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Study of Bioconversion of Conjugated Linolenic Acid (CLNA) of *Ricinodendron heudelotii* (Bail.) Seed in Male Rats into Conjugated Linoleic Acid (CLA) Using UV-Vis Spectrometry and Gas Chromatography

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ABSTRACT

The present study evaluates the efficiency of conversion of CLNA (conjugated linolenic acids; α-eleostearic acid) of *Ricinodendron heudelotii* to CLA (conjugated linoleic acids; rumenic acid) in the liver, small bowel and serum of male rats using ultraviolet spectrometry (UV) and Gas Chromatography (GC) techniques. One milliliter of oil was orally administrated to 30 rats during 24 h. The spectra of *R. heudelotii* oil, linoleic acid, rumenic acid and α-eleostearic acid (α-ESA) were determined. The concentrations of α-ESA and CLA were determined after 0, 3, 6, 12 and 24 h of oral administration of oil in liver, small bowel and serum. Linoleic acid has a peak at 215 nm and rumenic acid at 232 nm. Spectra of standard α-eleostearic acid and *R. heudelotii* oil were bunk with a characteristic peak at 270 nm. In the serum, we did not find any trace of fatty acid with either UV or GC. UV and GC methods showed that α-ESA was gradually converted in rumenic acid in the liver and small bowel. The liver had a faster and higher enzyme activity than the small bowel. These results showed that the conversion of CLNA to CLA in rats using UV spectrometry is the most method to use.

Key words: *Ricinodendron heudelotii,* oil, rats, α-eleostearic acid, rumenic acid

INTRODUCTION

Conjugated fatty acids are suspected to have numerous physiological effects. These acids are generally found as diene, triene and tetraene conjugated.

Conjugated Linoleic Acids (CLA) are one of the most studied diene conjugated fatty acid. This group is derived from geometric and position isomers of linoleic acid. It is naturally represent in meat and dairy products of ruminants (Singh and Sachan, 2011; Meraz-Torres and Hernandez-Sanchez, 2012). Rumenic acid (c9, t11-CLA) is one of the CLA isomers most widespread in nature and most biologically active. This acid represents about 80-90% of the total CLA of dairy products (Ha *et al*., 1987; MacDonald, 2000). Rumenic acid has several preventive properties such as inflammatory diseases, atherosclerosis and several types of cancer (Park *et al*., 1997;

Kelley *et al.*, 2007; Nakamura *et al.*, 2008). CLA has antidiabetic and antiadipogenic effects (Khanal and Dhiman, 2004). Aydin *et al.* (2006) and Sahin *et al.* (2008) showed that dietary containing CLA decreased the body weight.

Conjugated Linolenic Acid (CLNA) is a triene conjugated acid; isomer of α-linolenic acid. CLNA that has numerous physiological properties such as anti-carcinogenic, anti-obese activity (Noguchi *et al.*, 2001; Kohno *et al.*, 2002; Tsuzuki *et al.*, 2004; Tsuzuki and Kawakami, 2008; Hennessy *et al.*, 2010). It also alter the lipid metabolism in animals (Chardigny *et al.*, 2003; Yang *et al.*, 2005; Yamasaki *et al.*, 2006; Koba *et al.*, 2007; Lam *et al.*, 2008), suppress the growth of tumor cells (Suzuki *et al.*, 2001; Igarashi and Miyazawa, 2005; Yasui *et al.*, 2005). These acids are readily found at high levels in some seed oils: punicic acid (c9, t11, c13-18:3; 83.0%) in pomegranate seed oil, α-eleostearic acid (c9, t11, t13-18:3) in tung (67.7%) in bitter gourd (56.2%) and snake gourd seed oils, catalpic acid (t9, t11, c13-18:3; 42.3%) in catalpa seed oil, calendic acid (t8, t10, c12-18:3; 62.2%) in pot marigold seed oil and jacaric acid (c8, t10, c12-18:3) (Iwabuchi *et al.*, 2003; Takagi and Itabashi, 1981).

R. heudelotii in particular is an uncultivated specie in Central, East and West Africa and Madagascar forests which belong to the Euphorbiaceae family. It is popularly known as njansan in Cameroon; sanga sanga in Congo; eguessang in Gabon; betratra in Madagascar and Okwe in Nigeria. Kernels are used as spices in many dishes in Cameroon (Tchiegang *et al.*, 1997). Its lipids content is more than 50%. The fatty acids composition of this oil indicates a high content of eleostearic acid (51.1%) (Tchiegang *et al.*, 1997; Kapseu and Tchiegang, 1995). *R. heudelotii* oil has hypocholesterolemic effect in rats compared to maize oil (Tchankou Leudeu *et al.*, 2009).

Tsuzuki *et al.* (2003, 2004) and Yuan *et al.* (2009b) showed that α-ESA is converted to CLA in rats and mice. CLA and α-ESA have useful physiological roles; therefore it is important to examine the conversion of α-ESA of *R. heudelotii* oil to CLA *in vivo*.

Many analytical procedures have already been developed to follow the conversion α-ESA to CLA including GC, GC-electron, HPLC, GC-EI/MS, C-NMR (Tsuzuki *et al.*, 2003, 2004; Yuan *et al.*, 2009b). These classical methods are generally time-consuming. The instrumentation is cumbersome and requires highly skilled operators. High-speed, nondestructive analysis, requires minimum or no sample preparation and "easy-to-use" analytical techniques capable of providing a straight forward assessment of variety authenticity are therefore urgently needed (Yang and Irudayaraj, 2001; Roussel *et al.*, 2003; Poulli *et al.*, 2006; Bernuy *et al.*, 2009; El-Abassy *et al.*, 2009; Kadamne *et al.*, 2009).

R. heudelotii almonds are one of the oil bearing materials rich in oil containing more than 52% of α-eleostearic acid. To the best of our knowledge, studies have not been carried out on the conversion of *R. heudelotii* oil by rats. The aim of the present study was to investigate the metabolism of α-ESA (with a trans Δ-13 bond) from *R. heudelotii* in rats and to compare the relative conversion efficiency of this fatty acid into CLA using UV-vis spectrometry and gas chromatography.

MATERIALS AND METHODS

Oil: *R. heudelotii* seeds were purchased from local market (Douala, Cameroon). The lipids from kernels were obtained by press extraction using the screw KOMET press, type DD85G, manufactured in 1991; number 200.666 (Germany). Before extraction, kernels were crushed in a mill brand KOMET, type crusher, manufactured in 1991 and number 200.666 (Germany). The extraction of oil of *R. heudelotii* was made by cold pressing with head diameters oil outlet 6, 8, 10 and 12 cm.

Animals and diet: This study was conducted in conformity with the policies and procedures detailed in the Animal Experiment Guidelines of Ngaoundere University (Cameroon).

Twelve week-old weaned male albinos Sprague Dawley rats (Laboratoire de Biochimie et de Technologie Alimentaires, ENSAI, University of Ngaoundere-Cameroon) weighing between 200 to 250 g were randomly distributed into five experimental groups of 6 animals in polycarbonate cages in a controlled environment with a temperature of 23°C, 12 h light-and-dark cycle (light at 7: 00 a.m.) (Ranhotra *et al*., 1997; Portillo *et al*., 2001). During the acclimatization period of 1 week, the rats received tap water and a standard diet (AIN, 1977) *ad libitum*. The study was conducted between September 2010 and January 2011.

Identification of fatty acids of diet, *R. heudelotii* and pure fatty acids

Gas chromatography: The fatty acids composition of oils from the diet and oil from *R. heudelotii* seeds was analyzed by GC in the laboratory of Biochemistry of Nutrition (ISV, Catholic University of Louvain-Belgium) according to Focant *et al*. (1998) method. The methyl-esterification of the fatty acids, either in the free form or included in triglycerides was performed by treating 500 mg of each sample with 10 mL of KOH (0.1 M) in methanol during 1 h at 70°C, followed by addition of 4 mL HCl (1.2 M) in methanol and further incubation during 15 min at the same temperature. The extraction of the Fatty Acid Methyl Esters (FAME) was done after addition of 20 mL of hexane and 10 mL of demineralized water and addition of undecanoic acid methyl-ester as internal standard.

The apparatus, Thermo Finnigan, type TRACE GC (Milan, Italy) equipped with a Flame Ionization Detector (FID) was used to separate the ester methylated fatty acids. The capillary column used was RESTEK Rt-2560 (100 m length, 0.25 mm inside diameter, 0.2 mm film thickness) (Bellefonte, PA, USA). Hydrogen was used as a carrier gaz. The oven was initially set at 80°C, programmed to 225°C at 20°C min^{-1} and held constant for 15 min. Fatty acid peaks were identified using mixtures of pure methyl ester standards purchased from Larodan fine chemicals (Limhamn, Sweden). The content of each fatty acid was expressed as percentage of the total fatty acids detected.

UV spectrometry: The commercial standards of CLNA (α-eleostearic acid: c9, t11, t13-18:3) CLA (rumenic acid: c9, t11-18:2), ω-6 (linoleic acid: c9, c12-18:2) were purchased from Larodan fine chemicals (Limhamn, Sweden).

R. heudelotii oil and standards of fatty acids were determined by UV spectrometry in the laboratory of Biochemistry of Nutrition, Institute of Life Sciences of the Catholic University of Louvain (Belgium). A UV spectrometer (S2000, Ocean Optics, Dunedin, Florida) was used to measure the absorbances between 200 and 300 nm, with a 1 nm resolution. The samples were diluted in pure hexane and analyzed in triplet with a transparent quartz vial of 3 mL and 1 cm optical path to both sides. The spectra were acquired using a software based OII 32 (S2000, Ocean Optics, Dunedin, Florida). For each sample, ten spectra were averaged to provide the final spectrum.

Gavages and sacrifice of rats: After the acclimatization period, each rat receives 1 mL of njansan oil via a stomach tube. Before and 3-24 h after administration, rats were killed after

anesthesia with diethyl ether. Blood samples from the heart were immediately collected in tubes. Serum was separated by centrifugation at 3000 rpm for 5 min (4°C) and was then stored at -20°C until analysis. After blood collection, the liver and the small bowel were removed.

Extraction and determination of lipid contents of blood and organs: Liver and small bowel lipids were extracted with chloroform/methanol (2/1, v/v) as described previously by Bligh and Dyer (1959). The concentration of serum lipids was determined by the method of Folch *et al.* (1957). Lipids obtained from each sample were divided into two, one part for analyses by UV spectrometry and other by GC.

Characterization of lipid extracts and identification of metabolites: The fatty acids composition of lipid extracts of small bowel and liver were determined by GC using the chromatograph Thermo Finnigan, type TRACE GC (Milan, Italy) and by UV spectrometer (S2000, Ocean Optics, Dunedin, Florida).

Estimation of the conversion rates of α-ESA into c9, t11-18:2: The conversion rate of α-ESA into c9, t11-18:2 was estimated according to Kraft *et al.* (2006) and Kuhnt *et al.* (2006). The net changes of CLNA (α-ESA) and c9, t11-18:2 were calculated. This net change represents the difference in CLNA (α-ESA) and c9, t11-18:2 between rats that have received oil containing α-ESA and rats fed with a control diet.

The conversion rate of α-ESA into c9, t11-18:2 was estimated according to the following formula:

$$\text{Relative conversion rate} = \frac{\text{c9, t11-18:2 (treatment-control)}}{\text{c9, t11-18:2 (treatment-control)+CLNA (treatment-control)}}$$

Statistical analysis: Results were expressed as means±standard deviation. For each group, the result obtained was the mean of 6 rats. Results were analyzed using a one-way analysis of variance. Duncan's Multiple Range test was performed to evaluate differences between groups. Differences between means were considered to be significant at $p<0.05$.

RESULTS

Characterization of oil from *R. heudelotii* and oil from control diet: Table 1 gives the fatty acids composition of the *R. heudelotii* oil and control diet. The major fatty acid of *R. heudelotii* is α ESA acid (50% of total fatty acids). Oleic acid (7.27%) and linoleic acid (32.20) were also present.

The experimented diet was rich in polyunsaturated fatty acids, especially linoleic acid (33.97% of total fatty acids). In this diet, we did not find the presence of diene and triene conjugated fatty acids.

UV spectra of pure fatty acids and oil from *R. heudelotii* seeds: Figure 1 shows the spectra of pure linoleic acid (c9, c12-18:2 (ω-6)), rumenic acid (9c 11t-18:2), α-eleostearic acid (c9, t11, t13-18:3) compare to the spectrum of *R. heudelotii* oil.

Linoleic acid showed an absorption band with a peak at 215 nm which illustrated the presence of non conjugated double bonds.

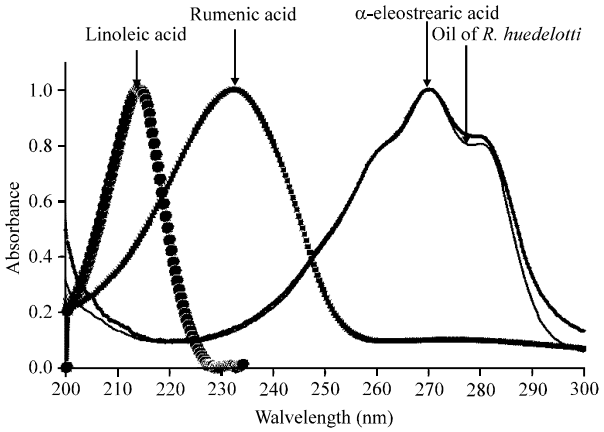

Fig. 1: Spectra of pure fatty acid and oil of *R. heudelotii*

Table 1: Fatty acids composition of diet and oil of *R. heudelotii*

Fatty acids	*R. heudelotii* oil (%)	Diet oil (%)
C10:0	0.08	0.05
C14:0	0.06	4.43
C15:0	0.00	0.21
C16:0	6.47	18.53
C16:1-9c	0.00	5.59
C17:0	0.08	0.18
C18:0	6.54	3.98
C18:1-9c	7.27	19.94
C18:1-11c	0.37	1.74
C18:2-9c, 12c (ω-6)	32.20	33.97
C20: 0	0.14	0.38
C18:3-9c,12c, 15c (ω-3)	0.18	2.98
C18:2-9c, 11t (rumenic acid)	0.00	0.00
C18:3-9c, 11t, 13t (α-ESA)	50.00	0.00
C20:4-5c, 8c, 11c, 14c	0.00	1.00
C20:5-5c, 8c, 11c, 14c, C17c	0.16	3.64
C22:5-7c, 10c, 13c, 16c, 19c	0.00	0.66
C22:6-4c, 7c, 10c, 13c, 16c, 19c	0.00	2.74

The standard diet was fed to all rats during the 1-week conditioning period; Carbohydrate: 58.62%, Crude fiber: 3.10%, Crude protein: 18.11%, Mineral mix: 4.52, Vitamin mix: 1.11, Maize oil: 4.02

Rumenic acid, showed an absorption band at 232 nm, characterizing the presence of conjugated double bonds. It is a conjugated diene fatty acid; isomer of Conjugated Linoleic Acid (CLA).

The spectrum of α-eleostearic acid overlaps that of *R. heudelotii* oil, with a profile characterized by three absorption bands whose peaks were located at 260, 270 and 280 nm.

Linoleic acid has a peak at 215 nm; rumenic acid at 232 nm and α-eleostearic acid at 270 nm.

Characterization of lipid extracts from liver and small bowel with respect to time

Spectrometry UV: Figure 2 and 3 show the UV absorption spectra (200-300 nm) of the lipid extracts of small bowel and liver of rats from 0 h to 24 h. These figures showed three peaks at

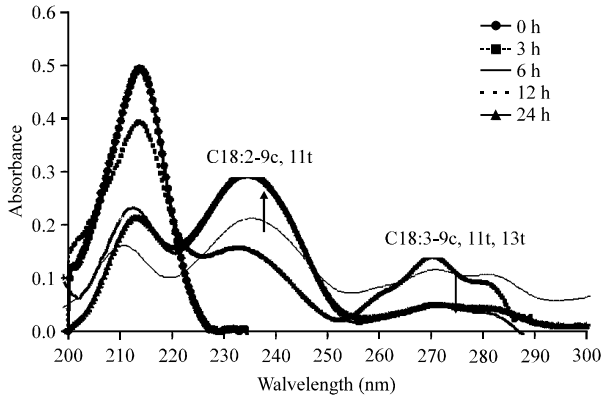

Fig. 2: Spectra of lipid extracts of liver

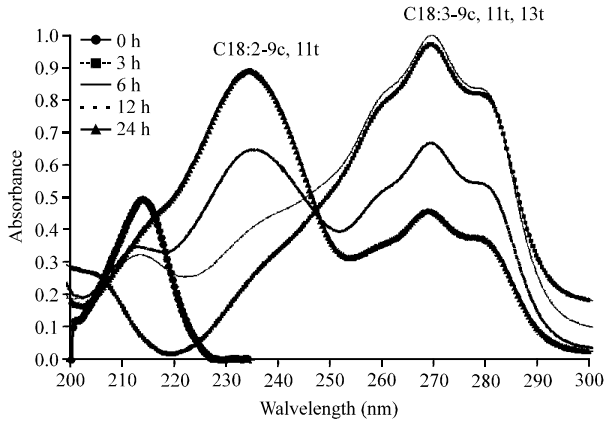

Fig. 3: Spectra of lipid extracts of small bowel

215 nm, 232 and 270 nm representing respectively linoleic acid, rumenic acid and α-ESA. There were no conjugated fatty acids in rat tissues immediately before administration of the oil, but only the peak of linoleic acid appeared.

In the livers of rats, 3 h after administration; in addition to the peaks of the α-ESA and linoleic acid, the peak of rumenic acid appears (Fig. 2). In the other hand in the small bowel, after 3 h of administration, the peaks of α-ESA and linoleic acid appeared, but there is not appearance the peak of rumenic acid. This peak begins to appear after 6 h (Fig. 3).

The absorbance at 270 nm decreases gradually in the liver and small bowel in favor of the absorbance at 232 nm. Thus in the liver, at 0 h the absorbance at 270 nm was 0, at 3 h, it was 0.141±0.001 and 0.054±0.001 at 24 h. At 232 nm the absorbances of oils were 0 at 0 h and 0.156±0.001 and 0.300±0.001, respectively after 3 and 24 h. These results showed that α-ESA was metabolized in rumenic acid in both organs but it is not complete after 24 h.

In serum, we did not find any trace of fatty acids.

Gas chromatography: There was no conjugated fatty acid in rat tissue immediately before administration of njansan oil. The conjugated fatty acids were detected 3 h after oil administration (Table 2). Fatty acid concentrations differed between organs. The liver and small bowel c9 t11-CLA

Table 2: Small bowel mucosa and liver fatty acid concentrations (μg mL^{-1} of ester methyl solution) obtained by GC with respect to the oral administration time

	0 h		3 h		6 h		12 h		24 h	
Fatty acids	B*	L**	B*	L**	B*	L**	B*	L**	B*	L**
C10:0	0.2	0.19	0.17	0.13	0.15	0.12	0.14	0.08	0.19	0.14
C14:0	1.33	0.96	1.81	0.64	2.03	0.81	1.62	0.65	1.99	0.49
C15:0	0.31	0.30	0.27	0.25	0.26	0.21	0.25	0.24	0.28	0.22
C16:0	23.94	21.74	27.95	22.67	27.76	30.06	24.55	28.54	29.13	21.76
C16:1-9c	3.46	2.64	6.82	1.42	7.45	4.27	4.24	3.25	6.98	1.75
C17:0	0.31	0.39	0.22	0.47	0.22	0.34	0.24	0.40	0.22	0.31
C18:0	5.67	9.94	7.27	13.10	4.68	11.90	4.72	8.61	3.30	8.80
C18:1-9c	36.44	19.16	31.27	24.60	32.44	19.94	33.68	25.70	32.79	20.88
C18:1-11c	2.45	2.97	1.89	2.26	2.67	2.37	2.31	2.39	1.93	2.02
C18:2-9c, 12c (ω-6)	22.58	22.29	16.10	17.62	15.11	12.46	23.27	16.08	19.75	21.67
C20:0	0.34	0.00	0.22	0.18	0.19	0.00	0.26	0.05	0.15	0.00
C18:3-9c, 12c, 15c (ω-3)	0.33	0.34	0.38	0.15	0.37	0.17	0.32	0.25	0.38	0.26
C18:2-9c, 11t (rumenic acid)	0.00	0.00	0.31	0.65	0.39	1.36	0.65	1.89	0.58	1.85
C18:3-9c, 11t, 13t (α-ESA)	0.00	0.00	2.64	0.24	2.52	0.42	1.15	0.25	0.14	0.22
C20:4-5c, 8c, 11c, 14c	1.43	10.35	2.06	10.97	0.78	9.62	1.36	6.88	1.08	10.13
C20:5-5c, 8c, 11c, 14c, 17c	0.10	0.39	0.04	0.18	2.54	0.14	0.09	0.11	0.12	0.27
C22:5-7c, 10c, 13c, 16c, 19c	0.24	1.17	0.11	0.58	0.10	0.62	0.26	0.37	0.21	0.62
C22:6-4c, 7c, 10c, 13c, 16c, 19c	0.95	7.15	0.46	3.88	0.32	5.19	0.88	4.25	0.79	8.62

*: Small bowel. **: Liver

concentrations increased with time while α-ESA concentration decreased. c9, t11-18:2 was detected in all tissues examined in α-ESA groups, suggesting that α-ESA could be metabolized into c9, t11-18:2.

In serum, we did not find the presence of fatty acids. CLA was detected in rat tissues (small bowel and liver) 3 h after oral administration and the CLA produced from α-ESA administration was present as the c9, t11-CLA isomer only, suggesting that it was synthesized through an enzymatic reaction.

Therefore, the combination of the results obtained from GC and UV spectrometry analyses provides good evidence that CLAs in all tissues examined in rats fed diet with the α-ESA acid is rumenic acid (c9, t11-18:2) but not all the α-ESA was absorbed.

Conversion rates of α-ESA into 9c, 11t-18:2

Comparison of lipid extracts of small bowel and liver: The liver recovery of α-ESA in *R. heudelotii* oil treated rats was 73% 3 h after administration and 89% after 24 h (Fig. 4). In small bowel, the conversion of α-ESA was 15% after 3 h and 66.34% after 24 h. A comparison of enzyme activity between liver and small bowel suggested that the liver had a faster and higher enzyme activity than the small bowel.

Comparison of the conversion rate of lipid extracts by UV and GC in liver: The comparison of conversion percentage of the α-eleostearic acid to rumenic acid by chromatography and UV spectrometry is summarized in Fig. 5.

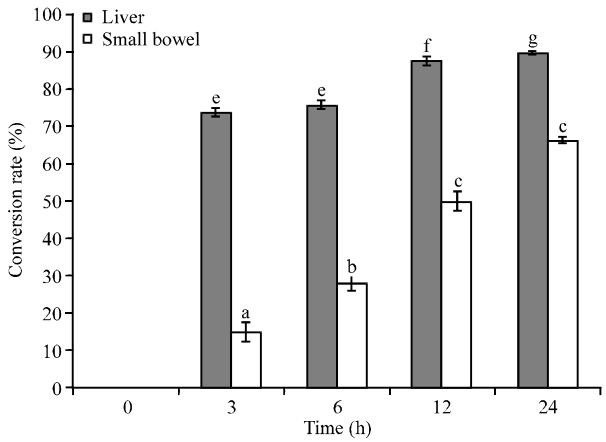

Fig. 4: Comparison of the conversion rate of α-eleostearic acid to rumenic acid in the small bowel and liver, values are Means±SEM (n = 6), Values not sharing a common letter are significantly different at $p<0.05$

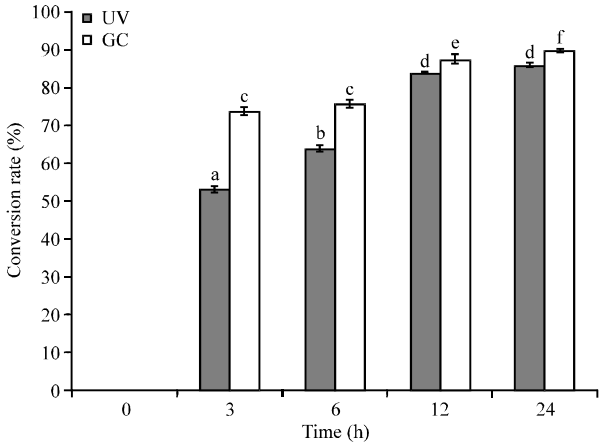

Fig. 5: Comparison of the conversion rate of α-eleostearic acid to rumenic acid using UV and GC methods, values are Means±SEM (n = 6), Values not sharing a common letter are significantly different at $p<0.05$

DISCUSSION

The proportion of α-ESA found in oil (50%) is lower than that found by Tchiegang *et al.* (1997) (51.1%) and (52.61%). CLA was not detected in this oil.

The experimented diet was rich in polyunsaturated fatty acids, especially linoleic acid (33.97% of total fatty acids) because corn oil was used for it formulation. Corn oil contains 56.5% of linoleic acid (FAO, 1981).

The spectrum of α-eleostearic acid overlaps that of *R. heudelotii* oil, with a profile characterized by three absorption bands whose peaks were located at 260, 270 and 280 nm. According to Karleskind (1996), 270 nm represents the conjugated triene. The fatty acids profile of *R. heudelotii* obtained by GC, indicate that α-eleostearic acid with 50% of total fatty acids is the major fatty acid. The conjugated triene fatty acid found in *R. heudelotii* oil is one of the isomers of Conjugated Linolenic Acid (CLNA) and precisely eleostearic acid.

Conjugated diene (rumenic acid) was detected in small intestine and liver in *R. heudelotii* groups, suggesting that conjugated triene (α-eleostearic acid) can be metabolized into conjugated diene. Tsuzuki *et al.* (2003) also identified rumenic acid in the tissues of rat groups fed with α-ESA. Yuan *et al.* (2009b) indicated that both α-eleostearic acid and punicic acid are metabolized into 9c, 11t-18:2 in mice. Tsuzuki *et al.* (2004, 2006) speculated that α-ESA could be converted into rumenic acid by a Δ-13-saturation reaction carried out by an NADPH-dependent enzyme which is either a novel enzyme recognizing conjugated trienoic acid or the enzyme active in the leukotriene B4 reductive pathway. Recently, Moise *et al.* (2004, 2007) described a retinol saturase which carries out the saturation of the 13-14 double bond of the all-trans-retinol to produce all-trans-13, 14-dihydroretinol. All-trans-retinol is another enzyme that can be recognized in the conversion of the α-eleostearic acid to rumenic acid. The enzyme involved in this conversion is still not clearly identified. However, further studies are warranted to provide a clearer understanding of the conversion mechanism of α-ESA and punicic acid into c9, t11-18:2.

The calculation of the conversion rate was an approximate estimation on the basis of the mean of net change of end-product levels (Kraft *et al.*, 2006). The more rapid conversion in the liver than in the intestine suggests that the enzyme is most represented in the liver than in the intestine. Tsuzuki *et al.* (2004) made the same observations in an *in vitro* study using liver and small bowel homogenates of rat. But it is contradictory to the results obtained by these same authors but *in vivo* because α-ESA was supplied continuously and the small intestine was the first organ to encounter α-ESA and may have absorbed it in large amounts, due to the large intestinal surface area. In a recent investigation Yuan *et al.* (2009a, b) showed that the conversion rate of α-ESA was 91.8% in adipose tissue; 88.4% in liver and 91.4% in spleen. The conversion rate of punicic acid is 76.2% in liver and 62.8% in the adipose tissue in mice (Yuan *et al.*, 2009b). The underlying reason for this is not clear. It is possible that the Δ 13-double bond saturation reaction activity differed among tissues.

There was a significant difference in conversion rate between UV and GC. The highest rates were obtained with GC. But GC technique was relatively time-consuming (24-48 h) compare to UV technique (15 at 30 min). The results obtained by means of spectrometry, without any sample treatment, are comparable with those obtained by means of chromatography technique, that requires a lot of treatment.

CONCLUSION

There is an evidence that α-ESA of *R. heudelotii* oil incorporated into various tissues through a diet could be effectively metabolized into c9, t11-18:2. The relative conversion rate of α-ESA was 89% in liver. The liver had a faster and higher enzyme activity than the small bowel. The conversion of CLNA into CLA in the tissues could be followed using spectrometry UV which is a very fast technique; it is easy to use. The observation that CLNA could be converted into c9, t11 CLA has gained increased importance since it had been demonstrated that c9, t11 CLA can exert many biological activities. The c9, t11-18:2 had been found naturally only in limited sources such as ruminant products at a very low level. Moreover, natural resources containing CLNA could be a potential dietary source of CLA and are expected to be useful as a functional food and nutraceuticals.

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