atoms and three double bonds [3, 10, 12, 13]. The bioactive molecules that are all CLnA-type fatty acids have demonstrated very good effects on human health, characterized not only by their antioxidant and anti-inflammatory effects, but also by their anti-cancer and anti-diabetic properties [11]. The presence of 3 double bonds in these molecules gives the oils from which they are derived specific characteristics. Like poly-unsaturated fatty acids (PUFA) rich oils, they have excellent drying properties, enabling them to dry by cross-linking at their double bonds with the oxygen present in the air. Compared with other PUFAs, CLnA-type fatty acids are more polymerizable the more their double bonds are conjugated. Furthermore, the conjugated double bonds present in these fatty acids give them very high melting points compared to the equivalent fatty acid with three non-conjugated double bonds (linolenic acid, $T_f = -11\text{ }^\circ\text{C}$) and even compared to fatty acids of the same chain length with one (oleic acid, $T_f = 16\text{ }^\circ\text{C}$) or two (linoleic acid, $T_f = -5\text{ }^\circ\text{C}$) double bonds.

These fatty acids are present in varying proportions in oils, the most abundant being punicic acid, α -eleostearic acid, catalpic acid and licanic acid. β -Eleostearic acid is present only in small quantities or is almost non-existent in unprocessed vegetable oils [14]. The case of β -eleostearic acid is all the more specific in that all 3 double bonds are conjugated and all three are in *trans* configuration. This makes this fatty acid even more reactive than α -eleostearic acid, which is already extremely reactive and has made a major contribution to the industrial use of Tung oil, in which it represents the majority. β -eleostearic acid is also more efficient as antioxidant than α -eleostearic acid due to its higher *trans* content. Moreover β -eleostearic acid also offers a very high melting point of $72\text{ }^\circ\text{C}$. This melting point, even higher than that of a saturated fatty acid also bearing 18 carbon atoms (stearic acid, $T_f = 70\text{ }^\circ\text{C}$), is a major feature for such a polyunsaturated acid with 3 double bonds. It thus

offers the benefits of a polyunsaturated fatty acid, while providing the temperature stability of a saturated one. Such a polyunsaturated fatty acid, which is solid at room temperature, could prove to be a raw material of choice, particularly in the world of food formulation, where manufacturers are looking for palm oil substitutes (the melting point of palmitic acid is $62\text{ }^\circ\text{C}$).

It's also important to note that this fatty acid is very rare and virtually absent in nature. Studies have shown that β -eleostearic acid can be synthesized from α -eleostearic acid [15–17]. This synthesis was achieved from Tung oil (*Aleurites fordii*) by isomerization of α -eleostearic acid to β -eleostearic acid. The methods of preparation and purification of β -eleostearic acid described in the literature were carried out under drastic conditions with long preparation times. Thus, to convert α -eleostearic acid to β -eleostearic acid, Cao et al. modified tung oil (solid) by adding iodine (I_2) [18]. This modified oil was then saponified using 150 mL of a 0.3 M KOH/ethanol solution under nitrogen at a temperature of $25\text{ }^\circ\text{C}$ for 24 h. The medium was then acidified with a 1 M solution of H_2SO_4 to pH 1.0 [18]. For the preparation of β -eleostearic acid, Yurawecz et al. used I_2 at room temperature for 10 days to promote the isomerization of α -eleostearic acid unsaturations [19]. The rarity of β -eleostearic acid and catalpic acid may explain the lack of information on their uses in the literature. However, no green process has been reported in the literature for obtaining them. The production of CLnA is generally difficult due to the obstacles encountered in the chemical synthesis of the isomers to obtain high-purity products [20].

In a previous study, we investigated the effects of solar radiation on the composition and properties of the native oil of *Ricinodendron heudelotii*. This is a plant species found in Central, West and East Africa, which has considerable development potential as a non-timber forest product, and for which the emergence of a value chain is being considered [21]. Analysis of the fatty acid composition of native oil and oil treated with solar radiation (natural and/or artificial) has shown that treatment results in the isomerization of α -eleostearic acid. As a consequence, α -eleostearic acid is partially converted into its various isomers β -eleostearic acid (C18:3 9t, 11t, 13t) and catalpic acid (C18:3 9t, 11t, 13c). Isomerization led to the formation of 5 times more β -eleostearic acid than catalpic acid. [14]. Exposure of the native oil to visible radiation (λ 300–800 nm) with a power of $250\text{--}765\text{ w/m}^2$ and a temperature of $55\text{ }^\circ\text{C}$ for twelve hours converted 63% of the α -eleostearic acid chains into 51.4% β -eleostearic acid and 11.6% catalpic acid. This previous study opened up the possibility of using *Ricinodendron heudelotii* oil to achieve the production of β -eleostearic and catalpic acids by isomerization of α -eleostearic acid chains [14]. This promising process, which achieved α -eleostearic

acid isomerization simply by exposure to sunlight and without any chemical treatment, has produced satisfactory results with the conversion of 63% of the α -eleostearic acid chains (after a 12-h exposition to artificial solar radiation), but can still be optimized. The aim of the present study was to set up an eco-fractionation process for free fatty acid synthesis and β -eleostearic acid enrichment from *Ricinodendron heudelotii* oil triglycerides. The term eco-fractionation refers here to a gentle, chemical-free and environmentally-friendly process for obtaining fractions concentrated in molecules of interest from vegetable oil. This process comprises a step of enzymatic hydrolysis of *Ricinodendron heudelotii* oil triglycerides in aqueous medium for the production of free fatty acids, followed by a mechanical fractionation step by centrifugal separation of these free fatty acids and taking advantage mainly of the high melting point of β -eleostearic acid.

Materials and methods

Materials

Ricinodendron heudelotii seeds came from the Sinfra region (Côte d'Ivoire). They were collected during the summer of 2015. The seeds were shelled by hand and the kernels used for oil extraction.

Methods

Extraction and modification of *Ricinodendron heudelotii* oil

Finely ground kernels (120 g) (laboratory grinder, Waring Blender, Waring Products, Torrington, USA) were added to 350 mL cyclohexane before vigorous agitation for 1 min. After centrifugation at $10000\times g$ for 15 min, the lipid extract contained in the upper organic phase was recovered. The process was repeated, adding the same volume of cyclohexane. The lipid extracts collected from successive extractions were mixed and filtered through a funnel with a glass fiber filter containing anhydrous sodium sulfate. The cyclohexane was then removed using a rotary evaporator at 50 °C and a reduced pressure of 130 mbar to obtain the native *Ricinodendron heudelotii* oil. The oil was extracted using a solvent for ease of use at laboratory scale, but for larger volumes or for future industrial development, the oil could be extracted from the seeds using a solvent-free mechanical tool such as a single-screw press.

This oil was then modified by inducing the isomerization of α -eleostearic acid according to the radiation treatment described by Nikiema et al. [14]. Briefly, the extracted native oil was exposed to artificial solar radiation in a SUNTEST

CPS + type device (ATLAS, Mount Prospect, USA). The oil was exposed for 12 h in permanent immersion in a chamber regulated between 30 and 55 °C with an irradiance range of 250–765 W/m² (300–800 nm) and behind a filter simulating a 3 mm-thick pane of glass, corresponding to a total exposure of around 30,000 kJ/m².

Release of fatty acids from oil by enzymatic hydrolysis

The two types of *Ricinodendron heudelotii* kernel oil (native and modified) were enzymatically hydrolyzed in aqueous medium, using the method developed by Mouloungui and Mechling for the hydrolysis of vegetable oils [8, 22]. These triglyceride hydrolysis reactions were carried out in the presence of *Candida rugosa* lipase (Lipolyve CC, LYVEN, Colombelles, France). This lipase consists of lyophilized proteins with an activity of 30,000U/g. In recent years, a number of authors have demonstrated that *Candida rugosa* lipase was a non fatty acid-specific lipase when used in the hydrolysis of vegetable oil triglycerides [23, 24]. Enzymatic hydrolysis of oil triglycerides was carried out in a 1-L jacketed reactor thermostated at 40 °C, the optimum temperature for lipolytic lipase activity. The reaction medium (pH between 6 and 7) was stirred with a rotor/stator fitted with a 2mm round-hole stator (Model L4RT, Silverson Machines LTD, Chelsam, England).

The oil/water ratio used for hydrolysis reactions was 1:1 (v/v). Native or modified oil (500 mL) and water (400 mL) were introduced into the reactor. Five grams of lipase (1% of the oil mass to be hydrolyzed) were prepared in 100 mL of water under stirring (400 rpm) for 10 min. This enzyme solution was then added to the reactor containing the oil/water mixture. Incubation for hydrolysis lasted 2 h at a speed of 1000 rpm [8, 22].

Fatty acids concentration by centrifugal separation

After incubation at 40 °C, the reaction medium was centrifuged at 15 °C for 15 min at 5000g in a laboratory centrifuge (6K15, Sigma Laboratory Centrifuges, Osterode am Harz, Germany). Such cold centrifugation (carried out at a temperature of 15 °C, i.e. below ambient) on the native oil hydrolysate enabled fatty acids to be concentrated by removal of the aqueous phase. The same cold centrifugation, on the radiation-modified oil hydrolysate, not only removed the aqueous phase but also split the organic phase in two, thus obtaining two different concentrates. The organic phases obtained for the various hydrolyses were separated from the aqueous phases and stored at 4 °C.

Profiles of partial glycerol esters and triglycerides by HPSEC-ELSD

High Performance Steric Exclusion Chromatography coupled to an Evaporative Light Scattering Detector (HPSEC-ELSD) (Ultimate 3000, DIONEX, Sunnyvale, USA) was used to monitor the hydrolysis of oil triglycerides and the formation/conversion of mono and diglycerides. Analysis conditions are described below. The eluent used was tetrahydrofuran (THF) at a flow rate of 0.8 mL/min. Oven temperature was maintained at 35 °C. An ALLTECH Model 3300 ELSD detector was used. It was set at 35 °C with a gas (air) flow rate of 1.6 mL/min. The detector gain was set to 1. Identification and quantification of the various partial esters (monoglycerides MG, diglycerides DG), triglycerides (TG) and free fatty acids (FA) were based on comparison of the retention times obtained with those of commercial standards: Trilinolein, 1,3-dioleoyl-glycerol, 1-linoleoyl-rac glycerol and linolenic acid (Sigma-Aldrich, Saint-Louis, Etats-Unis).

Fatty acid profiles of oils and free fatty acid concentrates by GC

The fatty acid profile of the products was determined by fatty acid derivation in accordance with the international standard [25].

The product (15 mg) was solubilized in 1 mL TBME (Tert-Butyl Methyl Ether). A 100 µL fraction of this solution was methylated with 50 µL TMSH (Tri Methyl Sulfoxonium Hydroxide) at 0.2 mol/L in methanol. The resulting fatty acid methyl esters were analyzed by gas chromatography (GC). The instrument used was a Varian 3900. One microliter of the solution was injected into this chromatograph, under the following parameters: injector in split mode, ratio 1/100, 250 °C (55 min); the column was a CP-Select CB for FAME, fused silica, wall-coated open tubular, 50 m × 0.25 mm, film thickness 0.25 µm; the carrier gas was helium with a flow rate of 1.2 mL/min. Oven temperature: 185 °C for the first 40 min, then increased by 15 °C/min to 250 °C, and held at 250 °C for 11 min.

The identification of fatty acid methyl esters was based on the comparison of retention times obtained with those of a commercial mixture of 37 fatty acid methyl ester standards (FAME Mix 37, Sigma Aldrich) and those obtained by fatty acid profiling of tung oil and catalpa oil (*Catalpa bignonioides*). It was thus possible to identify CLnA methyl esters in native oil and in free fatty acid concentrates.

Characterization by FTIR-ATR analysis

Infrared spectra of products were obtained using a Fourier transform infrared spectrometer (Spectrum 65, Perkin Elmer, Waltham, USA) coupled with a Golden Gate™ Single Reflection Diamond ATR System accessory (Specac, Orpington, England) with the following specifications: Single Crystal Diamond 45°–2 mm × 2.

Spectra were collected three times from different fractions of each product at a resolution of 4 cm⁻¹ over the range 4000 to 600 cm⁻¹ with 16 scans. Measurements were carried out at room temperature, which was regulated at 22 °C.

Hydrolysis of the oil was followed by the disappearance of the band at 1742 cm⁻¹ corresponding to ester bonds between glycerol and carboxylic acids, and the appearance of the band at 1706 cm⁻¹ (characteristic band of the C=O bond in carboxyl groups) reflecting the appearance of free fatty acids.

With regard to oil isomerization, the 1000 to 900 cm⁻¹ region was particularly studied as it corresponds to the characteristic bands of the different arrangements of *cis* and *trans* double bonds in the various fatty acids.

Statistical analysis

Analyses of variance were performed in Excel to compare the compositions of the different oils and lipid concentrates obtained. For the two oils studied, the means were compared using Duncan's test with a probability of 5%. Samples were composed of 3–9 experiments.

Results and discussion

The aim of this work was to develop a synthesis and enrichment process from *Ricinodendron heudelotii* oil in order to isolate a lipid concentrate rich in β-eleostearic acid as simply as possible. This involved eco-fractionating the CLnA-type fatty acids present in *Ricinodendron heudelotii* oil, modified or unmodified, in order to concentrate α-eleostearic acid, β-eleostearic acid or catalpic acid in free form. The process is illustrated in Fig. 1.

Several studies have shown that pre-treating kernels at temperatures of up to 90 °C for at least 1 h prior to oil extraction improves oil yield [26–31]. After extraction, the oil extracted from the kernels could be processed by exposure to artificial solar radiation, a step already demonstrated by Nikiema et al. [14].

The development of the process in question concerned the following stages: enzymatic hydrolysis and centrifugation to obtain fatty acid concentrates.

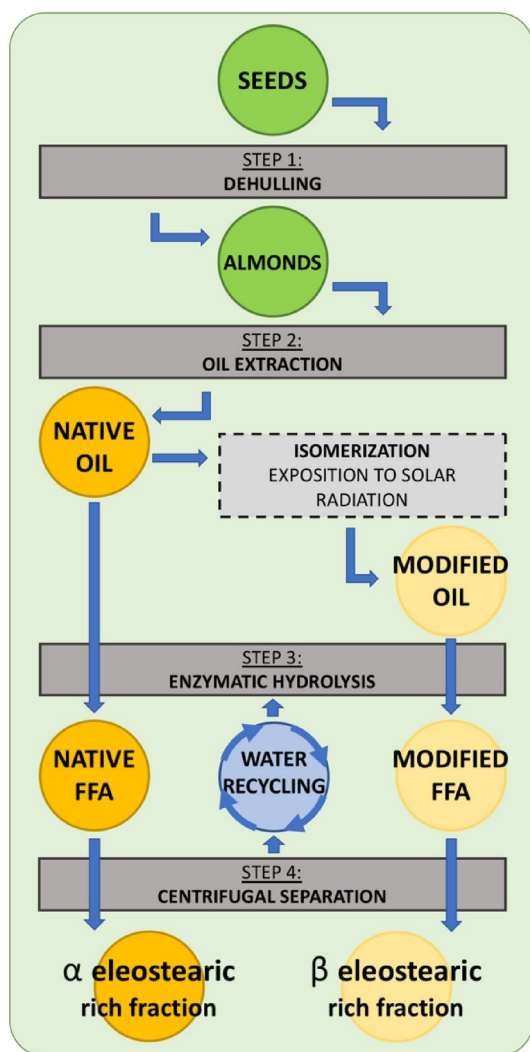


Fig. 1 Process for the extraction, isomerization, hydrolysis and concentration by centrifugal separation of free fatty acids from *Ricinodendron heudelotii* oil

Production of free fatty acids by enzymatic hydrolysis

After two hours of hydrolysis reaction, the reaction medium was an emulsified homogeneous mixture of water, lipase, glycerol, partial glyceride residues and free fatty acids released in situ. The mixtures obtained after hydrolysis of native and modified oils were then centrifuged for 15 min at 10,000 \times g and 15 °C.

After hydrolysis of native oil and modified oil organic and aqueous phases were thus separated by centrifugation prior to analysis by gas chromatography.

Different samples (1, 5, 10, 15, 30, 60 and 120 min) were also taken during hydrolysis, in order to monitor the hydrolysis kinetics of the native and modified oils using HPSEC-ELSD analysis. These kinetic profiles are shown in Fig. 2.

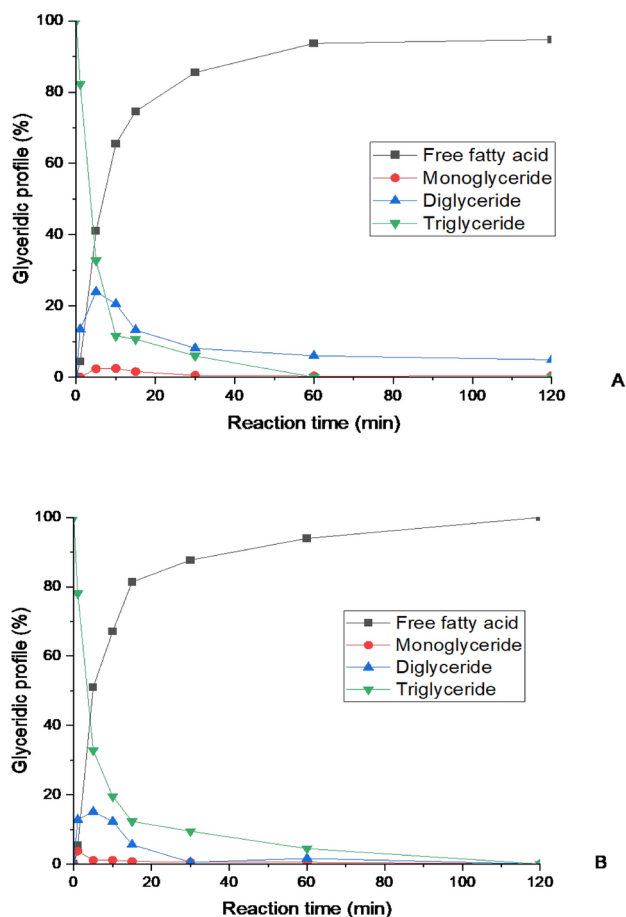


Fig. 2 Kinetic monitoring (2 h) of enzymatic hydrolysis of native oil (A) and modified oil (B) from *Ricinodendron heudelotii*

Kinetic monitoring of the reactions by HPSEC-ELSD analysis showed that hydrolysis of both types of oil was rapid and complete. This complete hydrolysis was the result of the cleavage of almost all the complex ester bonds in the triglycerides of the different oils, without any prior induction period. The breaking of ester bonds and release of free fatty acids and glycerol occurred mainly within the first twenty minutes of hydrolysis.

The hydrolysis was also monitored by FTIR-ATR analysis, which showed the disappearance of the band at 1742 cm^{-1} (characteristic band of C=O bond in esters) corresponding to ester bonds between glycerol and carboxylic acids, and the appearance of the band at 1706 cm^{-1} (characteristic band of C=O bond in carboxyl groups) reflecting the appearance of free fatty acids (Fig. 3).

In-depth analysis after 30 min of hydrolysis reaction of the native oil gave a composition of 85.5% free fatty acids (FFA), 0.5% monoglycerides (MG) and 8.1% diglycerides (DG), see Fig. 2. Hydrolysis of the modified oil under the same conditions gave similar results, with 87.7% free fatty acids after 30 min, 0.5% monoglycerides and 2.4%

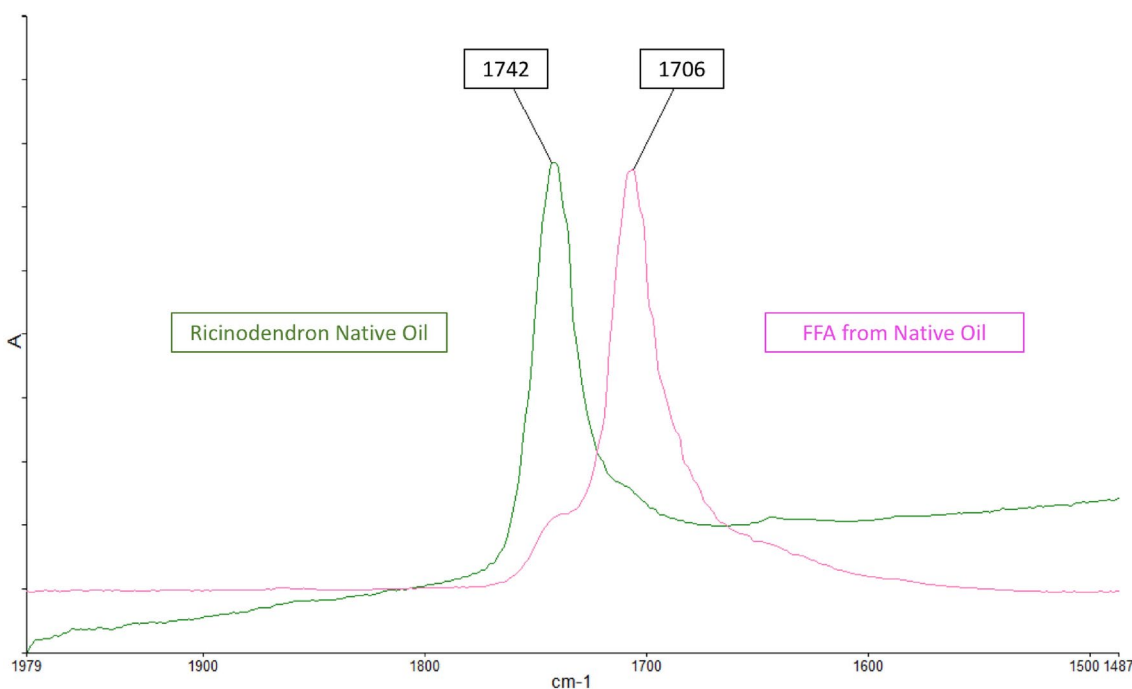


Fig. 3 Absorption Spectra of *Ricinodendron heudelotii* oil and free fatty acids (FFA) medium after one hour of enzymatic hydrolysis

diglycerides. The formation of these intermediate partial glycerides was the result of the breaking of ester bonds on triglycerides. Unfortunately, we were unable to compare these results with other work on this species, as this is the first report on the development of such an enzymatic hydrolysis process on *Ricinodendron heudelotii*. After 1 h, the enzymatic hydrolysis reaction of native and modified *Ricinodendron heudelotii* kernel oils produced a medium composed of over 90% free fatty acids. HPSEC-ELSD analysis quantified the total free fatty acid content after one hour at 93.70% for the native oil and 93.95% for the modified oil. After two hours of hydrolysis, the fatty acid content rose to 94.75% (native oil medium) and to almost 100% for the modified oil medium. In all cases, the triglyceride content was below 5%, even after a single hour of hydrolysis, making it possible to consider a single hour of hydrolysis for such a process.

These results showed that *Candida rugosa* lipase (Lipolyve CC, LYVEN, Colombelles, France) had a high lipolytic capacity without any selectivity, thus confirming previous results revealing that *Candida rugosa* lipase was non-specific [23, 24]. This lipase was therefore non-discriminatory on the fatty acids of the native oil, which was composed of 84% polyunsaturated chains including 60.1% α -eleostearic chains and 1.1% β -eleostearic chains, and on the modified oil, which was composed of 79.6% polyunsaturated chains including 11.8% α -eleostearic chains, 31.9% β -eleostearic chains and 7.4% catalpic chains.

Production of special CLnA-enriched concentrates

Melting properties of CLnAs

The separation of fatty acids by fractionation undertaken in this work was based on the difference in melting temperature (T_f) of the fatty acids derived from the two oils studied. Table 1 summarizes the melting temperatures of fatty acids with a carbon chain of 18 carbon atoms, with or without double bonds.

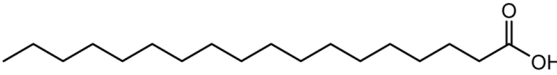
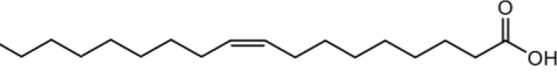
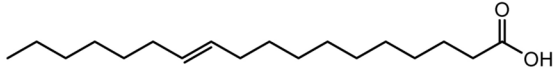
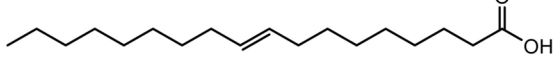
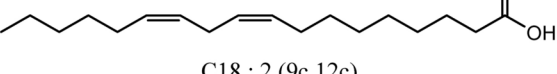
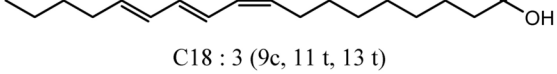
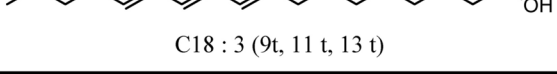
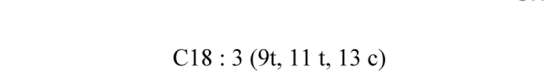
Generally speaking, the results of the studies compiled in this table revealed that the melting temperature of the various C18:n fatty acids (n =number of double bonds) was a function of the presence and nature of the double bonds, compared with stearic acid (C18:0) taken as the reference molecule.

According to the studies of Dijkstra and Seniha Güner et al., conjugated polyunsaturated fatty acids are high-melting compounds, such as α -eleostearic acid ($T_f=49^\circ\text{C}$), β -eleostearic acid ($T_f=72^\circ\text{C}$) and catalpic acid ($T_f=32^\circ\text{C}$), while oleic acid ($T_f=16.2^\circ\text{C}$) and linoleic acid ($T_f=-5^\circ\text{C}$) are liquid compounds at room temperature. [33–35].

As a basic rule, the parameters affecting the melting point of a sample are ionic bonds, intermolecular interaction forces, molecular size, molecular shape and the presence of double bonds [36].

In the case of our study, the fundamental differences highlighted between the various fatty acids were the presence of "cis" and "trans" double bonds in the molecules and

Table 1 Comparison of melting temperatures of C18 fatty acids

Free Fatty Acid	T _f (°C) [32]	Molecular Structure
Stearic Acid	70.1	 C18(0)
Oleic Acid	16.2	 C18 : 1 (9c)
Vaccenic Acid	44.1	 C18 : 1 (11t)
Elaidic Acid	45.5	 C18 : 1 (9t)
Linoleic Acid	-5.0	 C18 : 2 (9c,12c)
α -eleostearic Acid	49.0	 C18 : 3 (9c, 11 t, 13 t)
β -eleostearic Acid	72.0	 C18 : 3 (9t, 11 t, 13 t)
Catalpic Acid	32.0	 C18 : 3 (9t, 11 t, 13 c)

the combination of these configurations to give conjugated systems.

The introduction of *cis* or *trans* double bonds into the aliphatic chains of these free fatty acids (C18) leads to an increase in substrate fluidity. Indeed, the introduction of unsaturation into the molecules leads to a radical reduction in the T_f value compared with that of stearic acid (T_f=70.1 °C) taken as a reference. This decrease is all the more pronounced in the case of a "*cis*" bond than a "*trans*" bond. T_f=16.2 °C for oleic acid C18:1 (9c), T_f=44.1 °C for vaccenic acid C18:1 (11t) and elaidic acid C18:1 (9t) with T_f=45.5 °C.

In the case of linoleic acid C18:2 (9c, 12c), the presence of the two non-conjugated *cis* double bonds results in a marked increase in the fluidity of the molecules. This explains the sharp drop in melting temperature, T_f=−5 °C.

When it comes to conjugated double bonds, as in the case of CLnA with three conjugated double bonds (α -eleostearic acid and catalpic acid with 2 *trans* and 1 *cis*, β -eleostearic acid with 3 *trans*), the T_f values depend on the nature of the configurations of these double bonds. Thus, catalpic acid (2t, 1c) has a T_f=32 °C, α -eleostearic acid (1c, 2t) has a T_f=49 °C and β -eleostearic acid with (3t) has a T_f=72 °C. These values clearly show that the melting temperature increases with the number of *trans* bonds in the configuration.

This is because conjugation in these molecules results in less variation in torsion angles, and thus greater flatness (and parallelism) of chains with *trans* double bonds than fatty acids in *cis* conformation. This results in more intermolecular bonds, leading to a higher melting temperature. Taking into account the differences in these T_f values, we separated

Table 2 Comparison of the fatty acid profile of native oil before and after hydrolysis

Fatty acids (%)	Native oil	Free fatty acids concentrate from hydrolysed native oil
Palmitic acid C16:0	3.8 ± 0.0b	4.2 ± 0.0a
Stearic acid C18:0	5.1 ± 0.1a	4.8 ± 0.0b
Oleic acid C18:1n-9	7.1 ± 0.1b	7.6 ± 0.2a
Linoleic acid C18:2n-6	22.8 ± 0.0a	22.2 ± 0.0a
α-eleostearic acid C18:3c, t, t	60.1 ± 0.2a	60.0 ± 0.1a
Catalpic acid C18:3t, t, c	0.0 ± 0.1a	0.0 ± 0.1a
β-eleostearic acid C18:3t, t, t	1.1 ± 0.0a	1.2 ± 0.0a
Total PUFA (Polyunsaturated fatty acids)	84.0	83.4
Total CLnA (Conjugated linolenic Acids)	61.1	61.2

the free fatty acids obtained from hydrolysis of modified *Ricinodendron heudelotii* oil by cold centrifugation.

After enzymatic hydrolysis of both types of oil at 40 °C, the crude reaction media were centrifuged directly at 15 °C. This temperature was chosen to lower the temperature of the medium and promote crystallization of high-melting fatty acids such as β-eleostearic acid. This crystallization will facilitate the separation of the various fatty acids.

Processing of native oil hydrolysate

After centrifugation, the reaction medium obtained from the hydrolysis of *Ricinodendron heudelotii* native oil was a biphasic system. It comprised an upper organic phase solid at 15 °C and a lower liquid phase made up of water, glycerol and lipase. Note that for economic, environmental or even just efficiency reasons, it is possible to recycle this enzyme by simply re-using the aqueous phase for new oil hydrolysis reactions. Glycerol enrichment of the aqueous phase used as an enzyme preparation poses no problem for further hydrolysis reactions as long as the viscosity still allows sufficient mixing and agitation.

Table 2 shows the fatty acid profiles of the native oil and the concentrate obtained after hydrolysis of the latter. These

results showed that enzymatic hydrolysis had no impact on fatty acid composition and did not lead to conversion by isomerization of α-eleostearic acid under the conditions operated. Thus, the proportions of the various fatty acids remained similar after triglyceride hydrolysis. This result confirmed those already reported in a previous study [14].

Fractionation of modified oil hydrolysate

Figure 4 shows the modified oil hydrolysis reaction medium after 2 h of enzymatic hydrolysis at 40 °C followed by centrifugation at 15 °C.

The reaction medium resulting from hydrolysis of the modified oil was, after centrifugation, a three-phase system (Fig. 4). It consisted of a liquid lower aqueous phase containing the hydrolysis enzyme and released glycerol, a white solid organic interfacial fraction and a yellow liquid upper organic phase. Enzymatic hydrolysis of 500 g of oil gave the following mass distribution after splitting into 5 centrifuge pots and centrifugal fractionation at 15 °C: 327 g upper organic fraction and 141 g interfacial organic fraction. Manual recovery of the fractions thus resulted in a loss of 6.5% by mass of the initial material. This loss may seem significant but it is inherent in the handling of small quantities at laboratory scale.

This material assessment then had to be corrected by the residual water content of the interfacial organic fraction, which, solidified at 15 °C, trapped a mass of water corresponding to 22% of its mass.

The fatty acid profiles of the two lipid phases (upper and interfacial) are shown in Table 3. These two organic fractions had very different fatty acid compositions. They also both had fatty acid profiles that differed from those of the reaction medium after enzymatic hydrolysis of the modified oil.

In fact, hydrolysis of the radiation-modified oil, rich in over 30% β-eleostearic acid, resulted in two other fractions with very different compositions. The upper fraction contained slight variations in saturated fatty acids (palmitic acid and stearic acid) and monounsaturated fatty acids (oleic

Fig. 4 Fractionation of the crude reaction medium of modified oil after enzymatic hydrolysis, obtained by centrifugation at 15 °C (15 min at 10000×g)

Upper fraction (yellow, liquid)

Interfacial fraction (white, solid)

Aqueous phase enriched with glycerol

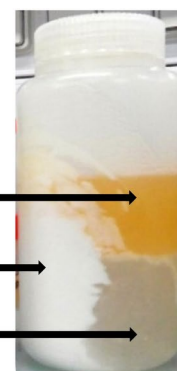


Table 3 Free fatty acid (FFA) composition of modified oil after hydrolysis and hydrolysate fractionation

Fatty acid (%)	Oil modified by artificial sunlight		
	FFA medium after hydrolysis	Centrifugal fractionation at 15 °C	
		Upper phase	Interfacial phase
Palmitic acid C16:0	5.9 ± 0.6	8.1 ± 1.9	2.1 ± 0.3
Stearic acid C18:0	6.6 ± 1.4	9.9 ± 3.3	2.3 ± 0.2
Oleic acid C18:1n-9	7.8 ± 0.6	8.9 ± 1.6	2.5 ± 0.4
Linoleic acid C18:2n-6	28.5 ± 1.1	34.5 ± 0.7	8.7 ± 0.9
α -eleostearic acid C18:3c, t, t	11.8 ± 1.7	11.0 ± 0.3	2.4 ± 0.3
Catalpic acid C18:3t, t, c	7.4 ± 0.6	6.0 ± 3.3	1.8 ± 0.2
β -eleostearic acid C18:3t, t, t	31.9 ± 1.1	21.0 ± 5.2	80.3 ± 2.3
Total PUFA (Polyunsaturated fatty acids)	79.6	72.5	93.2
Total CLnA (Conjugated linolenic acids)	51.1	38.0	84.5

Note: Values after centrifugal fractionation are reported with an uncertainty at a 95% confidence level

acid). On the other hand, there was a significant variation in polyunsaturated fatty acid content, particularly linoleic acid, which increased by 6% to 34.5% of total FFA, and β -eleostearic acid, which decreased by more than 10% to 21%.

In contrast, the interfacial fraction was characterized by a very high β -eleostearic acid content of over 80%, representing an almost 2.5-fold increase in total content.

These results showed the production of different lipid concentrates, rich in CLnA-type fatty acids. A first concentrate composed of 21% β -eleostearic acid, 11% α -eleostearic acid and 6% catalpic acid, and a second concentrate composed of 80.3% β -eleostearic acid.

The two fractions obtained are opposed in terms of physical properties. And yet, these two fatty acid concentrates are composed mainly of polyunsaturated fatty acids (PUFA). The upper phase, which is a yellow liquid at room temperature, contains over 70% PUFA, and in particular almost 35% linoleic acid, which has a melting point of -5 °C. Conversely, the interfacial fraction is a white solid at room temperature, consisting of over 80% β -eleostearic acid with a melting point of 72 °C. For texturizing applications, this β -eleostearic acid, despite carrying 3 unsaturations, behaves like a saturated fatty acid.

FTIR-ATR analysis of CLnA concentrates

The free fatty acid concentrate obtained from native oil composed of 60% α -eleostearic acid (C18:3 9c, 11t, 13t) and the interfacial fraction from hydrolysis of radiation-modified oil, composed of 80% β -eleostearic acid (C18:3 9t, 11t, 13t), were analyzed by FTIR-ATR.

The analyses were carried out at 22 °C directly on the free fatty acid concentrates, without prior preparation.

The ATR method enabled us to visualize, in the 1000 to 900 cm^{-1} region, bands characteristic of various *cis* and *trans* group arrangements in the different fatty acids. This ATR analysis method was therefore perfectly suited to the analysis of our samples, which were composed of different CLnAs: α -eleostearic acid and catalpic acid with 2 *trans*-conjugated double bonds, and β -eleostearic acid with 3 *trans*-conjugated double bonds.

The FTIR-ATR spectrum of free fatty acid concentrate of the native oil (containing 60% α -eleostearic acid) showed two absorption bands, a weak one at μ (C=C) \approx 966 cm^{-1} and a strong one at μ (C=C) \approx 991 cm^{-1} (Fig. 5). As described in the work of Colthup, they correspond to the presence of *trans* and *cis* double bonds in the molecules of these oils with specific configurations. In our case, a band at 991 cm^{-1} associated with a weaker one at 966 cm^{-1} corresponds to a majority of *cis-trans-trans* conjugated double bonds [37].

Analysis of samples composed of unsaturated fatty acids with a *trans* double bond configuration, at high absorbance, produced a characteristic intense peak near 966 cm^{-1} [38].

The FTIR-ATR spectrum of the free fatty acid concentrate of the radiation-modified oil (interfacial fraction), composed of 80% β -eleostearic acid showing 3 *trans* double bonds, showed a band of higher intensity and slightly shifted at μ (C=C) \approx 963 cm^{-1} characterizing the higher content of molecules with three conjugated *trans* double bonds. In this abundance of conjugated systems and different combinations of double bonds (*cis-trans-trans*, *trans-trans-trans* and *trans-trans-cis*), there are interactions between *cis* bonds and the oscillating C-H *trans* vibration. These results confirmed those obtained by Schönemann and Edwards on Tung seed oil, an oil composed of around 70–86% α -eleostearic acid [39].

Second order derivative of FTIR-ATR Absorption Spectra (Fig. 6) were also used to refine this analysis, revealing slight differences that correspond perfectly to Colthup's work [37]. The second order derivative spectrum of the native oil showed the 991 cm^{-1} and 966 cm^{-1} bands pattern specific to the *cis-trans-trans* configuration, whereas the second order derivative spectrum of the modified concentrate rich in β -eleostearic acid showed a band shifted to 994 cm^{-1} and characteristic of three *trans-trans-trans* conjugated double bonds, and a band at 986 cm^{-1} characterizing two conjugated *trans* double bonds.

This centrifugal fractionation of the oil resulted in a lipid concentrate with a previously unseen content of β -eleostearic acid. Centrifugal fractionation at 15 °C mainly crystallized β -eleostearic acid, unlike the other fatty acids present in the fractions. Once solidified, separation between

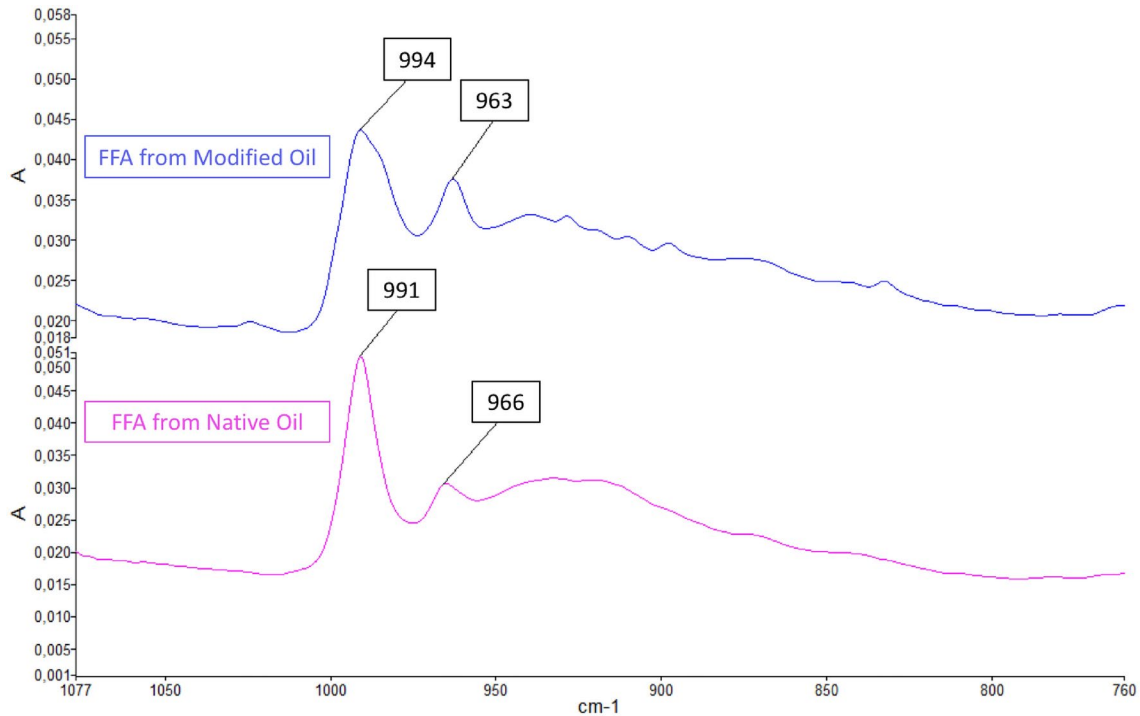


Fig. 5 FTIR-ATR Absorption Spectra of *Ricinodendron heudelotii* free fatty acids from native oil and modified oil

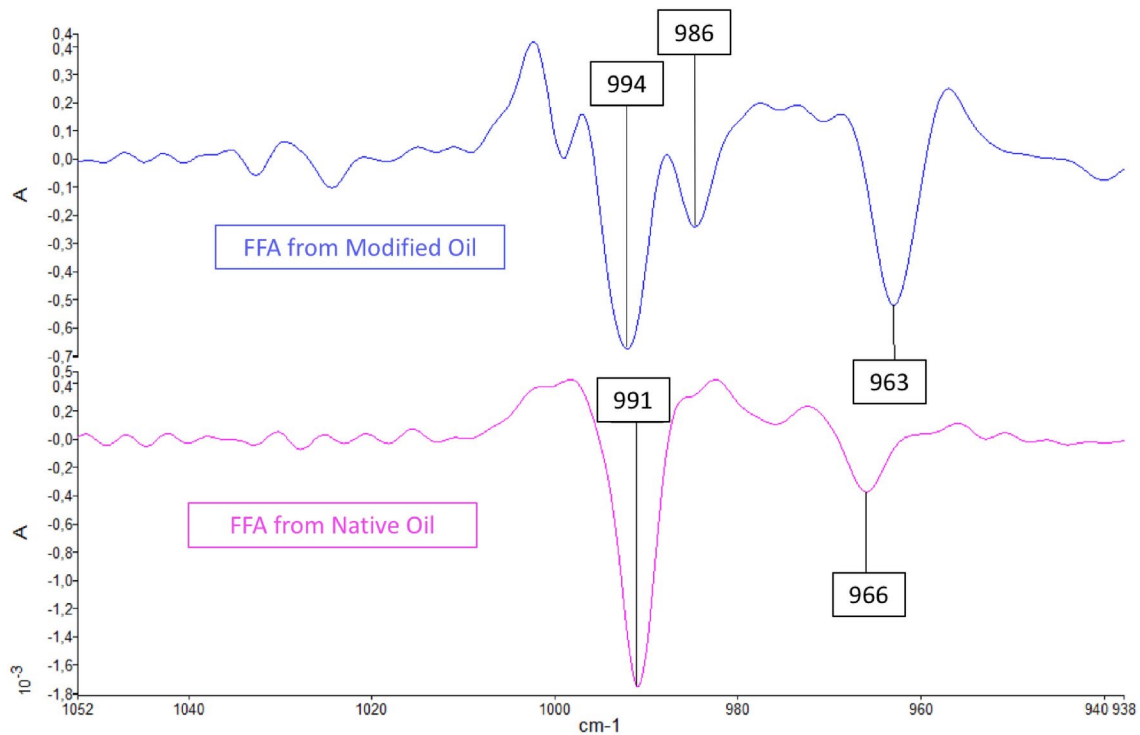


Fig. 6 Second order derivative of FTIR-ATR Absorption Spectra of *Ricinodendron heudelotii* free fatty acids from native oil and modified oil

β -eleostearic acid and the other acids (α -eleostearic acid, catalpic acid and linoleic acid) took place under the action of centrifugal force. This force caused accelerated decantation of β -eleostearic acid molecules from the mixture of

solid and liquid fatty acids. Thus, a process using such a sequence of treatment and processing steps (extraction of oil from *Ricinodendron heudelotii* kernels, treatment of the oil with radiation, enzymatic hydrolysis of the oil and

centrifugal concentration) could constitute an original way of obtaining lipid fractions enriched in CLnA and particularly in α -eleostearic acid and β -eleostearic acid.

Conclusion

Two-hour kinetic monitoring of enzymatic hydrolysis of native oil extracted from *Ricinodendron heudelotii* seeds by *Candida rugosa* lipase (Lipolyve CC, LYVEN) revealed almost complete hydrolysis of triglycerides after only the first hour of reaction at 40 °C, with a free fatty acid yield of almost 94%. The free fatty acid concentrate obtained after hydrolysis had the same fatty acid composition as the original oil, with α -eleostearic acid accounting for over 60% of the major fatty acid content.

Enzymatic hydrolysis of the modified oil after treatment with artificial sunlight using the same lipase also led to complete hydrolysis of the triglycerides after just 1 h's incubation at 40 °C, making it possible to consider a single hour's hydrolysis in such a *Ricinodendron heudelotii* oil treatment process. Hydrolysis of this modified *Ricinodendron heudelotii* oil and centrifugal eco-fractionation yielded two CLnA-rich lipid fractions with highly original compositions. One was mainly composed of 34.5% linoleic acid and 21% β -eleostearic acid, while the other contained over 80% β -eleostearic acid.

The original composition of this β -eleostearic acid-rich fraction derives all its potential industrial interest from the physical properties of this fatty acid. With this type of fatty acid as the main constituent of a lipid composition, it is possible to achieve saturated fat characteristics while still being composed of polyunsaturated fatty acids. This makes it a molecule of choice in industry, particularly for applications intended to replace palm oil or hydrogenated oils. It is also possible to target specific molecules for the synthesis of oleochemical specialties, for example partial esters, which also offer excellent surfactant properties.

The use of a photochemical treatment followed by enzymatic hydrolysis of triglycerides was an effective method for fractionating *Ricinodendron heudelotii* oil to obtain β -eleostearic acid, α -eleostearic acid and linoleic acid.

This process made it possible to diversify and eco-efficiently fractionate conjugated polyunsaturated fatty acids from *Ricinodendron heudelotii* oil, depending on the type of fatty acid required. Once again, *Ricinodendron heudelotii* was proving to be an important and original source of polyunsaturated fatty acids, particularly those of the CLnA type, such as α -eleostearic acid, β -eleostearic acid and catalpic acid.

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