

## The use of tris (tetraphenylimidodiphosphate) of praseodymium chemical shift reagent in proton NMR for the evaluation of the argan oil fatty acids autoxidation and the analysis of the argan pulp fatty acids

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**Abstract:** Proton NMR is a method of molecular investigation that has its limitations when applied to complex molecules or molecules with many nearly equivalent sites. Previous studies have resorted to the use of paramagnetic chemical shift reagents, having as formula tris (tetraphenylimidodiphosphate) of lanthanides  $\text{Ln}((\text{tpip})_3)$ . The use of reagent  $\text{Pr}(\text{tpip})_3$  in proton RMN has allowed us to evaluate the autoxidation of fatty acids mixture (stored 6 and 12 months after oil extraction) by the dosage of saturated and unsaturated acids on the one hand, and that of oleic and linoleic acids on the other. We note between 6 and 12 months of storage at 4°C a decrease in the percentage of unsaturated acids (76% to 63%) and an increase in the percentage of saturated acids (24% to 36%). The results show that the oleic acid maintained the same percentage (35%) as it is not easily oxidized whereas, for the linoleic acid, we observe a decrease in percentage from 22.5% to 18.5% (slow autoxidation at 4°C). We also used this NMR method for the analysis of the argan pulp fatty part. The GC analysis shows that it contains very few unsaturated fatty acids and that the main fatty acids are myristic (C14:0) and palmitic (C16:0) acids. The proton NMR with  $\text{Pr}(\text{tpip})_3$  allowed us to confirm these results. This method that does not require derivation has proven to be interesting, simple and efficient.

**Keywords:** Proton NMR analysis;  $\text{Pr}(\text{tpip})_3$ ; GC analysis; Argan oil; fruit pulp of arganier; Fatty Acids (FA).

### Introduction

The Argan tree is a plant species originating in and endemic to southern Morocco. A small argan population is found in the area of Béni Snassène (Berkane), 20 km away from the sea, and in the valley of Oued Grou (Khemisset) 80 km eastward of Rabat. Argan tree can be found at sea level and up to 1500 m above sea level, particularly appreciating the humid and oceanic air. The fruit of the argan tree is quite rich as it is constituted of the seeds, which are used in the human diet, as well as its pulp that is destined for cattle feeding. Traditionally extracted in a hand-press way and nowadays by mechanical cold-press, the oil has high dietetic value, unsaturated fatty acids being the major components. The oleic and linoleic acid make up 80% of the fatty acids, whereas linolenic acid is present only as in the form of traces. The unsaponifiable matter is rich in the tocopherol (vitamin E) allowing better conservation of the oil and

ensuring a good dietary intake of antioxidants<sup>1-10</sup>. The phenolic fraction known for these antioxidant properties is present with a not negligible content. The chemical composition of argan oil gives it nutritional, cosmetic and therapeutic properties<sup>11-15</sup>. The study of the pulp chemical composition shows a low fatty content. However, it is richer in cellulose, proteins and essentially in carbohydrates<sup>6,16</sup>.

This work is divided into two parts:

The first part deals with the evolution of the degradation of an argan oil FA mixture (oil extracted with hexane) by proton <sup>1</sup>H NMR using  $\text{Pr}(\text{tpip})_3$  as chemical shift reagent. The second part deals with the analysis of the pulp fatty part the by CG and by NMR with  $\text{Pr}(\text{tpip})_3$ .

Previous studies<sup>17</sup> have shown that the organic ligand praseodymium chelates produce chemical shifts that are as important europium chelates but in

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the opposite direction. Our choice was focused on the tetraphenylimidodiphosphinate (inorganic ligand) chelate with praseodymium (Pr) as rare earth instead of europium for two essential reasons, the first is due to its strong complexation privileged with carboxylic acids than with the other usual functions and the second resides in the fact that the chelate  $\text{Pr}(\text{tpip})_3$  reacts with the acids by inducing chemical shifts toward the strong fields (on the right of the TMS) which would avoid any overlap of the proton signals of our acid mixture with those of the phenyls of the ligand tetraphenylimidodiphosphinate (tpip).

For the first part, we followed the autoxidation evolution of the FA mixtures from an argan oil extracted with hexane in order to avoid any ambiguity related to the extraction method. Our analyzes were carried out on two samples at 6 and 12 months after the extraction of the oil on a Bruker AM 500 Spectrometer located at the Pierre and Marie Curie University in Paris.

Firstly, and for qualitative analysis, we took the sample of the FA mixture extracted 6 months before without and with  $\text{Pr}(\text{tpip})_3$  chelate in  $\text{CDCl}_3$  as a solvent.

$^1\text{H}$  NMR spectrum without chelate does not give any differentiation as to the number of unsaturations nor the length of the acids carbon chains that are present. However, the  $^1\text{H}$  NMR spectrum with chelate gives more information and details. The addition to the mixture of fatty acids in  $\text{CDCl}_3$  solution of  $\text{Pr}(\text{tpip})_3$  allowed the resolution of almost all the signals, the identification of the FA mixture constituents, and the determination of the position of the double bond.

Secondly, we sought to quantitatively evaluate the evolution of the unsaturated acids degradation constituting the FA mixtures by determining of the percentages of saturated acids by the ratio of the integration of the hydrogens  $\text{H}_8$ s to those of the hydrogens  $\text{H}_6$ s on the one hand, and the percentages of oleic and linoleic acids, and this through the integration of their characteristic protons ( $\text{H}_{11}$  and  $\text{H}_{14}$ ) on the other. The analyzed the regions of the spectra are those between (2.4ppm and 1.5ppm) and (0.05ppm and -1ppm).

This study was conducted on three FA samples: of 6 months, 12 months, and a commercial (extracted in a hand-press) oil taken as a reference. The results obtained allowed us to have an idea on the progress of the fatty acids degradation according to the duration of the conservation (samples stored at  $4^\circ\text{C}$  in bottles hermetic to air and light).

These results showed between samples 2 and 3, a decrease in the percentages of unsaturated acids and an increase in saturated acids. An unchanged percentage is noted for the oleic acid (35%) as it is less susceptible to oxidation. We also note a percentage decrease for linoleic acid (from 29% to 18.5%). The

results of these mixtures allowed us to evaluate the degradation of FA in 12 months, especially the most unsaturated acid (i.e linoleic acid). It is noteworthy that we did not notice degradation products since they are in small quantity and their detection by  $^1\text{H}$  RMN with reagents  $\text{Pr}(\text{tpip})_3$  is not privileged.

The second part of this work will be devoted to the characterization of FA in argan pulp. The GC analysis of the methyl esters indicates a low unsaturated fatty acid content. The dominant fatty acids consist mainly of myristic and palmitic acids. These results are in agreement with those obtained by the proton NMR with  $\text{Pr}(\text{tpip})_3$ . The latter method has already been used for the study of carboxylic acids of synthetic and natural origin [18-19].

## Materials and methods

### Samples

The argan fruit used in this study comes from the valley of Oued Grou (Khemisset) at 80 km east of Rabat, Maroc and it was harvested ripe in July. The two parts of the fruit used in this study are the seed and the pulp. The dried pulp is separated from the nuts (seed and shell), and the seed is obtained by breaking the shell. Both processes are carried out manually.

### Preparation of the argan oil

The extraction of the oil made from the crushed seed is carried out under reflux for 8 hours by Soxhlet using hexane as solvent. After drying with anhydrous sodium sulphate and evaporation of the solvent, an oil yield of 50% is obtained.

### Saponification of argan oil

5 g of oil is mixed with 100 ml of potassium hydroalcoholic solution (1N). We heat under reflux for 1h 30min. After the addition of 200 ml of distilled water, the unsaponifiable matter is extracted with ethyl ether, and two fractions are obtained:

- The aqueous fraction which contains the fatty acids (FA) in the form of potassium salts.
- The organic fraction containing the crude unsaponifiable.

The saponification in this study is necessary because the reagent  $\text{Pr}(\text{tpip})_3$  reveals only the fatty acid proton signals with which it forms a complex. The  $\text{Pr}(\text{tpip})_3$  has a reactivity towards carboxylic acids that is highly preferred compared to the other usual functions.

### Treatment of the aqueous phase, obtainment of the argan oil fatty acids

The aqueous phase is acidified by a solution of hydrochloric acid at 30% until acid pH in order to release the fatty acids which are then extracted with hexane. The hexane phase is washed with distilled water until neutral pH, dried with anhydrous sodium sulphate, filtered and then evaporated. Free fatty acids

are thus obtained. Samples of FA mixtures obtained are placed in airtight and light-tight bottles and stored at 4°C before being analyzed.

#### <sup>1</sup>H NMR analysis of the argan oil FAs mixture

All <sup>1</sup>H NMR spectra were recorded using a BRUKER AM instrument at 500MHz with TMS as an internal reference. The FA mixtures to be analyzed are directly prepared in the NMR tube. The experiment is carried out at room temperature.

-Analysis without Pr (tpip)<sub>3</sub> reagent: 5 mg of the FA mixture is added to 0.5 ml of CDCl<sub>3</sub> plus TMS.

-Analysis with Pr (tpip)<sub>3</sub> reagent: To the previous mixture, weighed amounts of Pr (tpip)<sub>3</sub> are gradually added until a molar rapport equals 1.

#### Preparation of the argan pulp lipid extract

The pulp is dried in an oven (60°C) for 48h. It is finely ground and then dried again in an oven for 48h. 30 g of crushed pulp is extracted with hexane in a Soxhlet apparatus for 8 hours; after evaporation of the solvent, a semi-fluid lipid extract is obtained with a yield of 9%.

#### Transesterification in a basic medium, obtainment of fatty acid methyl esters (FAMES).

The lipid extract is dissolved in methyltertiobutylether, and a methanolic solution (0.2 M) of sodium methoxide is added. The FAMES are obtained after stirring and adding a sulfuric acid solution.

#### Gas Chromatography analysis of argan pulp (FAMES)

The analysis is carried out on a Varian 3700 chromatograph equipped with an evaporator- injector (T: 220°C), a capillary column PB 20 (25m x 0.22mm, ID: 0,25 μm), a flame ionization detector (240°C), and integrator recorder Varian 4270. The vector gas is helium (flow: 1ml/min), and the oven temperature is programmed between 140 to 170°C at 6°C/min. The FAMES in hexane solution was injected at a concentration of 1 mg/ml.

#### Saponification of the argan pulp lipid extract

10 g of the argan pulp lipids are mixed with 100 ml of potassium hydroalcoholic solution (1N). We heat under reflux for 1h. After the addition of 150 ml of distilled water, the unsaponifiable matter is extracted with ethyl ether, and two fractions are obtained:

-The aqueous fraction which contains the fatty acids (FA) in the form of potassium salts.

-The organic fraction containing the crude unsaponifiable.

#### Treatment of the aqueous phase, obtainment of the argan pulp fatty acids

The aqueous phase is acidified by a solution of hydrochloric acid at 50% until acid pH in order to release the fatty acids which are then extracted with hexane. The hexane phase is washed with distilled water until neutral pH, dried with anhydrous sodium sulphate, filtered and then evaporated. Free fatty acids are thus obtained. The sample of the fatty acids mixture obtained is placed in an airtight and light-tight bottle and stored at 4°C before being analyzed.

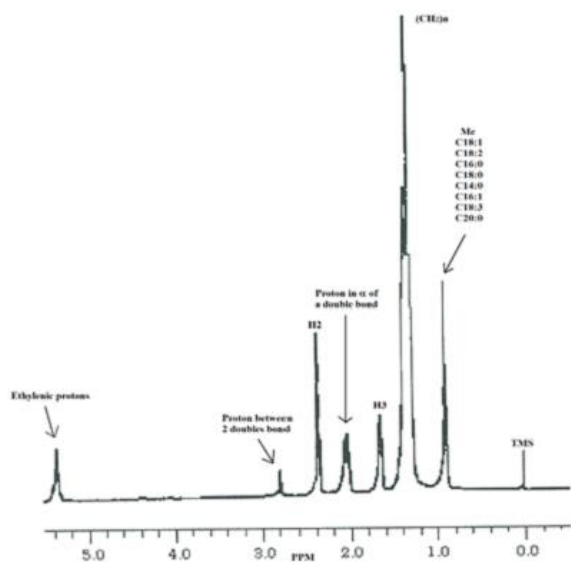
<sup>1</sup>H NMR analysis of argan pulp FAs: Same protocol as the fatty acid mixtures of argan seed oil

## Results and Discussions

### First Part

<sup>1</sup>H NMR analysis of the FAs mixture (6 months after the oil extraction) in CDCl<sub>3</sub> solution

The proton NMR spectrum of the FAs mixture (Fig.1) in CDCl<sub>3</sub> solution shows that the resonances of the ethylene protons, the protons of the methylenes located between two double bonds and the protons of the methylene's in α of a double bond appear into three signals, which excludes the possibility of determining by <sup>1</sup>H NMR the position of the double bonds and their configuration.

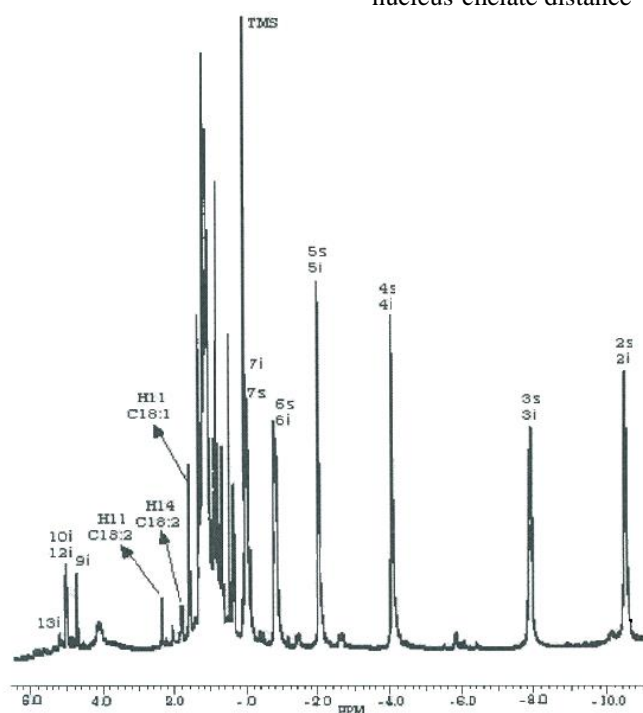


**Figure 1.** <sup>1</sup>H NMR spectrum of the fatty acids mixture (6 months after the oil extraction) in CDCl<sub>3</sub> solution at 500MHz

### $^1\text{H}$ NMR analysis of fatty acids mixture in $\text{CDCl}_3$ solution with $\text{Pr}(\text{tpip})_3$

In the presence of  $\text{Pr}(\text{tpip})_3$  (molar ratio close to 1), the  $^1\text{H}$  NMR spectra (Fig.1 and Fig.2) indicates

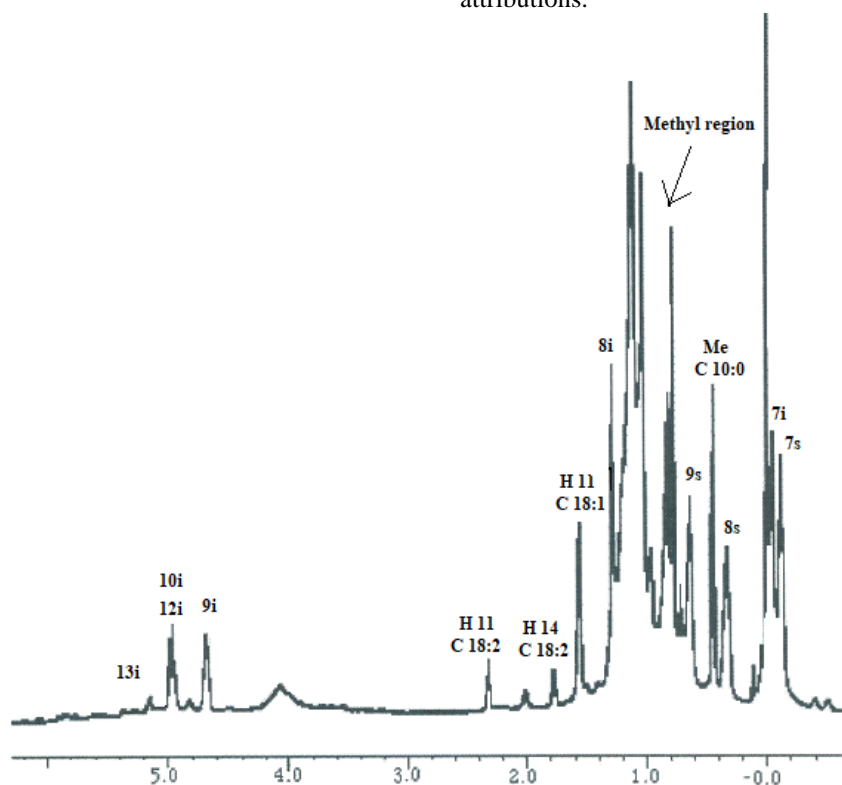
many signals. The majority of these overlapped in the absence of the chelate (Fig. 1) and went through an important differentiation in its presence. The induced chemical shifts of these signals are a function of the nucleus-chelate distance <sup>20-22</sup>.



**Figure 2.**  $^1\text{H}$  NMR spectrum of the fatty acids mixture (6 months after the oil extraction) in  $\text{CDCl}_3$  solution with  $\text{Pr}(\text{tpip})_3$  at 500MHz  
i: unsaturated; s: saturated

For clarity purposes, the spectrum (Fig. 2) will be split

into several regions to provide more details on the attributions.



**Figure 3.** Spectrum between (0-5ppm) of the fatty acids mixture in  $\text{CDCl}_3$  solution with  $\text{Pr}(\text{tpip})_3$  at 500MHz

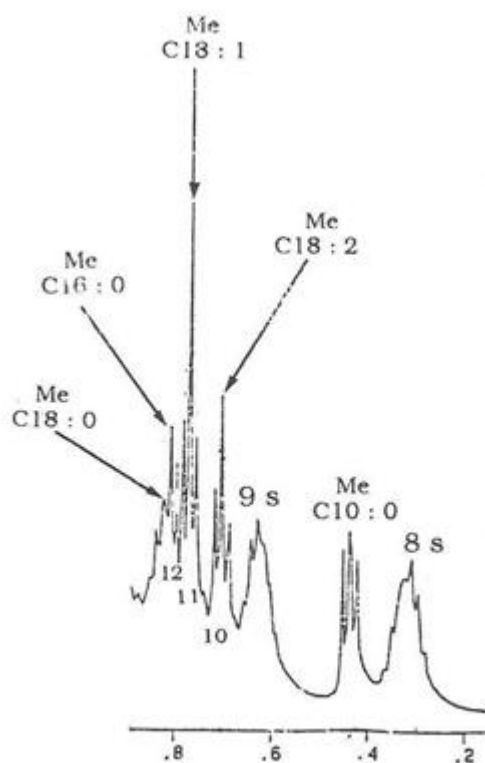
Previous studies 18-19 on known FA mixtures have shown that the relative values of the induced shifts for all the signals are independent of the respective concentrations of the different acids. The decanoic acid was added as a reference to any mixture examined because of its methyl group signal which appears to be isolated between the methylene groups signals in the position 8 and 9.

We, therefore, proceeded in the same fashion in the study of the FAs mixture of argan oil. The induced shifts will be normalized concerning the group Me C10:0 and will permit the observation of similar values.

### <sup>1</sup>H NMR analysis of the different regions of the spectrum (Fig. 2)

#### Analysis of the spectrum between (0.31 and 0.85) ppm

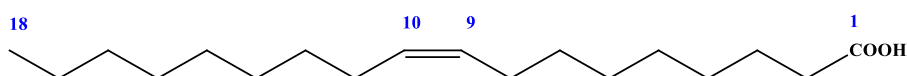
The partial spectrum (Fig a) indicates two signals in the form of distinct and well-resolved triplets at  $\delta = 0.71\text{ppm}$  and  $\delta = 0.77\text{ ppm}$  which correspond to the resonance of methyl groups protons of the C18: 2 and C18: 1 acid. These are the most represented in argan oil and the oleic acid being the major component. These attributions were confirmed through adding the C18:2 acid to our mixture. The signal at **0.71ppm** is assigned to the C18: 2 methyl and at **0.77 ppm** to the C18: 1 methyl.



**Figure a.** Spectrum between (0.31 and 0.85) ppm of the fatty acids mixture In CDCl<sub>3</sub> solution with Pr(tpip)<sub>3</sub> at 500MHz

At around **0.83 ppm** two less intense signals appear partially overlapped. The separate addition of C16: 0 and C18: 0 allowed us to assign the signal at **0.81 ppm** to methyl C16: 0 and that at **0.84 ppm** to methyl of C18: 0. The signals of methylene groups of the positions 8 ( $\delta = 0.33$ ) and 9 ( $\delta = 0.63\text{ ppm}$ ) concerning the acid function in the various saturated acids of the mixture appear in quintuplet form (Fig. a).

They are distinct and isolated from other signals, whereas the signals of the methylene groups of the positions 10, 11, and 12 concerning the acid function in the different saturated acids overlap with the signals of the group's methyl of all the acids present in the mixture. The signal at  $\delta = 0.44\text{ ppm}$  is that of the protons of the methyl group of decanoic acid (C10: 0) added as an internal reference.



Oleic acid structure

### Analysis of the spectrum between (1, 56 to 2, 32) ppm

The signal separation of protons H8 and H11 from oleic acid is noted. They correspond to the protons in  $\alpha$  of the double bond (positions 9, 10). The signal which appears at about **1.57 ppm** (Fig. e)

corresponds to the protons of the methylene group in  $\alpha$  of the double bond (position 11) concerning the carboxylic function of the acid C18: 1. The signal of these last protons which appeared at **2.15 ppm** (without chelate Fig. f) is shielded in the presence of the chelate and appears at **1.57 ppm**.

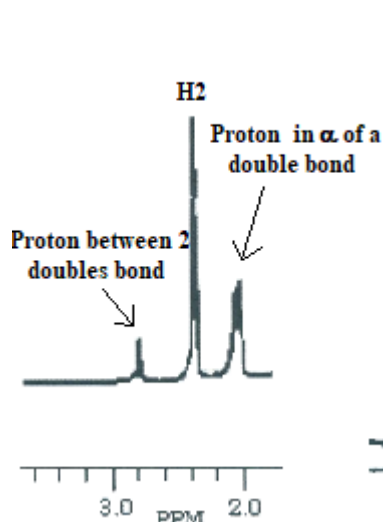


Figure f. Spectrum (2-2,8 ppm) without chelate

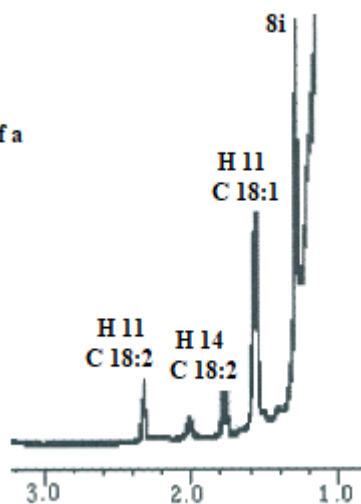
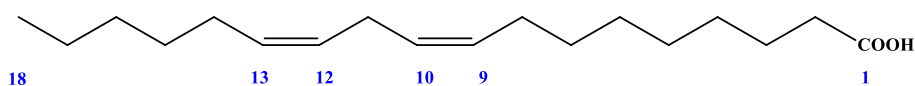


Figure e. Spectrum (1,56-32) ppm with chelate

Similarly, in the presence of the chelate, the protons H8 and H14 signals of linoleic acid are separated. The second signal which appears isolated at around **1.8 ppm** (Fig. e) thus corresponds to the methylene group protons in  $\alpha$  of the double bond (position 14) concerning the carboxylic function of C18: 2 acid. The third isolated signal appearing in

triplet form at around **2.30 ppm** (Fig. e) corresponds to the methylene group protons H11 of C18: 2 acid which was at **2.8 ppm** (Fig. f) in the absence of chelate. The latter protons located between two double bonds are shifted to the strong fields. The complexation more influences them with the chelate.



Linoleic acid structure

### Analysis of the spectrum between (4,7 to 5,2) ppm

The signals of the ethylene protons H9 ( $\delta = 4.7$  ppm), H10 ( $\delta = 5$  ppm), H12 ( $\delta = 5$  ppm) and H13 ( $\delta = 5.2$  ppm), overlapped in the absence of chelate (Fig. h), separate when the chelate is added (Fig. g) by

shifting to the strong fields. A relatively important intensity is noted for proton signals H9 (2H) and H10 (2H) belonging to both oleic (C18: 1) and linoleic acid (C18: 2), whereas the signals of protons H12 (1H) and H13 (1H) belong to linoleic acid only.

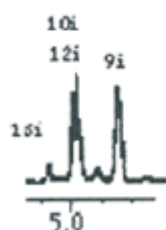


Figure g. Spectrum (4,7-5,2) ppm with chelate

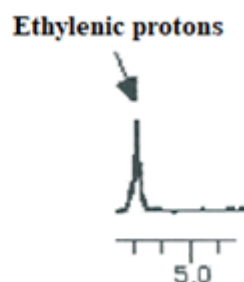


Figure h. Spectrum at 5,35 ppm without chelate

The induced shifts of the characteristic proton signals of FAs present in the mixture were normalized concerning the induced shifts of the methyl group of

C10: 0 (added as an external reference). They are listed in Table I.

**Table I.** Induced chemical shifts of the FAs characteristic signals normalized concerning the induced shifts of the methyl group of C10: 0 (added as an external reference) in the presence of the chelate.

Compounds	Site	Me		8-CH <sub>2</sub>		11-CH <sub>2</sub>		14-CH <sub>2</sub>	
		$\delta$	$\Delta\delta / \Delta\delta_{C_{10:0}}$	$\delta$	$\Delta\delta / \Delta\delta_{C_{10:0}}$	$\delta$	$\Delta\delta / \Delta\delta_{C_{10:0}}$	$\delta$	$\Delta\delta / \Delta\delta_{C_{10:0}}$
C10 :0		0,89	1						
C14 :0		0,89	0,39	1,26	1,7				
C16 :0		0,89	0,15	1,26	1,7				
C18 :0		0,89	0,11	1,26	1,7				
C18 :1		0,89	0,24	2,01	1,69	2,01	1,05		
C18 :2		0,89	0,42	2,04	1,69	2,77	1,05	2,1	0,66

$\delta$ : Chemical shifts in the absence of chelate

$\Delta\delta / \Delta\delta_{C_{10:0}}$ : Induced chemical shifts normalized concerning the induced shifts of the C10: 0 methyl group (added as an external reference).

Calculation of induced chemical shifts of some characteristic protons of linoleic acid

#### For methyl groups

Without chelate

$$\delta_{C_{10:0}} = 0,89 \text{ ppm} \quad \Delta\delta_{C_{10:0}} / \Delta\delta_{C_{10:0}} = 1$$

With chelate

$$\delta_{C_{10:0}} = 0,44 \text{ ppm}$$

Without chelate

$$\delta_{C_{18:2}} = 0,89 \text{ ppm}$$

With chelate

$$\delta_{C_{18:2}} = 0,71 \text{ ppm} \quad \Delta\delta_{C_{18:2}} / \Delta\delta_{C_{10:0}} = 0,18/0,45 = 0,4$$

#### For methylene groups 14-CH<sub>2</sub>

Without chelate

$$\delta_{C_{18:2}} = 2,1 \text{ ppm}$$

With chelate

$$\delta_{C_{18:2}} = 1,8 \text{ ppm} \quad \Delta\delta_{C_{18:2}} / \Delta\delta_{C_{10:0}} = 0,3/0,45 = 0,68$$

#### For methylene groups 11-CH<sub>2</sub>

Without chelate

$$\delta_{C_{18:2}} = 2,77 \text{ ppm}$$

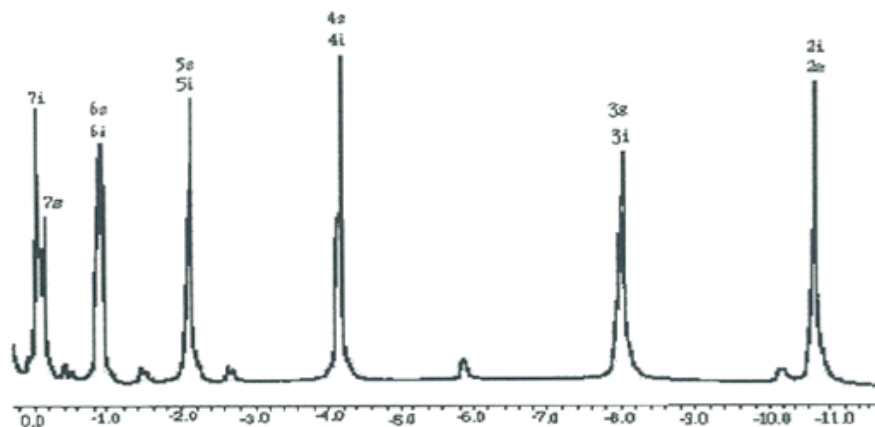
With chelate

$$\delta_{C_{18:2}} = 2,3 \text{ ppm} \quad \Delta\delta_{C_{18:2}} / \Delta\delta_{C_{10:0}} = 0,47/0,45 = 1,05$$

#### Analysis of the spectrum between (-11 to 0)

**Spectrum** (Fig. 4) shows signals that are almost entirely distinct from one another. Indeed, the closer the hydrogens are to the COOH complexation site, the more critical the shift towards strong fields. The hydrogens in position 2 concerning the acid function (H<sub>2</sub>) go through the most significant shielding. This allows a separation of the hydrogen H<sub>4</sub> to H<sub>7</sub> signals.

The net splitting of the H<sub>7</sub> signal shows the presence of saturated fatty acids next to unsaturated fatty acids, which have their first unsaturation in position 9. The signal intensity of the methylene groups in position 7 in the unsaturated acids (7i) is relatively strong compared to that of the signal of the groups H<sub>7s</sub>, showing the preponderance of the unsaturated acids in the mixture.



**Figure 4.** Spectrum between (-11 to 0) of the fatty acids mixture in CDCl<sub>3</sub> solution with Pr(tpip)<sub>3</sub> at 500MHz

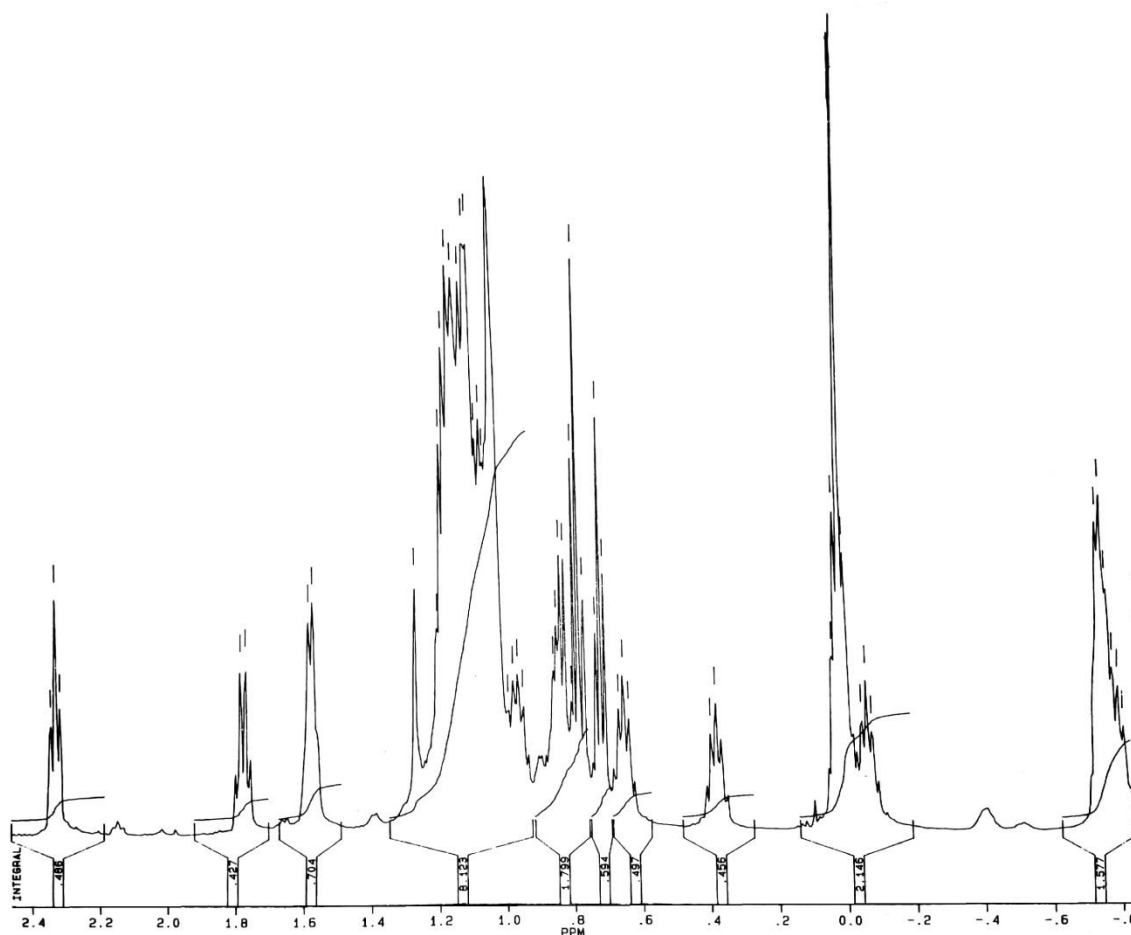
In the presence of chelate, two regions of the  $^1\text{H}$  NMR spectrum have provided us with important information:

- The methyl group region shows well-separated signals (Me of C18: 2 and C18: 1).
- For the other methyl groups, the values of the chemical shifts are close to one another, and their signals are overlapped as in the example of the C16: 0 and C18: 0 acid signals. The attributions were recognized by adding each of these latter acids.
- From the region of the methylene groups, we also obtained valuable information because the identification of the unsaturated acids of the mixture (C18: 2 and C18: 1 acid) was realized by the three signals isolated and separated from the methylene groups that are close to the double bonds.

### The autoxidation evaluation from three samples of argan oil fatty acid mixtures

#### Fatty acids quantitative determination of argan oil by $^1\text{H}$ NMR with $\text{Pr}(\text{tpip})_3$ (sample 1: FAs mixture from commercial argan oil) Fig. 5

The integrations of the different signals (Fig. 5) enabled us to estimate the percentage of saturated and unsaturated fatty acids in the FA mixture and that of each of the unsaturated acids. The intensity of the signals of the proton H3, H4, H5 or H6 is used as a reference since they correspond to the totality of the acids. Thus, the proportion of saturated acids is obtained by the ratio of the integration of the hydrogens H8s to those of the hydrogens H6s (protons with a clear integration).



**Figure 5.** Spectrum between (-1 and 2,4) of FAs mixture (from commercial argan oil) in  $\text{CDCl}_3$  solution with  $\text{Pr}(\text{tpip})_3$  at 500MHz

$$\% \text{ saturated acids} = \frac{0,456}{1,577} = 28,9 \%$$

$$\% \text{ insaturated Acids} = 100 - 28,9 = 71,1 \%$$

We can estimate the proportions of each of the unsaturated acids by the ratio of the integration of

their characteristic protons (H11 for C18: 1 and H11 or H14 for C18: 2) to that of H6 hydrogens.

$$\% \text{ C18: 1} = \frac{0,704}{1,577} = 44,7 \%$$

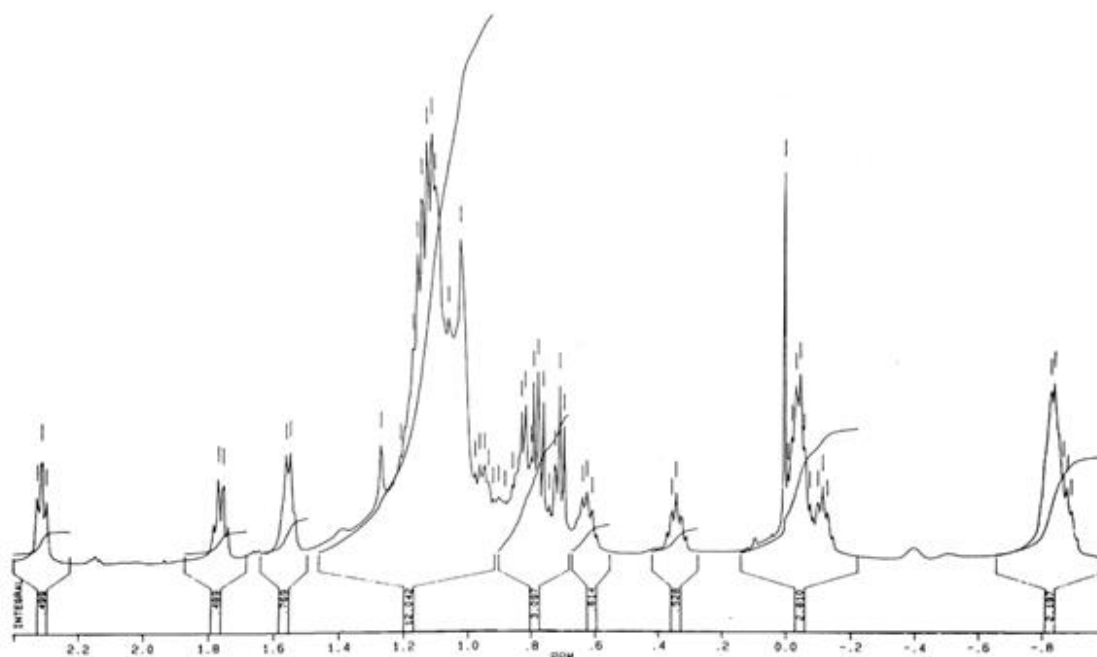


$$\% C18:2 = \frac{0,427}{1,577} = 27,1 \%$$

$$\% \text{ average } C18:2 = 29 \%$$

$$\% C18:2 = \frac{0,486}{1,577} = 30,8 \%$$

**Fatty acids quantitative determination of argan oil by  $^1\text{H}$  NMR with Pr (tpip) $_3$  (sample 2: FAs mixture 6 months after oil extraction) Fig.6**



**Figure 6.** Spectrum between (-1 and 2,4) of FAs mixture (6 months after oil extraction with hexane) in  $\text{CDCl}_3$  solution with Pr (tpip) $_3$  at 500MHz

Similarly, from the integrations of the different signals of the spectrum (Fig 6), we can estimate the

proportions of saturated and unsaturated acids and the proportions of each of the unsaturated acids.

$$\% \text{ saturated acids} = \frac{0,528}{2,197} = 24 \%$$

$$\% \text{ unsaturated Acids} = 100 - 24 = 76 \%$$

$$\% C18:1 = \frac{0,769}{2,197} = 35 \%$$

$$\% C18:2 = \frac{0,489}{2,197} = 22,25 \%$$

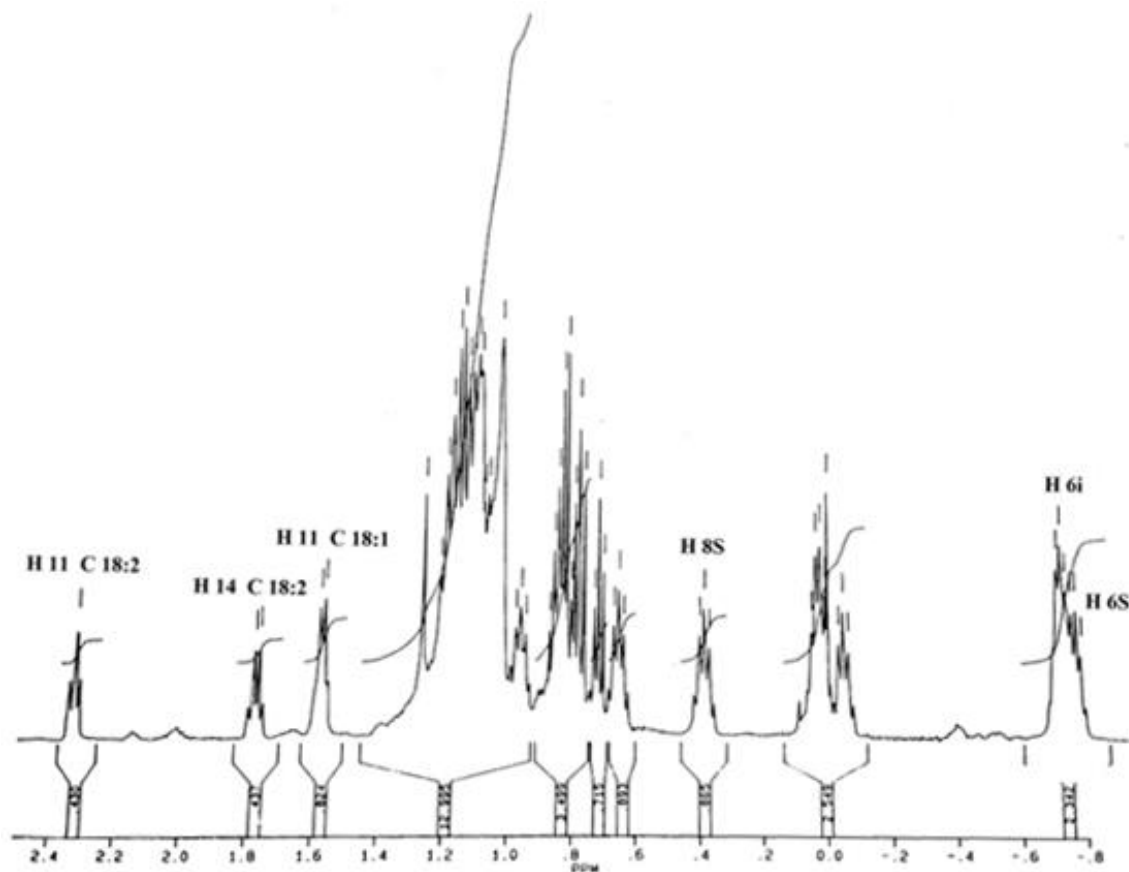
$$\% \text{ average } C18:2 = 22,5 \%$$

$$\% C18:2 = \frac{0,499}{2,197} = 22,7 \%$$

**Fatty acids quantitative determination of argan oil by  $^1\text{H}$  NMR with Pr (tpip) $_3$  (sample 3: FAs mixture 12 months after oil extraction) Fig. 7.**

proportions of saturated and unsaturated acids and the proportions of each of the unsaturated acids.

A third sample of FA mixture 12 months after extraction of oil (Fig. 7) allow us to estimate the



**Figure 7.** Spectrum between (-1 and 2,4) of FAs mixture (12 months after oil extraction with hexane) in CDCl<sub>3</sub> solution with Pr (tpip)<sub>3</sub> at 500MHz

$$\% \text{ saturated acids} = \frac{0,865}{2,342} = 36,9\%$$

$$\% \text{ insaturated Acids} = 100 - 36,9 = 63,1\%$$

$$\% \text{ C18:1} = \frac{0,824}{2,342} = 35 \%$$

$$\% \text{ C18:2} = \frac{0,437}{2,342} = 18,7 \%$$

$$\% \text{ average C18:2} = 18,5 \%$$

$$\% \text{ C18:2} = \frac{0,430}{2,342} = 18,4 \%$$

**Comparative study of the composition in the percentage of saturated and unsaturated acids of three argan oil fatty acid mixture analyzed by <sup>1</sup>H**

**NMR with Pr(tpip)<sub>3</sub> and five argan oil fatty acids mixture of the literature analyzed by GC.**

**Table II.** Composition in the percentage of saturated and unsaturated acids of three argan oil fatty acid mixtures analyzed by <sup>1</sup>H NMR with Pr (tpip)<sub>3</sub> and five argan oil fatty acid mixtures of the literature analyzed by GC.

Sample FAM % FA	1	2	3	4	5	6	7	8
	EAO 0 month this study	HO 6 months this study	HO 12 months this study	HO [1]	EAO [6,7]	EMPO 0 month [6,7]	EMPO 6 months [6,7]	EMPO 12months [6,7]
SFA	28,9	24	36,9	20,5	18,5	18,6	18,8	18,8
USFA	71	76	63,1	79,9	80,8	80,9	80,8	80,5
Oleic Acid C18:1	44,7	35	35	46,9	47,7	48,1	48,4	48,2
Linoleic Acid C18:2	29	22,7	18,5	31,6	32,9	32,5	32,1	32,1

SFA: Saturated Fatty Acids

USFA: Unsaturated Fatty Acids

EAO Edible Artisanal Oil

HO (oil extracted with hexane)

EMPO Edible Mechanical Press Oil

For sample (1,2,3) Analysis by <sup>1</sup>H NMR with Pr(tpip)<sub>3</sub>

For sample (4,5,6,7,8) Analysis by GC

Before beginning this comparative study, we specify that the samples analyzed, 1, 2 and 3, are mixtures of fatty acids (FAs) that were stored in airtight and light-tight bottles at 4°C, whereas the samples of the literature are FA mixtures that come from oils that are stored under these same conditions. The abundance of natural antioxidants (tocopherols and polyphenols) in the latter argan oils protected these FAs against oxidation throughout the storage of the oils <sup>2,3,6,7,23-25</sup>.

According to the results (Table II), we notice a difference of 10% between the composition of saturated and unsaturated acids of the artisanal edible oil (sample 1) compared to that of the artisanal edible oil of the literature (sample 5). This difference shows for sample 1 a degradation of the unsaturated acids in favor of the saturated acids, which can be explained by the mode of extraction and/or the time before analysis. This degradation is due to the autoxidation of these unsaturated acids. By comparing the samples (2 and 3) of FA mixtures stored 6 months and 12 months, we note a decrease in the percentages of unsaturated acids (76% to 63%) and an increase in saturated acids (24% to 36%). Throughout the storage and unlike the fatty acid mixture of the literature oils stored for 6 months and 12 months (Sample 7 and 8), the samples 2 and 3 did not have the natural antioxidants of argan oil.

It is also noted that the percentage of oleic acid remains unchanged (35%) because it is not easily autoxidized whereas that of linoleic acid decreases from 22.7% to 18.5% (slow autoxidation at 4°C). In fact, the more the double bonds the FAs contain, the more easily auto-oxidized they are <sup>26,27</sup>. These results are in line with the work of Howard who reported that the autoxidation speed of acid increases with its degree of unsaturation <sup>28</sup>. By comparing the

composition of the FAs of the oil extracted with hexane (sample 4) with that extracted by the mechanical press (sample 6), a small difference is observed.

We note that for the edible mechanical press oil (samples 6,7 and 8) a similar FAs composition for 0, 6 and 12 months of storage at 4°C. These oils, which come from roasted seeds and respect the right manufacturing and hygiene practices, can be conserved for a period reaching 24 months at 4°C <sup>6,7</sup>. We can conclude that the storage at 4°C for 6 months and 12 months of FA mixtures (sample 2 and 3) led to the autoxidation of unsaturated acids and especially the most unsaturated, in this case, linoleic acid. On the other hand, the storage of oils at 4°C for the same duration (samples 7 and 8) led to the same composition of FA. The latter is not affected by the storage thanks to the activity of natural antioxidants such as phospholipids and tocopherols that protect against the degradation of the fatty acids. Indeed, a molecule of tocopherol can protect 103 to 106 molecules of unsaturated FAs <sup>25</sup>.

In our case, we evaluated the degradation of the fatty acid mixture stored for a period of up to one year. We have noticed an incomplete autoxidation of unsaturated acids. It would have been necessary to evaluate this degradation for two years and to compare with the results of the literature to highlight the role of the natural antioxidants of argan oil. This work could also have been approached by using <sup>1</sup>H NMR in the presence of chelate for fatty acid mixtures from oils stored at 4°C for periods of up to 2 years in order to compare with the literature results of the Gas Chromatography.

## Second Part

### Analysis of the argan pulp fatty acids by Gas Chromatography (GC)

The GC analysis of the argan pulp fatty acid methyl esters lead to the chromatogram shown in Fig. 8.

Table III shows the composition as a percentage of the argan pulp FAs. In the fat part of the pulp, we have highlighted the preponderance of myristic and palmitic fatty acids (81% of the total FA) and the low unsaturated FAs content (less than 9%). In order to confirm these results, we, therefore, used proton NMR analysis.

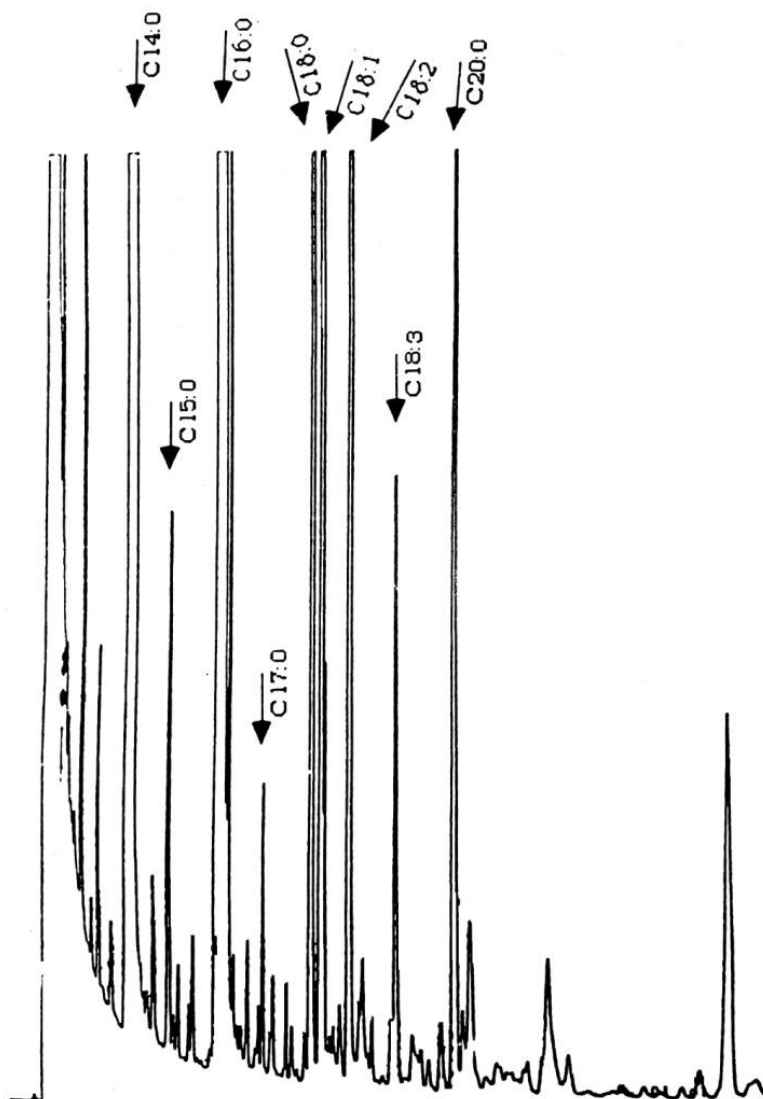


Figure 8. Chromatogram (GC) of fatty acids méthyl esters of argan fruit pulp

Table III. Fatty acids composition of the argan pulp lipid extract (% p).

Fatty Acids		% Fatty acids of the pulp in this study	Fellat-Zarrouk [16]	Z. Charrouf [6]	Retention time (mn) in this study
Myristic	C14:0	51,9	4,3	14-22	4.49
Palmitic	C16:0	31	18,4	27-31	7.01
Stearic	C18:0	2,5	6,3	6-7	11.00
Oleic	C18:1	3	42	15-18	11.43
Linoleic	C18:2	2,7	18,8	20-23	12.45
Linolenic	C18:3	0,4	1	-	14.10
Arachidic	C20:0	1,2	-	-	16.29
Other minority acids		7,3	1,5	-	-

The results obtained by GC (Table III) differ from those of the literature as Z. Charrouf <sup>6</sup> notes a similar content in saturated acids (myristic and palmitic) and unsaturated (oleic and linoleic), whereas Fellat-Zerrouck <sup>16</sup> notes that the oleic and linoleic acids constitute the major components.

These disparities could come partly from the substantial genetic variability of the argan tree but very probably from the various pulp manipulations (fruit collection, the preparation time of the raw material, different extraction stages ...). Rich in water and being the outer part of the argan fruit, the pulp is more fragile than the seed due to the degradation by biological and chemical agents and therefore more exposed to thermal degradation and oxidation. These results show that the pulp requires a different treatment than the one currently used in order to preserve the compounds to be extracted <sup>6,29</sup>.

#### Analysis of the argan pulp fatty acids mixture by <sup>1</sup>H NMR

The spectrum NMR <sup>1</sup>H of the FAs mixture in CDCl<sub>3</sub> solution (Fig. 9) is a characteristic spectrum of

saturated long-chain FAs mixture quite similar to that obtained for a mixture of acids (C 10: 0, C 12: 0, C 14: 0, C 16: 0, C 18: 0). The spectrum comprises, in addition to the undifferentiated resonance signal of all the methyls groups ( $\delta = 0.88\text{ppm}$ ), a signal ( $\delta = 2.35\text{ppm}$ ) due to the methylene groups protons in  $\alpha$  (position 2) relative to the carboxyl group, a signal at  $1.63\text{ppm}$  due to the methylene groups protons in  $\beta$  (position 3) relative the carboxyl group, and a signal close to  $1.29\text{ppm}$  assignable to the protons of all other methylenes starting from position 4 concerning the carboxylic acid function.

In the ethylenes protons area, only one signal of low intensity is observed at  $5.40\text{ppm}$ , confirming the results of GC analysis (low unsaturated FAs content), the spectrum does not give more information relating to the composition in unsaturated acids. For this reason, we use the method of analysis by <sup>1</sup>H NMR with the Pr(tpip)<sub>3</sub> as chemical shift reagent.

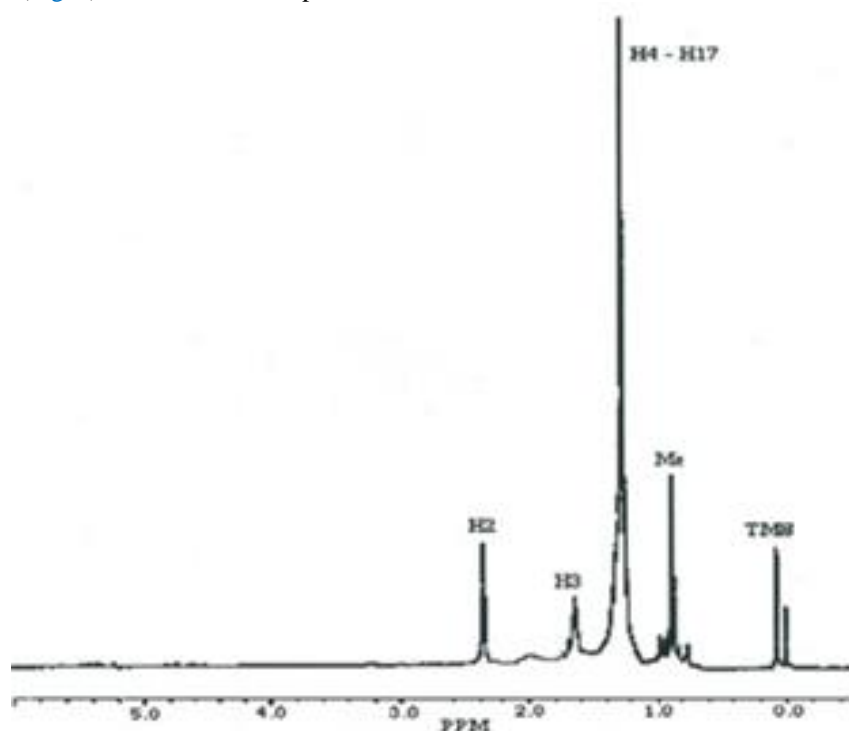
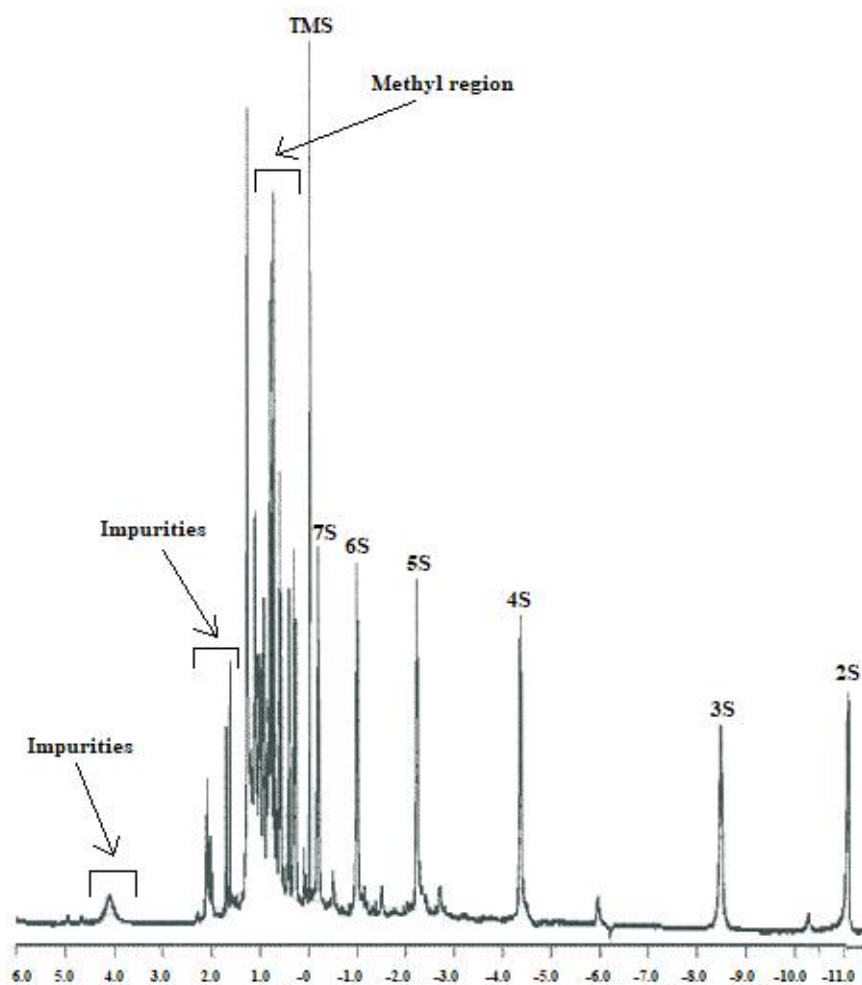


Figure 9. <sup>1</sup>H NMR spectrum of argan pulp's fatty acids mixture in CDCl<sub>3</sub> at 500MHZ

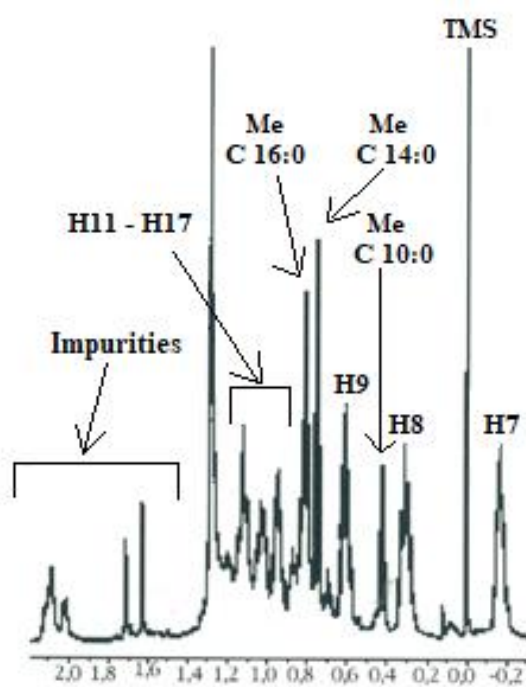
#### Analysis of the argan pulp FAs mixture by <sup>1</sup>H NMR with chelate Pr(tpip)<sub>3</sub>

The addition of Pr(tpip)<sub>3</sub> (molar ratio close to 1) to the argan pulp FA mixture (Fig.10 and Fig.11) shows signals almost all distinct from one another.

We note the chemical shifts of the different methylene protons from the <sup>1</sup>H NMR spectra of the argan pulp FAs mixture recorded in CDCl<sub>3</sub> solution (Fig. 9) and in the presence of the chelate Pr(tpip)<sub>3</sub> (Fig.10 and Fig.11).



**Figure 10.**  $^1\text{H}$  NMR spectrum of the argan pulp fatty acids mixture with the Pr (tpip) 3 at 500MHZ



**Figure 11.**  $^1\text{H}$  NMR spectrum between (-0,2 and 2,5) of the argan pulp FAs mixture with the Pr(tpip)3 at 500MHZ (reference added: C10 acid: 0)

These results in Table IV, allow the following interpretations:

-With chelate, the signals of methylene protons H4 to H13 initially overlapped, are separate.

-The signals of the methylene protons H14 to H17 are the only ones to remain relatively overlapped in the presence of the chelate. Indeed, they are the furthest away protons from the complexation site.

The signals of the methyl groups provide the most important information. Initially overlapped and resonant at  $\delta = 0.88$  ppm, these signals separate from one another during the addition of the chelate because they are unequally distant from the carboxyl group (positions 14, 16 and 18) and, thus allow the identification of the present acids.

**Table IV.** Chemical shifts of the fatty acids methylene groups without and with the chelate Pr(tpip)<sub>3</sub>.

Proton N°		$\delta$ (ppm)	
		without chelate	with chelate
C10 :0 C14 :0 C16 :0 C18 :0	H2	2,38	-10,36
	H3	1,73	-7,8
	H4	1,28	-4,01
	H5	1,28	-2,02
	H6	1,28	-8,4
	H7	1,28	-0,09
	H8	1,28	0,36
	H9	1,28	0,64
	H10	1,28	0,84
C14 :0 C16 :0 C18 :0	H11	1,28	0,95
	H12	1,28	1,03
	H13	1,28	1,13
C16 :0 C18 :0	H14	1,28	1,13
	H15	1,28	1,13
C18 :0	H16	1,28	1,13
	H17	1,28	1,13

The induced shifts of the signals are normalized concerning the induced shift of the saturated acid

methyl groups C10:0 and allow the analysis of the argan pulp FAs mixture. They are listed in Table V.

**Table V.** Induced chemical shifts of fatty acid methyl groups normalized concerning the induced shift of the saturated acid methyl groups C10:0.

Me	$\delta_0$ (ppm)	$\delta$ (ppm)	$\Delta\delta$ ( $\delta_0 - \delta$ )	$\Delta\delta / \Delta\delta_{C10}$
C 10	0,88	0,42	0,46	1
C 14	0,88	0,75	0,14	0,31
C 16	0,88	0,81	0,07	0,15
C 18	0,88	0,84	0,05	0,11

The study of the methyl groups region of the spectrum (Fig.10 and Fig.11) in comparison with the results of Table V, shows two FA major compounds in our mixture: C14:0 myristic acid ( $\delta = 0.75$  ppm) and C16: 0 palmitic acid ( $\delta = 0, 81$  ppm). The minority presence of C18: 0 acid at  $\delta = 0.84$  ppm is probable. Other signals of very low intensity that are barely visible on the spectrum and overlapping with impurities can be attributed to H11 protons of C18:1(1.6 ppm), and H11 (2,3 ppm) and H14 (1.75 ppm) of C18: 2.

The proton NMR analysis with Pr(tpip)<sub>3</sub> of the argan pulp FAs mixture allowed us to determine two dominant saturated acids (palmitic and myristic) and a minority presence of unsaturated acids. In this case, the overlap of the methylenes in position 10 and 11

with methyls of myristic acid C14: 0 and palmitic C16: 0 makes the dosage of the majority acids impossible.

## Conclusion

The use of Pr(tpip)<sub>3</sub> as a proton NMR chemical shift reagent allowed us to evaluate the autoxidation of FAs mixture from commercial argan oil produced artisanally by hand –press and FA mixtures from oil extracted with hexane. The FAs mixture from the hexane extraction stored at 4°C was analyzed after 6 months, and another part of the same mixture was analyzed after 12 months. The results of our analyzes showed a decrease in the percentage of unsaturated acids from 76% to 63% and an increase in saturated acids from 24% to 36%. The analysis of the same

mixtures showed the unchanged percentage of oleic acid (35%) because it is not easily oxidized, whereas that of linoleic acid decreased from 22.5% to 18.5%, which corresponds to a slow autoxidation at 4°C.

The comparison with the results of the literature shows that for argan oils stored at 4°C, fatty acid mixtures have benefited from a high content of natural antioxidants (tocopherols and polyphenols) allowing oils to be well preserved along this storage period, unlike our stored fatty acids mixture which did not contain these natural antioxidants.

Concerning the FAs mixture of the argan tree fruit pulp, <sup>1</sup>H NMR analysis in the presence of the Pr(tpip)<sub>3</sub> chelate shows the presence of two major fatty acids, myristic and palmitic acids. These results are in agreement with results we obtained by GC but differ from those of the literature. This can be due to the important genetic variability of the argan tree and more likely to the different pulp manipulations (fruit collection, the preparation time of the raw material, different extraction stages ...) Rich in water and being the outer part of the argan fruit, the pulp is more fragile than the seed due to the degradation by biological and chemical agents and therefore more exposed to thermal degradation and oxidation. The use of the reagent Pr(tpip)<sub>3</sub> proved effective for the evaluation of the argan oil unsaturated acids autoxidation and the argan pulp fatty acid <sup>1</sup>H NMR analysis (following our GC results). This method is simple, fast, and does not require a derivation. It can be considered as an efficient structural analysis tool for the FAs of a natural mixture.

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