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Quick unreferenced NMR quantification of Squalene in vegetable oils

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This manuscript is aimed to the NMR complete and straightforward validation of the squalene content in food oils. For this purpose, more than 40 specific experiments, based on the addition of standard squalene, were run to have biunivocal correspondence between calculated and measured values. As any ^{1}H NMR signal is proportional to the proton magnetic momentum and to the number of isochronous spins, the NMR quantification can be achieved without tedious standard calibration. These experiments confirm: a) theoretical principles are fulfilled leading just a very slight under-evaluation of the squalene quantification; b) despite the intrinsic low sensitivity of the NMR, acceptable accuracy and sensitivity are reached over 2000ppm. Because of our previous focus on squalene isolated by several vegetal matrices we also point out how this substance is affecting the overall 1H NMR profile and why it is important for the analisys of the global composition of the oil. This study is specifically based on extra-virgin olive oil but it is extendable to any other vegetal oil.

^{1}H NMR, vegetal oils, Squalene, quantitative NMR

1. Introduction

Squalene (SQ) attracts a great biological interest as it is the third main component of the lipidic film whose fundamental role is to protect our skin from photoxydation (Nicolaides, 1974; De Luca et al. 2010)). As a matter of this fact, it has been demonstrated that SQ is not very susceptible to peroxidation and seems to act, in the skin, as a quencher of singlet oxygen, preventing lipid peroxidation caused by the exposure of ionizing radiations leading to the dangerous skin cancer (Kelly, 1999). Despite its known biological role, medicinal and cosmetic use of squalene took place only in the first years of this century. These uses followed some *in vitro* studies evidencing SQ detoxification activity against the cutis oxygen derived free radicals and also protection from the UV induced lipo-peroxydation. Besides, because of the chemical structure, SQ and its derivative squalane, are easily absorbed at the local/topical level. These features account for their use as: a) emollients, to recover the lipidic film; b) demulcents for delicate skin treatments; c) antiaging agent against the oxygen derived free radicals; d) antioxidant ointments able to protect cutis and the entire body from microbiological and oxidative burden. Further studies are actually in progress over the possible strategic role of SQ in the regulation of cell-cell chemical interactions to organize keratinocytes and fibroblasts.

Actually, since SQ is essential for biosynthesis of steroids and triterpenes and is also considered an intermediate in endogenous cholesterol synthesis, there was a concern that high SQ-intake from the diet could lead to cholesterol increase, which translates into a greater risk for development of atherosclerosis (Kohno et al., 1995). On the contrary, though, it has been also reported that SQ might lead to several beneficial effects reducing cholesterol and triglyceride levels in serum and protecting against a variety of cancers (Chan et al. 1996; Storm et al., 1993). This theory is

supported by several trials run over animals (Rao et al. 1998), as sharks, whose liver oil is very rich in SQ, are thought to face a lower cancer occurrence (Mathews et al. 1992). Moreover SQ is able to enhance the anti cancer activity of several drugs used against specific cell lines (Nagakawa et al. 1985) and it is also an effective adjuvant for cancer immunotherapy (Mesa & Fernandez 2004). In summary the regular uptake of EVOO is considered beneficial against cardiovascular threat, blood-pressure disorders, cancer challenges as well as microbial diseases and inflammatory events, moreover it is a main component of the lipidic film and is thought to preserve skin and retinas allowing also a stable cyto-architecture. This is the reason why it is suggested the consumption of at least 30 grams of SQ per day (especially from raw EVOO) and many medical ointments containing SQ are widely spread. The average SQ intake is 30 mg/day, whereas in Mediterranean areas, where the consumption of EVOO is higher and combined with other SQ containing food-stuff, this parameter is estimated to arise of one order of magnitude (Saitta et al., 2009).

Chemically speaking SQ is an isoprenoid compound with 30 carbon atoms, containing six double bonds and it is present mainly in the cod liver oil. SQ owes its name to the first isolation from the shark liver oil, but is ubiquitous in nature circulating in both vegetable and animal realms (Tsujimoto, 1916). Traditionally, SQ in foods, oils and fats was determined by titrimetric or chromatographic procedures (HPLC, GC, hyphenated chromatographic techniques) and some studies recommended SPE for sample preparation (Grigoriadou et al., 2007). In the last years for the SQ isolation from seeds, the supercritical fluid extraction has been preferred, but this method is still expensive at the industrial level (Popa et al., 2015). The most accredited methods for the determination of SQ involves sample saponification, extraction of the non-saponifiable matter with large volume of solvent, fractionation through chromatographic column and other treatment just before titration (AOAC. Official method of analysis, 1999). In the existing HPLC methods both official or not, the quantification of SQ by direct analysis is difficult and requires a pre-treatment step to eliminate the interfering substances. Usually, pre-treatment involves relatively high volumes of organic solvent, high capital cost and long elution time. In this scenario it is possible to account for the great relevance gained by NMR within the field of food analytical chemistry (Belton et al. 1996,1998) conveying these main advantages: a) Minimal chemical treatment; b) quick acquisition of a great amount of data; c) constant replication of any possible experimental error (Mannina et al. 2012). We would like to add that the nuclear magnetic detection is rigorously proportional to the number of spins so that the NMR spectrum shows 1 what is 1 regardless the analyzed sample or chemical groups (Derome et al. 1987), therefore, in principle, it is possible to quantify substances without any standard reference (absolute intensity) or quantify many species with just one reference (this is often the case in food analysis). We think this paper strongly support theoretical bases confirming the great importance of NMR in food analysis. The robust NMR quantification-confidence was actually well assessed by a brilliant paper estimating, for many different laboratories, the performance in terms of closeness to the “consensus slopes” of the calibration lines (Gallo et al. 2015), our contribution is about validating a specific metabolite (SQ) in similar matrices (vegetal oils) with just one 500 MHz machine, on the other hand this contribution can open up perspectives and limits of the NMR oil analyses.

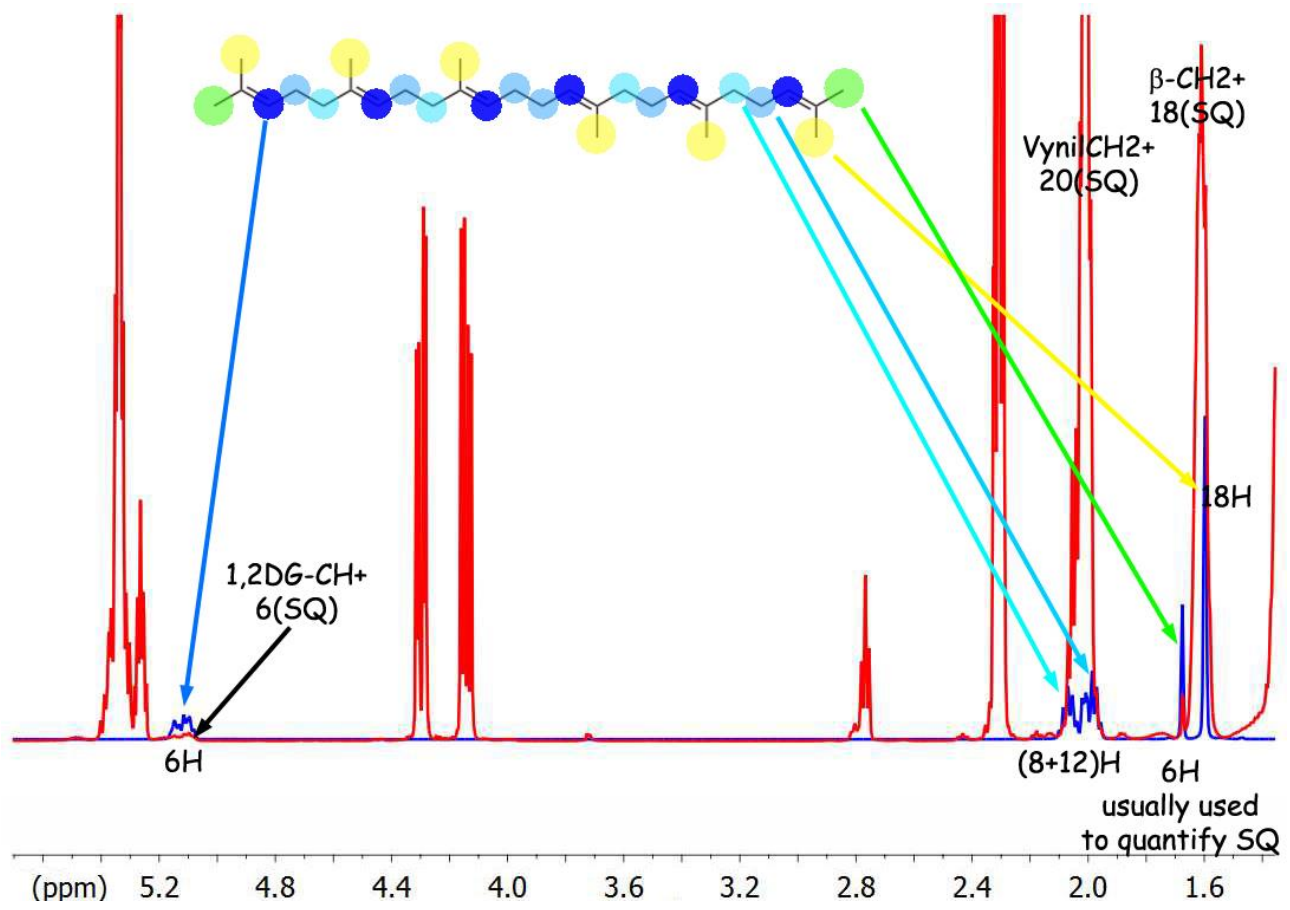
2. Experimental

All the oil samples were dissolved in $CDCl_3$ with traces of TMS standard reference. As it is recommended in many oil analyses (Girelli et al. 2015; Del Coco et al. 2014) we keep the oil to

$CDCl_3$ weight ratio equal to 13.5/86.5; it corresponds to put 122uL of oil into the 5mm NMR test tube together with 478uL of deuterated chloroform. All the samples were prepared in the same way and analyzed at the same temperature (T = 25 °C) by a 500 MHz Avance III NMR spectrometer equipped with an inverse, z-gradient probe (SMARTprobe). After the automatic tuning (atma) and shimming (topshim) lineshape of the tiny TMS signal is optimized by some iterative manual or automatic shimming. When the TMS line-shape was acceptable the 90° pulse was measured most of the times being always 8.4±0.1 us at -0.8 dB of transmission power. For all of the samples we have run 12ppm spectral with ^{1}H-NMR experiments with 64 repeated 90° pulses, 2 seconds of acquisition time, and 2.5 seconds of scan delay in order to match high sensitivity without any signal loss. It was first verified, by overlapping spectral profiles with different scans, that after the first 8 transients a steady state is reached yielding to consistent profiles and consistent integrations within the standard deviation. All the spectra, first processed by TOPSPIN, were finely analysed by ACD/NMR processor with multiple fid treatment to keep all the spectra consistent. All the spectra were automatically phased, baseline was corrected with the fid reconstruction method, profiles were referenced to the TMS frequency standard (δ=0.0 ppm) and simultaneously integrated for many NMR indicative bands. For the SQ specific study we have run also the DPFGSE-TOCSY 1D spectra (TOCSY1D) with excitation at 1.66ppm. Quantification of SQ as for other compounds is performed by integration of specific portions of the spectra with a specific kind of grid.

3. $\frac{mg}{g} = \frac{I_x M_x 6}{10000 * 887 * N° H_x}$ Discussion

The ^{1}H NMR spectrum of vegetal oils presents typically the triglyceridic (TGs) and diglyceridic (DG) signals of many common fatty acids (Rotondo et al. 2016). Just by enhancing the intensity it is possible to evidence some minor constituents of the fatty vegetal oils. ^{1}H NMR spectrum run over the standard SQ molecule presents six specific NMR resonances according to the six different chemical groups. The chemical shift (cs) of these signals is not really changed by the vegetal oil matrices which just overlap their spectral profile.



Beyond the internal standard addition experiments, it is confirmed by the selective DPFGSE TOCSY spectra exciting the 1.66ppm signals of doped oil samples. All these preliminary considerations are drawn to figure out that: a) the bare 1.66 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 to some chemically similar groups belonging to glyceridic or fatty acid moieties, therefore cannot be directly related to the SQ content, on the other hand, we know that those signal integrations are affected by the SQ content despite it was not really considered so far (Barison et al. 2010). We have to say though that in other fat matrices, other SQ signals are in some way mentioned and also used for quantitative considerations (Robosky et al. 2008, Borchman et al. 2013). Another outstanding question concerns the possible precision of the NMR experiments which also relays to the meaning of the many NMR data often used for statistical treatments and evaluations.

Briefly, any signal is proportional to the number of protons (N°H) in the chemical group 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 not-overlapped signal; indeed the integral area of the internal reference (α-C$\boldsymbol{H}_2$ of TGs, 1.30ppm) and the integral area of the component X to be determined are related by the Eq (1):

$$\frac{mg_x}{g} = \frac{10^3\, I_x M_x\, N°H_{ref}}{I_{ref}\, M_{ref}\, N°H_x} \quad (1)$$

where I_x and I_{ref} are the integral values for a nucleus related to the compound *x* and TGs, 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 reference value for α-C***H***$_2$-TGs integration:

$$\frac{mg}{g} = \frac{I_x M_x\, 6}{10000 * 887 * N°H_x} \qquad (2)$$

As it is known the isolated 1.66ppm signal coming from the terminal E-oriented methyl groups of SQ (6 protons), we can write an equation 3 for the SQ quantification

$$\frac{mg_{SQ}}{g_{EVOO}} = \frac{I_{SQ} * 410.7 * 6}{10000 * 887 * 6} \quad (3)$$

Small known aliquots of standard SQ are added to several extra-virgin olive oil (EVOO) and peanuts 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).

As we know EVOO basically contains around 5000ppm (Salvo et al. 2016 submitted evoo) of SQ, which can be also confirmed by the NMR analysis of the neat EVOO, the other obtained solutions corresponds to the internal standard addition which again can be validated. On the other hand the measurements on the AROs were achieved because it originally contains around 100ppm of SQ (Salvo et al. pist 2016 submitted) which is barely detectable by NMR. In this case it is possible to consider the SQ signal due just to the doping, even though within an EVOO like matrix; this is mainly done to explore the limits of quantification keeping in mind NMR does bear challenging sensitivity limits. Technically speaking, SQ quantification (spanning from 5700 to 11500ppm) by NMR over 10 Sicilian and Greek EVOO is feasible being the relative standard deviation (SD%) always below 15%. Simple 4 minutes spectra are always able to confirm the higher SQ content of the Sicilian over the Greek EVOOs confirming our previous finding by UPLC analysis (Salvo et al.). This is not the case of the ARO samples because these are really SQ poor so that the detection of the SQ cannot lead to an appropriate quantification. Doping these samples by standard SQ we can observe the limit of quantification is around 2000ppm as the original SQ content of AROs can be approximated to 0. Looking at the graph representation (Figure 1) of the real vs NMR detected SQ doping we can conclude it works pretty well, within the NMR sensitivity limits, starting with SQ rich matrices (EVOOs); on the other hand, as expected, the “real” quantification of the added SQ in vegetal oils is possible just above the limit of quantification (around 2000ppm) as demonstrated by the AROs runs represented by the figure 3b. These statements are validated by at least 5 different runs for both vegetal oils.

By the graphic representations of the expected against the real (added) value it is possible to say that in SQ measurements is acceptable above 1000ppm (below 15%) whereas the curve slope is very close to 45° meaning that theoretical hypotheses fulfil experimental records. More precisely the average slope is around 0.95 instead of 1 reporting a slight underestimation of the real 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, running three NMR experiments over three different EVOO samples and also in three different days (27 experiments) we were able to state that: a) inter-day variations are not really relevant provided that a good shimming is accomplished; b) there is a noteworthy (human?) uncertainty in samples prepared in the same way; c) Anyway, relative standard deviation measured in the "worst case scenarios" is kept below 10% for defined signals whose integration is above 1.5% of the reference 2.35ppm signal (α-CH_2 protons belonging to the fatty acids). (Rotondo's private records 2016). SQ 1.66ppm signal for many EVOO is just around this threshold being much more in the case of the Sicilian EVOOs (Salvo et al.2016), this is clearly evidenced by our three organic EVOO samples showing SD% values always close to 4%. Again we highlight that, because of the NMR sensitivity limits SQ quantification is not really precise, however it has been shown soundly accurate.

4. Conclusions

This paper is about the validation of the theoretical NMR principles as sound bases for a *real* quantification, even without expensive standard references or troublesome calibration curves. Of course to demonstrate this we did use traditional lab practices in order to get some important assessments. First of all, despite NMR is widely used for statistical analyses with global data treatment we have to issue some *caveat* about small integrations/bucketing which are non-sense numbers because of the offset standard deviation; our personal idea is not to use these misleading data (data-points, numbers). We also face the challenge of the SQ quantification in EVOO which is just within the limits of quantification; on another hand, unlike other experimental procedures the quick NMR detection is *per se* accurate and does not really need standard calibrations. Another crucial point we want to highlight is about the quantification of other compounds by NMR as the unsaturated fatty acids which were overestimated so far because of the SQ signal overlap. This is actually negligible most of the times, however this is not the case for Sicilian EVOO with high SQ content (usually >8000 ppm). We think that, beyond the NMR easy validation of straightforward quantitative analyses, this paper is full of suggestions concerning wisetreatment of NMR data concerning EVOO.

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