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Quantification of Squalene in Olive Oil Using ^{13}C Nuclear Magnetic Resonance Spectroscopy

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Abstract: In the course of our ongoing work on the chemical characterization of Corsican olive oil, we have developed and validated a method for direct quantification of squalene using ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy without saponification, extraction, or fractionation of the investigated samples. Good accuracy, linearity, and precision of the measurements have been observed. The experimental procedure was applied to the quantification of squalene in 24 olive oil samples from Corsica. Squalene accounted for 0.35–0.83% of the whole composition.

Keywords: squalene; olive oil; ^{13}C NMR analysis; quantification; Corsica

1. Introduction

Squalene—(*E*)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene—is a natural acyclic symmetrical triterpene. It is a key intermediate in the biosynthesis of sterols [1]. In the human body, squalene is synthesized and then converted into cholesterol. In medicine, squalene plays a major role in the reduction of cancer risks, particularly with regard to cancer of the pancreas and colon in rodents [2–4]. Squalene increases the stability of various emulsions (vaccines, pharmaceutical formulations) [5,6]. It is also useful at the surface of the skin, playing the role of protective barrier against Ultra-Violet (UV) radiations [7]. Hydrogenated squalene (i.e., squalane) is appreciated in cosmetics as emollient agent in creams and capillary serums [8].

The largest source of squalene for industrial purposes is from animal origin, provided by various species of shark [9]. According to the species, squalene represents up to 80% of the shark liver oil [10]. Various species of shark are now endangered as a result of their overexploitation.

Squalene is also widespread in the vegetable kingdom. Indeed, it is present in oil seeds and in green vegetables [11]. In olive oil, squalene represents 0.3% to 0.7% of the whole mass, accounting for 60–75% of the unsaponifiable fraction [12]. The presence of squalene confers to olive oil a great stability against auto-oxidation and photo-oxidation [13].

The Association of Official Analytical Chemists [14] recommended a method for extraction of squalene from natural matrices. Analytical techniques used in quantification of squalene in edible oils, in the presence of acylglycerols, fatty acids, phytosterols, and tocopherols have been recently reviewed [15]. Methods using a preliminary fractionation of samples, procedure that simplifies the analysis have been developed. Analysis of squalene in edible oils is predominantly achieved by chromatographic techniques (Gas Chromatography (GC) or Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)), after saponification of triglycerides, solvent extraction of the unsaponifiable fraction, and eventually isolation of the hydrocarbon fraction by Column Chromatography (CC) or Thin Layer Chromatography (TLC) [16–21]. The direct injection of olive oil in the injector port has been applied [22], as well as HPLC coupled with GC [23] or HPLC coupled to electrospray tandem mass spectrometry [24].

In parallel, ^{1}H and ^{13}C NMR have been widely used for identification and quantitative evaluation of triglycerides in olive oil (saturated fatty acid chains, mono-unsaturated, poly-unsaturated, stereochemistry of the double bonds, etc.) and for quality assessment and authentication [25–27]. Using the fingerprint technique, characteristic resonances of individual components of the unsaponifiable fraction (sterols, alcohols, tocopherol) have been identified using ^{1}H NMR, and the results allowed the determination of geographical origin of olive oil [28]. Similarly, the ratio of squalene vs. the other minor components of olive oil has been evaluated, and statistical analysis of the results gave useful information on the quality, authenticity, and origin of the investigated olive oil samples [25,29]. The content of squalene in human sebum (containing low proportion of triglycerides) has been measured using a 600 MHz spectrometer equipped with a cryoprobe [30]. Otherwise, quantitative analyses of two structurally close triterpenoid acids, as well as that of positional and geometric isomers of octadecadienoic acid with conjugated double bonds, have been performed using 2D NMR [31,32].

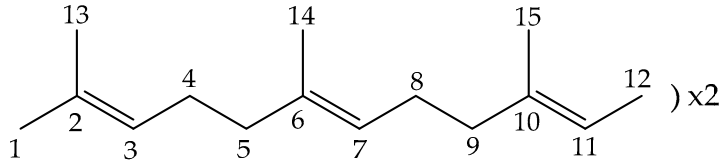
In previous works carried out in our laboratory, we demonstrated ^{13}C NMR spectroscopy was a powerful tool for the identification and quantitative determination of terpenes in natural matrices, mono and sesquiterpenes in essential oils [33] and fixed oil [34], diterpenes in cedar resins [35], triterpenes in solvent extracts from cork [36], or leaves from olive tree [37]. Taking into account that chromatographic techniques used to quantify squalene in olive oil needed laborious and time-consuming fractionation steps, the aim of the present study was to develop a method, based on ^{13}C NMR, and using a routine spectrometer (9.4 Tesla), that allowed the quantitative determination of squalene in olive oil, avoiding the fractionation steps.

2. Results and Discussion

2.1. ^{13}C NMR Data of Squalene and Olive Oil

The ^{13}C NMR spectrum of squalene displayed 15 resonances belonging to quaternary carbons (135.11; 134.90 and 131.26 ppm), ethylenic methines (124.42; 124.32 and 124.28 ppm), allylic methylenes (39.77; 39.74, 28.29; 26.78; 26.67 ppm) and methyl groups (25.71; 17.69; 16.05 and 16.01 ppm). The chemical shift values of our recorded spectrum (Table 1) fitted perfectly with previous data reported by Pogliani et al. [38]. However, it could be noted a difference of 1 ppm for carbon C3, probably due to a misprint in the paper [38].

Table 1. Structure, ^{13}C NMR chemical shifts, and longitudinal relaxation times (T_1) of carbons of squalene.



C	δ (SQ)	T_1	C	δ (SQ)	T_1
1	25.71	1.9	9	39.77 *	0.7
2	131.26	10.0	10	135.11	5.2
3	124.42	2.5	11	124.32 #	1.7
4	26.78	0.9	12	28.29	0.4
5	39.74 *	0.7	13	17.69	4.5
6	134.90	4.1	14	16.05 †	2.8
7	124.28 #	1.2	15	16.01 †	3.3
8	26.67	0.6			

δ (SQ): Chemical shift of carbons of squalene (ppm vs. tetramethylsilane (TMS)). Assignment has been done according to Pogliani et al. [38]. *, # and †: chemical shifts may be inversed. T_1: longitudinal relaxation times in s.

The ^{13}C NMR spectrum of a commercially available olive oil is more complex (Figure 1). Four parts may be distinguished: 172–174 ppm, esters; 124–134 ppm, ethylenic carbons; 60–72 ppm, carbons of glycerol; and 13–35 ppm, aliphatic carbons. In that spectrum, all the resonances with high intensity belong to the triglycerides. Twelve out of 15 resonances of squalene were observed. They were perfectly resolved, and therefore they could be used for quantitative determination of squalene in olive oil.

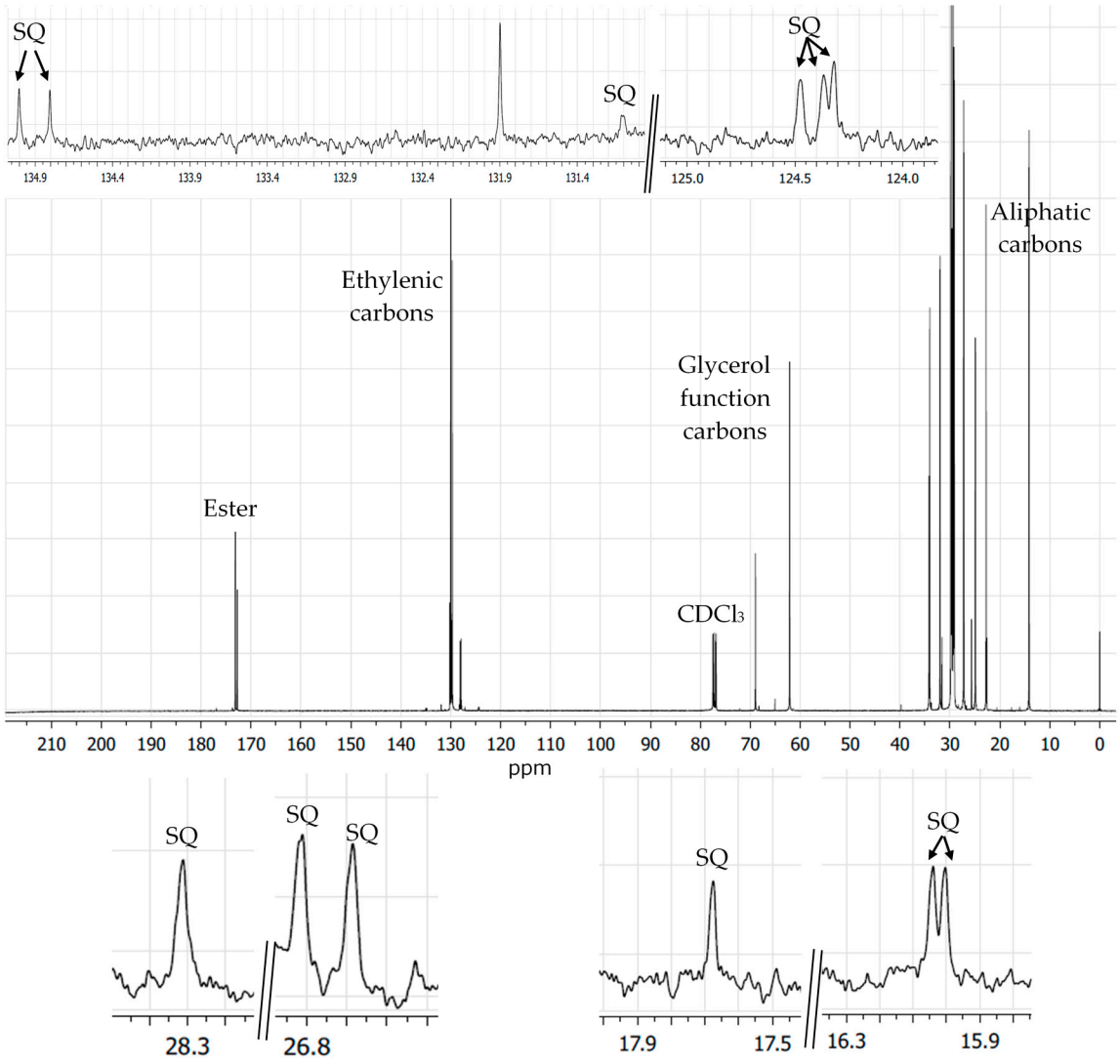


Figure 1. ^{13}C NMR spectrum of a commercially available virgin olive oil. SQ: squalene.

2.2. Validation of the Experimental Procedure for Quantitative Determination of Squalene Using ^{13}C NMR

In order to approach the physico-chemical properties of olive oil (viscosity for instance) the experiments for validation of the experimental procedure have been carried out using know quantities of squalene in trioleine (glyceryl tris octadec-9-enoate) that is the major triglyceride of olive oil accounting for 48–62% of the whole composition [39].

Several techniques have been developed for quantification of individual components of a natural mixture based on ^{13}C NMR spectroscopy. The standard sequence combines a 90° pulse angle, gated decoupling technique and requires waiting a period of $5T_1$ of the longest T_1 value, before applying another pulse. This sequence provides accurate result but is really time consuming. Otherwise, use of a paramagnetic relaxation reagent allows decrease of experimental time but induces a line width broadening. Quantitative determination can be led using a rapid train of short pulses

because a small flip angle provides less difference in the steady-state magnetization than a larger one in the presence of carbons having different T_1 values.

Owing to our experience in the analysis of complex natural mixtures containing nuclei with different T_1 values, a good approach is a compromise between the aforementioned procedures. For instance, quantification of various compounds has been performed in our laboratories, using this approach: carbohydrates in ethanol extract of *Pinus* species [40], triterpenes in cork extract [36] and olive leaf extract [37], and taxanes in leaf extract of *Taxus baccata* [41]. Quantitative determination of a component in a natural mixture is achieved by internal standardization by comparison of the areas of the resonances of that compound with those of an internal standard. In these conditions, it is obvious that quantitative estimation will be led from not fully relaxed spectra and that validation of the method should be performed before applying it to the analysis of mixtures [42]. The best conditions for the pulse sequence are those that reduce as far as possible the difference in the steady-state magnetization of nuclei with different T_1 values and that simultaneously allow a good S/N ratio in a short period of time. They could be selected using Becker's equation that allows the calculation of the S/N ratio as a function of the pulse angle and the ratio of longitudinal relaxation time to total recycling time [43].

Then, the theoretical parameters (precision, accuracy, linearity of measures) should be validated using pure squalene in trioleine before application of the method to the quantification of squalene in genuine olive oils. To carry out the validation of the method:

- $CDCl_3$ has been conserved as solvent and trioleine has been used as a model for olive oil;
- Longitudinal relaxation times have been measured for carbons of squalene by the inversion-recovery method. They ranged from 0.4 to 10.0 s, the highest values (4.1–10.0 s) being measured, as expected, for quaternary carbons (Table 1). T_1 values of vinylic methines and allylic methylenes ranged from 1.2 s to 2.5 s and from 0.4 s to 0.9 s, respectively. Finally, T_1s of the four methyl groups ranged from 1.9 s to 4.5 s. Quantitative analysis has been conducted with resonances of carbons not overlapped, perfectly resolved and with T_1 values comprised from 0.7 s to 4.5 s;
- Di-2-methoxyethyl oxide (diglyme) has been chosen as internal standard (T_1 value of its methylenes = 3.8 s) since its resonances do not overlap with those of triglycerides contained in olive oil.

The parameters of the pulse sequence have been determined using formula (1) for various T_1 values (0.7–4.5 s), and for a repetition delay of 3.7 s (acquisition time = 2.7 s; relaxation delay = 1.0 s) required for a 128 K data table. According to Becker et al. [43], we determined and plotted the percentage of recovered signal, expressed as S/N (%), as a function of the pulse angle α, using formula (1). Using a pulse angle of 30°, this procedure provided a small difference (3.6%) in the steady-state magnetization between carbons exhibiting different T_1 values and a reasonable time of analysis in spite of the utilization of a medium field spectrometer (3000 scans in less than 3 h) (Figure 2).

$$\frac{S}{N} = \frac{M_0 \times [1 - e^{(-D/T_1)}] \times \sin \alpha}{\sqrt{D} \times [1 - e^{(-D/T_1)} \cos \alpha]} \quad (1)$$

S/N: signal-to-noise ratio, M_0: initial magnetization, D: time between two pulses (in seconds), T_1: longitudinal relaxation time (in seconds), and α: pulse angle (in degrees).

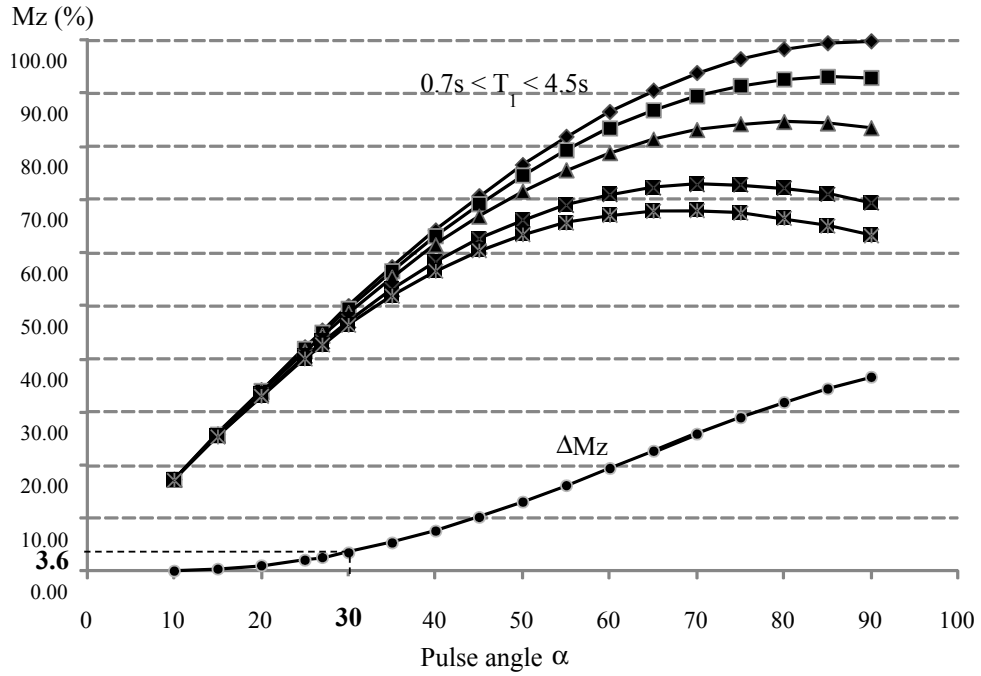


Figure 2. S/N (%) vs. flip angle α, plotted from formula (1) according to Becker et al. [43], for selected values of T_1 (0.7 s< T_1 < 4.5 s) and a minimum total recycling time τ of 3.7 s using a 128 K data table (acquisition time = 2.7 s and relaxation delay = 1.0 s).

Accuracy, precision and response linearity of this method have been validated by various experiments carried out on pure squalene by comparing the weighted quantities (0.37–1.66 mg) with those measured by NMR. From the ^{13}C NMR spectrum, the mass of squalene m_{SQ} (mg) was calculated using Formula (2). Relative errors between weighted and calculated masses are comprised between 0.0% and 10.3%, and therefore they demonstrated good accuracy of measurements (Table 2).

$$mSQ = 2 \times \frac{A_{SQ} \times M_{SQ} \times m_D}{A_D \times M_D} \times p_{SQ} \times p_D \quad (2)$$

The area A_{SQ} taken into account was the mean value of the areas of selected protonated carbons. A_D is the mean value of the areas of the two methylenes of diglyme. M_{SQ} is the molecular weight of squalene. M_D is the molecular weight of diglyme and m_D is the amount of diglyme. p_{SQ} and p_D: purity of squalene and of diglyme, respectively.

Table 2. Quantitative determination of squalene by ^{13}C NMR spectroscopy using diglyme as internal reference.

A_D	0.9901	0.9955	0.9959	1.0074	1.0011	0.9845	1.0168	1.0139
A_{SQ}	0.0734	0.1220	0.1668	0.1970	0.2253	0.2689	0.3129	0.3431
m_w (mg)	0.37	0.55	0.74	0.92	1.10	1.29	1.47	1.66
m_c (mg)	0.33	0.56	0.74	1.00	1.03	1.22	1.44	1.58
ER (%)	10.3	−0.7	0.0	−9.1	6.8	5.5	2.2	4.8

A_D and A_{SQ}: Mean areas of selected carbons of diglyme and squalene, respectively; Mass of diglyme (m_D): 1.49 mg; m_w: weighted mass of squalene (mg); m_c: calculated mass of squalene (mg) using formula (2); ER: relative error (%) between m_c and m_w; Molecular weight of squalene: 410.7 g·mol^{-1}.

Then, we drew the calibration line for the quantification of squalene. The straight line was plotted by expressing the ratio of the mean value of areas of the resonances of squalene selected carbons (A_{SQ})

with those of diglyme (A_D) as a function of the weighed mass of squalene (m_w). We observed a good linearity of the measurements because the linear determination factor (R^2) is 0.996 (Figure 3).

Finally, the spectrum of the sample containing 0.55 mg of squalene has been recorded five times. The repeatability, calculated with a confidence interval of 99% (Student's *t*-test) was equal to 0.56 mg ± 0.04 mg, i.e., 0.56 mg ± 6.8% which indicates a good precision of measurements.

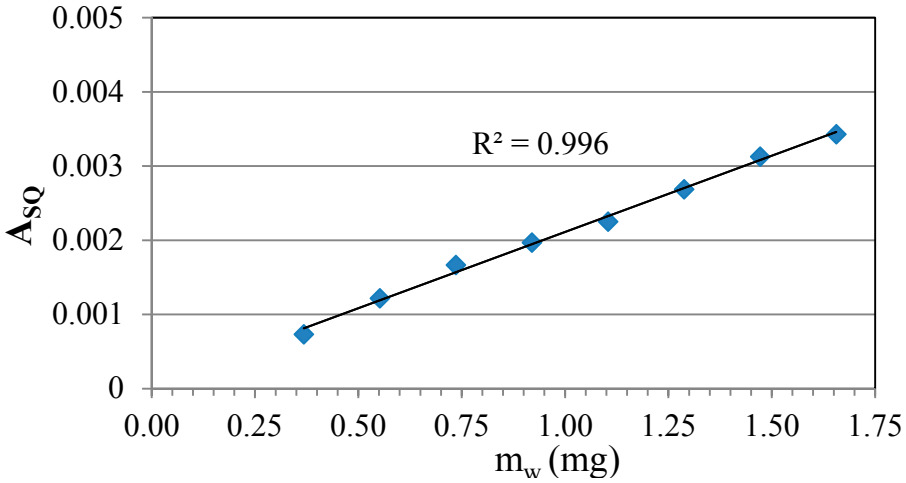


Figure 3. Calibration line of squalene. m_w = weighted mass of squalene.

The experimental procedure developed to quantify squalene in triolein exhibited good accuracy, precision and linearity of measurements. Analysis time with a routine spectrometer (9.4 Tesla) is not prohibitive since a single analysis requires three hours. Therefore, this procedure could be applied for quantification of squalene in olive oils of Corsican origin.

2.3. Quantification of Squalene in Various Olive Oil Samples of Corsican Origin

Twenty-four olive oil samples from various localities in Corsica and from various olive varieties have been analyzed using ^{13}C NMR, according to the experimental procedure previously described. In the ^{13}C NMR spectrum of olive oil (Figure 2), eight out of 12 of the protonated carbons of squalene were observed. All of these resonances were perfectly resolved and did not overlap with resonances of other components of olive oil, and their relaxation times were between 0.7 s and 4.5 s. The mass of squalene in every olive oil sample has been calculated using Formula (2), taking into account the mean areas of these resonances. Then, the mass percentages of squalene have been calculated using Formula (3), which are reported in the Table 3.

$$\%C = \frac{m_{SQ}}{m} \times 100 \quad (3)$$

%C: percentage of squalene; m_{SQ}: calculated mass (mg) of squalene; m: mass of the olive oil sample.

Table 3. Quantification of squalene in olive oils from Corsica using ^{13}C NMR.

Sample	Olive Variety	Squalene (%)*
1	Zinzala	0.35
2		0.37
3		0.41
4	Sabine	0.35
5		0.35
6		0.40
7		0.42
8	Picholine	0.38
9	Germaine	0.40
10		0.43
11		0.44
12		0.49
13		0.51
14		0.51
15		0.83
16	Cortenaise	0.42
17		0.47
18	Capannacce	0.52
19	Germaine/Picholine	0.37
20	Germaine/Capanacce	0.44
21		0.67
22	Germaine/Sabine	0.47
23		0.49
24	Sabine/Picholine	0.46

*: percentages calculated using formula (3).

Among the 24 olive oil samples, 18 samples were obtained from olive of a single variety, the last six samples coming from olives of two varieties. From Table 3, it is observed that Corsican olive oils contained appreciable amount of squalene comprised between 0.35% and 0.52% for 22 samples out of 24. The two last samples exhibited higher contents (0.67% and 0.83%). These results are in agreement with those reported in the literature (0.3–0.7%) [12].

Although the number of samples from every locality and from every olive variety is limited, it seems that there is no direct relation between the content of squalene in a given olive oil sample and the variety of the olive. However, it could be observed that zinzala, sabine, and picholine olives produced an oil containing 0.35–0.42% of squalene. The olive oil from Germaine, Cortenaise and Capanacce varieties exhibited a slightly higher content of squalene (0.40–0.83%). Finally, olive oil coming from two varieties of fruits contained 0.37–0.67% of squalene.

3. Materials and Methods

3.1. Chemicals

Squalene, triolein and di-2-methoxyethyloxide (diglyme) were obtained from Sigma-Aldrich (St-Louis, MO, USA), Acros Organics (Geel, Belgium), and Jansen Chimica (Geel, Belgium), respectively. Olive oil samples were supplied by Mrs. Henneman (Chambre d'Agriculture de la Haute Corse, Bastia, Corsica, France).

3.2. NMR Experiments

3.2.1. Quantitative ^{13}C NMR Spectra

Quantitative ^{13}C NMR spectra were recorded on a Bruker (Wissembourg, France) AVANCE 400 Fourier Transform spectrometer operating at 100.13 MHz for ^{13}C, equipped with a 5 mm probe,

in $CDCl_3$ with all shifts referred to internal TMS. ^{13}C NMR spectra were recorded with the following parameters: inverse gated decoupling, flip angle 30°, acquisition time = 2.7 s for 128 K data table with a spectral width of 24,000 Hz (240 ppm), a relaxation delay D_1 = 1.0 s, composite pulse decoupling of the proton band, and a digital resolution of 0.366 Hz/pt. The internal reference used was diglyme. The number of accumulated scans was 3000 for each sample. Exponential line broadening multiplication (1 Hz) of the free induction decay was applied before Fourier transformation.

3.2.2. T_1 Measurements

The longitudinal relaxation times of the ^{13}C nuclei (T_1 values) were determined by the inversion-recovery method, using the standard sequence: 180°–τ–90°–D_1, with an acquisition time of 0.68 s (for 32 K data table with a spectral width of 25,000 Hz) and a relaxation delay D_1 of 20 s. Each delay of inversion (τ) was thus taken into account for the computation of the corresponding T_1 using the function $I_p = I_0 + pe^{-\tau/T_1}$ (Bruker microprogram; I_p and I_0 are populations of nuclear spins; p is a constant of integration).

3.2.3. Calibration Line

A weighted amount of 0.37–1.66 mg of squalene was diluted in 0.5 mL of $CDCl_3$ containing 1.49 mg of diglyme.

3.2.4. Quantification of Squalene in Olive Oils

A weighted amount of 140–150 mg of olive oil was diluted in 0.5 ml of $CDCl_3$ containing 1.53 mg of diglyme.

4. Conclusions

An experimental procedure, based on ^{13}C NMR spectroscopic analysis, was developed and allowed for the quantification of squalene in olive oil samples. An optimized pulse sequence (flip angle $\alpha = 30°$, inverse gated decoupling, total recycling time 3.7 s) was checked and led to reliable quantitative determination of squalene in olive oil samples from Corsica with an analysis time of less than three hours using a medium field NMR spectrometer (9.4 T). In the 24 olive oil samples investigated, squalene accounted for 0.35–0.83% of the whole composition.

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Author Contributions: A.B., F.T. and J.C. conceived and designed the experiments; A.-M.N. and M.P. performed the experiments; A.B. and F.T. analyzed the data; J.C. and M.P. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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