Research Article

Quick unreferenced NMR quantification of Squalene in vegetable oils†

Running Title: ^{1}H-NMR quantification of squalene in oils without reference

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Abstract

We present here a simple method for the rapid quantification of squalene in vegetable oils by NMR (Nuclear Magnetic Resonance). The method was validated by adding internal standards to several vegetable oil samples. Quantification was accomplished by exploiting the characteristic resolved signal of terminal methyl groups at 1.67 ppm, which allowed squalene quantification regardless of the hydrophobic matrix. Theoretical principles are fulfilled by the method and, despite the general belief that NMR displays intrinsic low sensitivity, acceptable accuracy (<4%) and reproducibility (<6%) can be reached when squalene is over 2000 ppm, even in "worst case scenarios". This method may be useful in the continuing efforts to rapidly generate accurate and complete quantitative data suitable for inclusion in the identification labels of vegetable oil products.

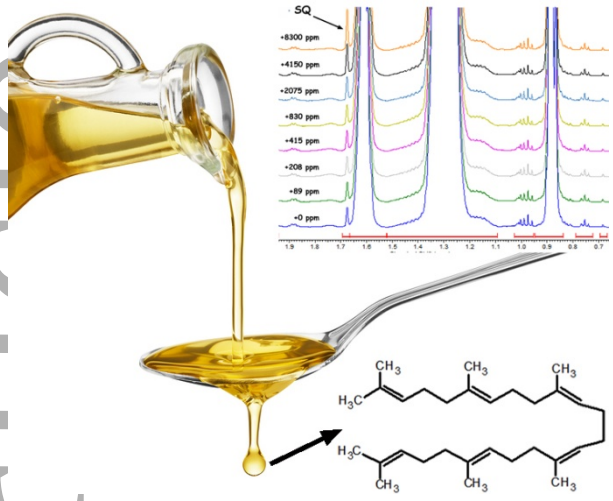
Practical applications

Unreferenced and accurate quantification of squalene in vegetable oils, was validated here by the analysis of several ^{1}H-NMR spectra of vegetable oils and of standard doped samples. Moreover, we found that squalene can affect the NMR spectra of other quantifiable species. These data may be useful to produce more accurate and precise analyses of vegetable oil using their 1H-NMR profiles.

Keywords: ^{1}H NMR, vegetable oils, Squalene, quantitative NMR, NMR unreferenced accuracy

Abbreviations: NMR, Nuclear Magnetic Resonance**, SQ,** squalene**, TG,** triacyl-glycerol**, DG,** diacyl-glycerol**, TOCSY,** total correlation spectroscopy**, TMS,** Tetra-Methyl-Silane**, EVOO,** Extra-virgin olive oil**, ARO,** peanut oil**, M_x,** molecular mass of compound X**, I_x,** signal integration relative to the compound X**, LOD,** limit of detection**, LOQ,** limit of quantification**, CV% or RSD,** percentage of coefficient of variation or relative standard deviation

Graphical abstract



Collectively, our data indicate that quantification of squalene in vegetable oils can be easily accomplished by ^{1}H-NMR, opening new potentialities in NMR-based fat analysis. The ability of squalene to alter NMR spectra should be taken into account, particularly in oils with a high squalene content, such as Sicilian olive oils.

1 Introduction

Squalene (SQ) is an unsaturated hydrocarbon, first found in the unsaponifiable fraction of shark liver oil by Dr Mizumaru Tsujimoto [1], the scientist who purified it for the first time and assigned to it the molecular formula $C_{30}H_{50}$. The compound was named squalene as sharks (*Squalidae* family) were the richest natural source of this substance [2]. SQ is a sesqui-terpene bearing six double bonds [3,4], and it is ubiquitous in both animal [5] and vegetable kingdoms [6].

SQ is attracting great biological interest, as it is the third main component of skin surface lipids (SSL). SSL generate a film able to protect cells from the external environment [7,8], particularly from photo-oxidation [9, 10, 11], which is related to skin cancer induction [12]. Despite the biological importance of SQ was suspected long ago, its cosmetic and pharmaceutical use has developed more recently after description of its protective effect on the *cutis* oxidative stress [13]. SQ and its saturate analogue, squalane, are easily administrable at the topical level [14]. As an important intermediate in endogenous cholesterol synthesis, it was feared that diets with high SQ-content could induce high cholesterol blood levels and therefore increase the risk of cardio-vascular diseases [10]. Conversely, other studies [11] demonstrated that SQ produces beneficial effects by reducing cholesterol and triglyceride serum levels and protecting against a variety of cancers [15]. Such a view is confirmed by several tests on animals [16, 17] and *in vitro* experiments [18]. Moreover, SQ is a potent vaccine adjuvant which is useful for influenza immuno-prophylaxis and cancer immunotherapy [19]. For this reason, dietary consumption of at least 30 mg of SQ per day is presently recommended and medical ointments containing SQ are popular all over the world. It has been suggested that high-level consumption of extra-virgin olive oil (EVOO) in Mediterranean regions increases SQ intake to up to 500 mg/day and that this might explain the low incidence of certain cancers in the Mediterranean populations [20, 21, 22].

Usually, SQ in foods, oils and fats is quantified through chromatographic and titrimetric procedures involving sophisticated [23], but also tedious and time consuming, steps [24, 25, 26]. In this scenario it would be useful to take advantage of the great relevance gained by NMR within the field of food analytical chemistry [27] for the following reasons: a) minimal chemical treatment; b) quick acquisition of a great amount of data; c) constant replication of any possible experimental error [28]. It should be pointed out that, in principle, nuclear magnetic detection is rigorously proportional to the number of spins [29]. Therefore it should be possible to quantify

substances either without any standard reference (i.e. by absolute intensity) or by using a nonspecific reference (this is often the case in food analysis). Robustness of NMR quantification is well documented [30] and our group was able to use this tool for many minor components of the extra-virgin olive oil [31-33]. Based on this scientific background, in the present paper we present an effective NMR method to quantify SQ in oil matter. Two different vegetable oils have been selected (olive oil and peanut oil) with the aim to appreciate the robustness of the method and to evaluate a reliable limit of detection in real matrix.

2 Materials and methods

2.1 Sample Preparation

All the oil samples were dissolved in $CDCl_3$ with traces of TMS standard reference. As recommended in many oil analyses [34], we have kept the oil to $CDCl_3$ weight ratio equal to 13.5/86.5; this corresponds to mixing 122 μL of oil and 478 μL of deuterated chloroform into a 5 mm test-tube for NMR.

2.2 NMR Analysis

All the samples were analyzed at the same temperature (T = 25 °C) by a 500 MHz Avance III NMR spectrometer equipped with an inverse probe with gradients (SMARTprobe). After the automatic tuning (atma) and shimming (topshim), lineshape of the TMS signal was optimized by some iterative manual or automatic shimming until line-shape was lower than 1.5 Hz. 90° hard pulse was calibrated most of the times being always 8.4±0.1 μs. ^{1}H-NMR experiments were run with a spectral width of 12 ppm, with 64 scans, 3.9 s of acquisition time (corresponding to 24K of recorded data points) and 3 s of time delay. By integration of several signals, using several ns (number of scans), it is possible to figure out that the stationary state (constant integration ratios) is fully reached after 16 scans. Differences among the relaxation times of the signals have to be considered as a possible source of error, however the relative integration of 1.67 ppm SQ signal in these experiments (recycling delay around 7 sec) showed limited deviations respect to the same experiment run with recycling delay over 15 sec (much more than 5 times the T1 of all the integrated resonances), it can be considered within the experimental intra-sample repeatability deviations. We would like to point out that the inversion recovery experiment states that the reference signal (2.33 ppm) shows T1 = 0.80(3) sec, whereas the squalene signal (1.77 ppm) shows T1 = 1.58(3) sec; this would require the recycling delay of 8 sec which is almost respected. Again we want to stress that the spectral profiles with several recycling delays up to 15sec do not change but for the $CHCl_3$ residual signal (T1 > 5 sec, see tables S3 and S4 and section 4 of the supporting information material).

2.3 NMR Processing and data treatment

All the spectra were processed by ACDLab/NMR with multiple fid treatment to keep all the spectra consistently aligned and processed. All the spectra were automatically phased and baseline was corrected with the fid reconstruction method. Signals were referenced to TMS frequency (δ=0.0 ppm) and simultaneously integrated for many NMR indicative bands (Table S1 and Fig. S1, S2 in supplementary data). For the SQ specific study we have run also the DPFGSE-TOCSY 1D spectra (TOCSY1D) with excitation at 1.68 ppm with the “seduce” shaped pulse width of 87msec (power level of 0.5mW). Quantification of SQ was performed by standard addition method.

3 Results

3.1 Signal assignment and experimental procedures

The ^{1}H NMR spectra of the two vegetable oils considered in the present study show triglyceridic (TGs) and diglyceridic (DG) signals of many common fatty acids [33, 35] as shown in Fig. 1 (grey line). ^{1}H NMR spectrum of the standard SQ molecule shows six specific NMR resonances due to the six different chemical groups (Fig.1, black line). Chemical shifts of SQ signals are not sensitive to the oil matrix, remaining unaffected when SQ is contained in vegetable oils (Fig. 1).

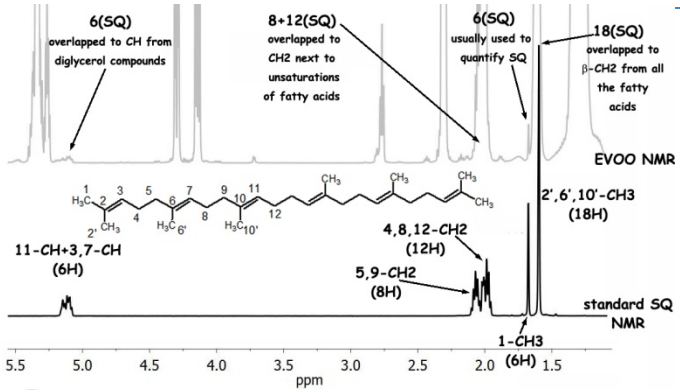


Figure 1. Bottom black line: ^{1}H-NMR profile of the pure standard squalene (SQ) in $CDCl_3$ along with the SQ molecular structure complete peak assignment; top gray trace is the comparison to the analogous spectrum of the whole EVOO (Tonda Iblea 2014) shows position and overlap of the ^{1}H internal SQ resonances

This is confirmed by the internal standard addition experiments and also by the selective DPFGSE TOCSY spectra exciting the 1.67 ppm signals of EVOO doped samples. All these preliminary considerations suggest that: a) the bare 1.67 ppm signal is the main SQ "marker" as it never looks overlapped to other known NMR oil signals; b) the other signals in EVOO are overlapped with some chemically similar groups belonging to glyceridic or fatty acid moieties, therefore cannot be directly related to the SQ content. On the other side, we find that these signal integrations are affected by the SQ content despite this has not been held into consideration so far [36]. We have to say, though, that indifferent fat matrices, other SQ signals are in some way mentioned and also used for quantitative purposes [37, 38]. Another outstanding question concerns the possible precision of the NMR experiments which also relays to the meaning of the many NMR data matrices often used for statistical treatments and evaluations.

3.2 Theoretical quantification of SQ

Quantification of SQ in EVOO is rationalized as follows. NMR signals are proportional to moles of protons (N°H) of the different chemical groups and to the number of molecules; consequently weight quantifications are inversely proportional to formula weights. Since triacyl derivates of fatty acids (TGs) are the main constituents of EVOO (more than 98%), theoretically it is possible to quantify any compound in EVOO with a neat non-overlapped signal [39] Thus, the ratio between the mass of the component X and the mass of TGs is related to the integral area of the α-C***H***$_2$ signal of TGs at 2.30 ppm and the integral area of the component X Eq (1):

$$\frac{g_X}{g} = \frac{I_X M_X N°H_{ref}}{I_{ref} M_{ref} N°H_X} \qquad (1)$$

where I_X and I_{ref} are the integral values of the signals representing compound X and TGs, respectively. Accordingly, M_X and M_{ref} are the molecular weight of compound X and TGs, and $N°H_x$ and $N°H_{ref}$ are the number of hydrogen atoms arising the signal of the compound X and of the α-C***H***$_2$-TGs, respectively.

By choosing 10000 as an arbitrary integral reference value for α-CH_2-TGs integration, equation 1 can be rewritten as:

$$\frac{mg}{g} = \frac{I_x M_x * 6}{10000 * 885 * N°H_x} \cdot 1000 \qquad (2)$$

As it is known [40], since the isolated 1.67 ppm signal comes from the terminal E-oriented methyl groups of SQ (6 protons), we can rewrite equation 2 for the SQ quantification as

$$\frac{mg_{SQ}}{g_{EVOO}} = \frac{I_{SQ} * 410.7 * 6}{10000 * 885 * 6} \cdot 1000 \qquad (3)$$

As it was noticed by careful reviewers, the choice of the triacylglycerol MW is a potential source of error. Actually, the chosen 885 value refers to the tri-oleyl-glycerol MW which is the main compound in both used oils. Here we would anyway like to note that all vegetable oils keep the average number of C atoms for the fatty acid very close to 18. For example the average molecular weight for EVOO is around 880. Using this latter number, instead of 885, might slightly improve the accuracy of the proposed method, since this number is slightly closer to the average vegetable oil molecular weight in many oil types. The maximum estimated error is below 1.4% for EVOOs or similar oils but can be up to 5% for other vegetable oils (changes of 45 in average MW are really extreme).

3.3 Experimental data:

Small known aliquots of standard SQ are added to extra-virgin olive oil (EVOO) and peanut oil (ARO) samples in order to obtain the so called

oil mother solutions doped by around 8000 ppm of SQ. By diluting these new samples with the original fresh oils (1:1 any time) it was possible to have the sample with around 250, 500, 1000, 2000, and 4000 ppm SQ doping (daughter solutions).

Provided that EVOO contains about 5000 ppm of SQ [41] and peanut oil (ARO) contains about 100 ppm of SQ [42, 43], we developed two calibration lines by standard addition method using EVOO and ARO as starting oil matrices (Fig. 2). NMR analysis of neat and SQ doped EVOO, provided the SQ quantification and evaluation according to equation (3);this led to the SQ quantification (7500 ppm) in EVOO *Tonda Iblea 2014* which is also consistent with the intersection of the y axis and the calibration line build up using the measured SQ for the doped EVOO samples. On the other hand, an analogous procedure was performed for ARO, since ARO originally contains around 100 ppm of SQ [42, 43], barely detectable by NMR. It is possible to consider that the SQ signal is due just to the standard SQ doping, even though within a vegetable oil matrix. After all, this is confirmed by the calibration line whose intersection with the y axis is reasonably around 0 (Fig. 2). This last analysis sets the limit of quantification (LOQ) around 2000 ppm; indeed this was the lowest SQ addition properly determined (within an acceptable standard deviation) by the addition of SQ on ARO samples. Lower values could not be evaluated. Looking at the graphical representation (Fig. 2) of the "really" added vs. NMR-detected SQ values, we can conclude that quantification works pretty well within the NMR sensitivity limits for SQ-rich matrices (EVOOs); on the other hand, as expected, the "real" quantification of the added SQ in vegetable oils is possible just above the limit of quantification (around 2000 ppm). These statements were validated by at least 5 different runs for both vegetable oils (see supporting information excel material).

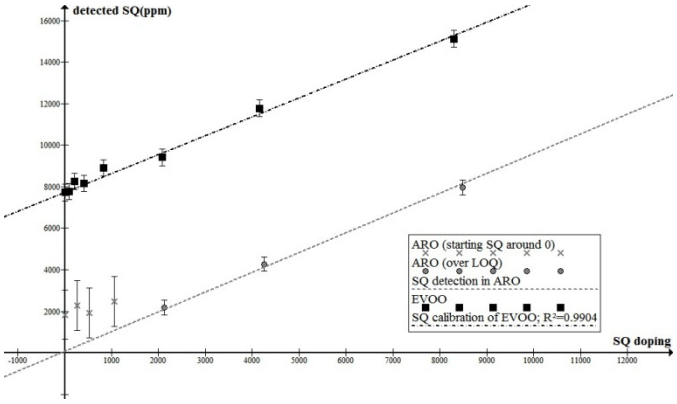


Figure 2. Quantification of SQ by NMR for EVOO (dark squares draw) and ARO (gray circles draw) against the known standard SQ addition. Graph shows the expected linear correlation.

4 Discussion

From the graphical representation of the measured against the real (added) values it is possible to conclude that SQ quantifications are acceptable above 2000 ppm (CV% below 0.15), whereas the curve slope remains always close to 45°, meaning that theoretical hypotheses fulfil experimental records. More precisely, the average slope is around 0.95, rather than 1, reflecting a slight underestimation of the actual value (95% detection). However, considering that the standard SQ purchased sample is declared at 98% of purity we can consider the underestimation really negligible.

In our laboratory, by observing nine NMR samples over three different EVOOs and also in three different days (27 experiments) we were able to conclude that: a) inter-day variations are relatively small (for squalene the highest standard deviation measured was 1.6%) ; b) there is a low degree of variability in samples prepared in the same way (for squalene, the highest standard deviation was 2.9%); c) anyway, relative standard deviations measured in the "worst case scenarios" remain below 10% for defined signals whose integration is above 1.5% of the reference 2.35 ppm signal (α-CH_2 protons belonging to the fatty acids; see section 3 of the supporting information material). The 1.67 ppm SQ signal for many EVOO is just around this threshold, being significantly higher in the case of the Sicilian EVOOs [41]. This is clearly evidenced by our three organic EVOO samples showing CV% values always below 4%. Collectively, our data indicate that, althought NMR quantification bears some sensitivity limits, it

appears soundly accurate even with unreferenced data.

5 Conclusions

In the first place, this paper provides an additional example of how NMR theoretical principles can provide a sound base for "real" quantification, even without expensive standard references or troublesome calibration curves. Secondly, data presented here provide a usable method to easily quantify SQ in vegetable oils. This is a novel application, since NMR was not previously reported as a possible quantitative method for SQ [25]. The method might be of wide interest, in view of the crucial role of SQ in several biological processes and its commercial importance in the pharmaceutical and cosmetic industries. Quantification by NMR was validated by using well-established analytical procedures, such as the addition of internal standards, and by observing a satisfactory correlation between expected and experimental values. Although NMR is widely used for statistical analyses with global data treatment [28, 34, 36, 38, 40], we have to issue some *caveat* about small integrations/bucketing becoming non-sense numbers because of the offset standard deviation; our approach is not to use these potentially misleading parameters (i.e. data-points, numbers). On the other hand, unlike other experimental procedures, such as liquid chromatography, NMR detection is simple and rapid, and does not really need standard calibrations.

An additional point raised by our data concerns the possibility to correct the NMR quantification in oils of compounds different from SQ; specifically we show that unsaturated fatty acids can be overestimated because of the ignored SQ signal overlap [36]. Although this is actually negligible most of the times, this is not the case for Sicilian EVOOs with high SQ content (usually >8000 ppm). Overall, beyond validation of NMR as a tool for simple and straightforward quantitative analyses, our data may be useful for the wise treatment of NMR data concerning EVOO. Our ultimate goal is to develop automated qNMR methods over most of the oil species to create high-throughput "spectra to labels" pathways.

6 Conflict of interest

The authors have declared no conflict of interest.

*The manuscript **does not** contain experiments using animals. The manuscript **does not** contain human studies.*

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