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ORIGINAL ARTICLE

High-Resolution NMR Spectroscopy: An Alternative Fast Tool for Qualitative and Quantitative Analysis of Diacylglycerol (DAG) Oil

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Abstract Multinuclear (^{1}H, ^{13}C, ^{31}P) and multidimensional NMR spectroscopy was employed for the analysis of diacylglycerol (DAG) oil and the quantification of its acylglycerols and acyl chains composition. A number of gradient selected two dimensional NMR techniques (TOCSY, HSQC-DEPT, HSQC-TOCSY, and HMBC) facilitated the assignment of the complex one dimensional ^{1}H- and ^{13}C-NMR spectra. In several cases, the aforementioned 2D-NMR techniques offered solid proof of earlier assignments based on chemical shift changes induced by the presence and relative positions of double bonds within the acyl chains. Integration of the appropriate signals in the NMR spectra of the three nuclei allowed the determination of DAG oil composition, which was found to be within the limits accepted for this oil, namely 1-monoacylglycerols 0.40–0.60%; 2-monoacylglycerols 0.40–0.50%; 1,3-diacylglycerols 57–62%; 1,2-diacylglycerols 28–32%; triacylglycerols 9–11%; saturated fatty acids 3–5%; oleic acid 37–45%; linoleic acid 49–53%; and linolenic acid 5–6.5%; tocopherols 0.24–0.27%. The compositional results obtained from the NMR spectra of the three nuclei were compared and discussed in terms of repeatability and ease of performance.

Keywords Diacylglycerol oil · NMR spectroscopy · Composition

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Introduction

Diacylglycerol oil (DAG oil) was introduced as a dietary fat by Kao Corporation of Japan in 1999 under the brand name ENOVA oil [1]. Since then, DAG oil has been commercialized in Japan, United States, Canada, Australia, and New Zealand as a cooking oil, and as an ingredient in baked goods, pizza, fats and oils, health bars, meal replacements, frozen entrees, soups, gravies and in other applications [2, 3].

DAG oil was manufactured enzymatically by lipase-catalysed esterification of glycerol with fatty acids from natural edible plant oils such as soybean, canola, and corn oil, and is composed largely of randomized diacylglycerols (DAG) [3, 4]. Effects of reaction parameters such as reaction time, temperature, enzyme type, enzyme load, substrate molar ratio and water content were studied extensively in the past [4]. DAG oil contains approximately 80% diacylglycerols (DAG), 20% triacylglycerols (TAG), 3% monoacylglycerols (MAG), <0.2% emulsifiers (polyglycerol esters of fatty acids), and antioxidants, mainly mixed tocopherols (0.075%). The phytosterol content ranged between 0.05 and 1.2%. The DAG component is produced as a mixture of 1,3-diacylglycerols (1,3-DAG) (~70%) and 1,2-diacylglycerols (1,2-DAG) (~30%). 1-monoacylglycerols (1-MAG) is the major component of the two MAG isomers, the 2-MAG counterpart being found in smaller amounts. The main constituent fatty acids of

DAG oil are oleic (OL) (20–65%), linoleic acid (LO) (15–65%), linolenic acid (LN) (~15%), and saturated fatty acids (SFA) (mainly palmitic and stearic acids) (~10%) [1]. The mixture of fatty acids was prepared in a way that matches the major fatty acid composition of the DAG and TAG constituents as closely as possible. *Trans* fatty acids should be completely absent in DAG oil.

The analysis of DAG oil and the quantification of its constituents have been carried out by employing conventional analytical techniques, such as gas chromatography, high performance liquid chromatography, and gas–liquid chromatography [1, 3, 5, 6]. The cost of the analysis using these methods is low and in addition no particular skill is required. However, these methods are time-consuming insomuch as lengthy sample pre-treatment and calibration with standards precede the analysis. In addition, the use of rather large volumes of organic solvents can cause environmental pollution.

Recently, high resolution ^{1}H- and ^{13}C-NMR spectroscopy has been largely employed as an alternative analytical tool for the analysis of complex mixtures such as vegetable oils without previous separation of their individual components [7, 8]. The usefulness of ^{1}H-NMR spectroscopy has been increasingly recognized for its non-invasiveness, rapidity and sensitivity to a wide range of compounds that can be detected in a single measurement (spectrum), whereas little or no need for sample pre-treatment is required. On the other hand, the potential of ^{13}C-NMR spectroscopy has been tested in several instances in determining the profile of the acyl chains with complex unsaturated systems made up of double and triple bonds, either isolated and/or conjugated in triacylglycerols of vegetable oils with different botanical origin [8, 9]. Another magnetic nucleus introduced recently in the analysis of vegetable oils is phosphorus-31 [10, 11]. ^{31}P-NMR spectroscopy is particularly useful in cases where extensive coupling in ^{1}H-NMR spectra render their interpretation a difficult task, and/or when quantitative ^{13}C-NMR experiments cannot be avoided; the latter measurements require lengthy accumulations and long relaxation delays to achieve a satisfactory signal to noise ratio for the insensitive ^{13}C-nuclei, and more importantly to account for by NOE effects and carbon long longitudinal relaxation times. The ^{31}P-NMR method is based on the derivatization of the labile hydrogens of hydroxyl and carboxyl groups of the oil constituents by the phosphorus reagent 2-chloro-4, 4, 5, 5-tetramethyldioxaphospholane (**1**) and the use of the ^{31}P chemical shifts to identify the phosphitylated compounds. Compound **1** reacts rapidly (~15 min) and quantitatively under mild conditions (within the NMR tube) with the hydroxyl and carboxyl groups [10].

In the present study, NMR spectroscopy will be used as a versatile, rapid and effective method to analyse DAG oil, and provide qualitative and quantitative information about the various classes of acylglycerols and acyl chains, as well as other minor compounds added and/or produced during the manufacturing process of DAG oil. The analysis of DAG oil and the quantification of its constituents will be made by using three different magnetic nuclei, namely, proton, carbon-13 and phosphorus-31, and a number of one and two dimensional NMR techniques. The results obtained by these spectroscopic methods will be compared in terms of the duration and ease of the analysis for a particular constituent, the repeatability of the analysis, destruction of the analyte, and the ability of each NMR technique to determine as many compounds as possible in a single experiment.

Materials and Experiments

Six samples of DAG oil (ENOVA) were purchased from US supermarkets. Pinacol, phosphorus trichloride, $Cr(acac)_3$, pyridine, cyclohexanol (99%), dimethylsulfoxide (99.9%), deuterated solvents (chloroform and dimethylsulfoxide), mono-, di- and triolein standards were obtained from Sigma-Aldrich (Athens, Greece). The preparation of the phosphorus reagent was slightly modified [12] from that described in the literature [13] in order to increase the yield of the product.

Sample Preparation for Spectroscopic Analysis

Samples of 10 and 20 mg of DAG oil were dissolved in $CDCl_3$ for recording one and two-dimensional ^{1}H-, and ^{13}C-NMR spectra, respectively. 100 mg was used for quantitative ^{13}C NMR. Samples of DAG oil for ^{31}P experiments were prepared as follows [10, 12]: a stock solution (10 mL) composed of pyridine and $CDCl_3$ in a 1.6:1.0 volume ratio containing 0.6 mg of chromium acetylacetonate, $Cr(acac)_3$, (0.165 μM) and 13.5 mg cyclohexanol (13.47 mM) was prepared and protected from moisture with 5-Å molecular sieves. 100 mg of the oil sample were placed in a 5-mm NMR tube. The required volumes of stock solution (0.4 mL) and the reagent **1** (50 μL) were added. The reaction mixture was left to react for about 15 min at room temperature. Upon completion of the reaction, the solution was used to obtain the ^{31}P-NMR spectra.

NMR Experiments

^{1}H- and ^{13}C-NMR experiments were conducted on a Bruker Avance III spectrometer operating at 600.20 and 150.93 MHz for proton and carbon-13 nuclei, respectively. ^{31}P-NMR experiments were carried out on a Bruker

AMX500 spectrometer operating at 202.2 MHz for the phosphorus-31 nucleus. All experiments were performed at 25 ± 1 °C. All spectra were processed by the Brüker Topspin software package.

One Dimensional (1D) NMR Spectra

^{1}H-NMR spectra were recorded with the following acquisition parameters: time domain 32 K; 90° pulse width 11 μs; spectral width 10 ppm; relaxation delay 2 s; acquisition time 2.73 s; 32 scans and 4 dummy scans were accumulated for each free induction decay. Base-line correction was performed carefully by applying a polynomial fourth-order function in order to achieve quantitative measurements upon integration of signals of interest. The spectra were acquired without spinning the NMR tube in order to avoid artificial signals, such as spinning side bands of the first or higher order. Chemical shifts are reported in ppm from TMS ($\delta = 0$).

^{13}C-NMR spectra were obtained with proton decoupling to exploit the nuclear Overhauser enhancement of the signal intensities of compounds of low concentration inherent in DAG oil. These spectra were recorded with spectral width of 200 ppm, using 64 K data points, a 90° excitation pulse of 21 μs; acquisition time 1.08 s and a relaxation delay of 5 s in order to avoid signal saturation. 128 scans were collected and zero-filled to 128 K. For all FIDs, line broadening of 1 Hz was applied prior to Fourier transform. Two quantitative ^{13}C-NMR experiments were performed; the first for the glycerol and olefinic carbons, and the second for the carbonyl carbons with repetition times (relaxation delays plus the acquisition time) five times the longitudinal relaxation times of the olefinic and carbonyl carbons of the acyl chains, respectively, measured by the *null method* [14] (see below). The NOE factors of the acyl chain carbons were measured by *the inverse gated decoupling method* [15] using relaxation delays between consecutive 90° pulses equal to ten times their longitudinal relaxation times.

Precautions were taken to obtain ^{31}P-NMR spectra free from NOE effects by using the inverse-gated decoupling technique. Typical parameters for quantitative studies were: 90° pulse width 18.8 μs; sweep width 50 ppm; relaxation delay 25 s; and memory size 16 K (zero-filled to 32 K). Line broadening of 1 Hz was applied, and drift correction was performed prior to Fourier transform. Polynomial fourth-order baseline correction was performed before integration. For each spectrum 32 transients were acquired. All ^{31}P chemical shifts are reported relative to the product of the reaction of **1** with water (moisture contained in all samples), which gives a sharp signal in pyridine/$CDCl_3$ at 132.20 ppm [10].

Two Dimensional (2D) NMR Spectra

Experimental details and pertinent references for most of the 2D pulse sequences used in this study can be found in Reference [16]. *^{1}H-^{1}H Total Correlation Homonuclear Spectroscopy* (H–H-TOCSY). These spectra were acquired in the phase sensitive mode with TPPI, using the DISPI2 pulse sequence for the spin lock. Typically, 16 dummy scans and 32 scans were collected for each of 512 increments with a spectral width of 10 ppm in both dimensions, 2,048 data points, spin-lock time of 80 ms, and a relaxation delay of 1.5 s. The data points in the second dimension were increased to 2 K real data points by linear prediction, and the spectra were zero-filled to a final size of 2 K × 2 K prior to Fourier transformation. A sine-bell squared window function was used in both dimensions. The combined experiment of the phase-sensitive *Gradient selected ^{1}H-^{13}C multiplicity-edited heteronuclear single quantum coherence* (HSQC-DEPT) [17] was performed with 512 × 512 complex points and a spectral width of 170 ppm for ^{13}C (F1) and 6 ppm Hz for ^{1}H (F2). Typical parameters for data acquisition included: 128 increments, 8 scans preceded by 16 dummy scans for each increment according to the echo-antiecho procedure, relaxation delay 1.8 s; delays of 3.45 ms (1/2 *J*) for multiplicity selection, and 1.725 ms (1/4 *J*) for sensitivity improvement were used. Carbon decoupling during proton acquisition was achieved by applying a GARP pulse train. Gradient strengths were 20 and 5 G/cm. The data were multiplied in ^{1}H with a sine weighting function. The ^{13}C time domain was doubled by forward linear prediction prior to a cosine window function. *Gradient selected ^{1}H-^{13}C heteronuclear multiple bond correlation* (gHMBC). The phase-sensitive gradient selected hydrogen-carbon HMBC experiment was performed using a low-pass *J*-filter (3.4 ms) and delays of 65 and 36 ms to observe long-range C–H couplings optimized for 3 and 7 Hz with 256 increments and 86 transients of 2,048 data points. The relaxation delay was 2.0 s. Zero-filling to a 2 K × 2 K matrix and π/2-shifted sine-square bell multiplication was performed prior to Fourier transform. The *HSQC-TOCSY* pulse sequence constructed by inserting in the basic gradient enhanced HSQC building block a phase-sensitive TOCSY transfer step with the TPPI method using the DISPI2 pulse train for the spin lock. The experiment was conducted with 1,024 × 256 complex points and a spectral width of 10 ppm for ^{1}H and 180 ppm for ^{13}C. Eight transients were collected for each point with 16 dummy scans. The mixing time was 80 ms and the relaxation delay 1.5 s. the spectra were zero filled to 2 K × 2 K and processed with Qsine-square bell in both dimension. *^{1}H diffusion ordered spectroscopy* (DOSY) experiments were performed using the STE bipolar

gradient pulse pair (stebpgp1s) pulse sequence. Typically for each experiment 32 scans of 16 data points were collected. The maximum gradient strength produced in the z direction was 5.35 Gmm^{-1}. The duration of the magnetic field pulse gradients (δ) was optimized for each diffusion time (Δ) in order to obtain a 2% residual signal with the maximum gradient strength. The values of δ and Δ were 1,800 μs and 100 ms, respectively. The pulse gradients were incremented from 2 to 95% of the maximum gradient strength in a linear ramp. The temperature was set and controlled to 298 K with an air flow of 400 l h^{-1} in order to avoid any temperature fluctuations due to sample heating during the magnetic field pulse gradients.

Results and Discussion

Spectroscopic Assignment

The identification and quantification of the various constituents of DAG oil by using NMR spectroscopy rely on the correct assignment of the 1D-NMR spectra. To achieve this objective we made use of the powerful arsenal of the 2D-NMR techniques described in the experimental part, and ^{1}H- and ^{13}C-NMR spectra recorded for mono-, di-, and triolein model compounds. The proton and carbon NMR spectra of model compounds were assigned undoubtedly using a number of homonuclear and heteronuclear 2D-NMR experiments. Splitting patterns and signal intensities from the 1D ^{1}H-NMR spectra of DAG oils, as well as available NMR data from literature were additional sources of information.

^{1}H-NMR Spectra

The ^{1}H-NMR spectra of the tricylglycerols of vegetable oils, and in particular of olive oil, have been reported in several instances [7, 8, 18–22]. Although these spectra are quantitative and easy to obtain, they suffer severe signal overlap in the aliphatic proton region. This problem is expected to become more serious for DAG oil due to the fact that it has a different composition from vegetable oils. Figure 1 compares the NMR spectra of olive oil and DAG oil. The higher concentration of MAG and DAG, the greater content of linoleic and linolenic acids, the smaller amount of oleic acid, and the significant quantities of tocopherols in DAG oil results in additional complexity of the proton resonances in the olefinic and aliphatic spectral region for this oil. For instance, the multiplets of the olefinic protons of the unsaturated fatty acids resonate at a very narrow range of chemical shifts (δ 5.40–δ 5.29) prohibiting thus their assignment. Nevertheless, the TOCSY spectrum of DAG oil allows the identification of other signals, and especially those of the glycerol backbone protons. The methine protons H2′ of the glycerol moiety for each of the acylglycerol species resonate at well known frequencies as deduced from our model compounds and literature data [18, 21, 23]; these protons constitute a good start to assign the remaining protons of the glycerol skeleton via the TOCSY experiments. This experiment creates correlations between all protons, even distant ones, within a given spin network as long as there are couplings between every intervening proton. The TOCSY spectrum of a DAG oil sample showing the connectivity amongst the glycerol protons is depicted in Fig. 2. It is interesting to note that the TOCSY spectrum reveals the backbone glycerol proton H1b′ of TAG, which was hidden under the strong signals of H1a′ and H1b′ protons of 1,3-DAG. Also, the signals of the glycerol segment of TAG were differentiated relative to those of DAG on the basis of their different diffusion coefficients obtained from the DOSY NMR spectrum of DAG oil. The heavier TAG showed smaller diffusion coefficient (4.5×10^{-10} m^2 s^{-1}) than that of the lighter DAG components (5.4×10^{-10} m^2 s^{-1}). The DOSY spectrum of DAG oil is available as Supplementary Material. The chemical shifts of the glycerol protons, and whenever possible the coupling constants, are summarized in Table 1. The remaining signals of the ^{1}H-NMR spectrum of DAG oil belong to the acyl chains protons. The ^{1}H spectrum of DAG oil shows distinct differences concerning the acyl chains resonances from the respective signals of olive oil (Fig. 1). The two magnetically equivalent protons next to carboxyl group for each acyl chain appear as a triplet (due to coupling with the neighboring methylene H3 protons) covering the range δ 2.35–δ 2.29 of equal coupling constants (average $^{3}J = 7.6$ Hz), but with different intensities reflecting the relative abundance of the acylglycerol entities in the mixture. The assignment of these protons was made in accordance to the acylglycerol class they belong, and their chemical shifts are depicted in Table 1. Further support to this assignment was given by performing a gradient selected HMBC experiment (see below). The assignment of the allylic (δ 2.04) and bis-allylic (δ 2.80) protons of the linolenyl chain was confirmed following the cross-peaks in the TOCSY spectrum (Fig. 2) all the way to the signal at δ 0.97 which belongs to the terminal methyl protons of the same chain [19–21, 23]. Accordingly, the signals at δ 2.80 are attributed to the bis-allylic protons of linoleyl chain, and the connectivity of this signal with that at δ 2.00 of the TOCSY spectrum (Fig. 2) establishes the correct assignment of the allylic proton of the chain. The allylic protons of the oleyl chain resonate at the same frequency with those of the linolenyl chain (δ 2.04) as deduced from intensity measurements and verified through a HSQC-TOCSY experiment (see below). A peculiar quartet was observed at the chemical shift expected for the terminal methyl protons

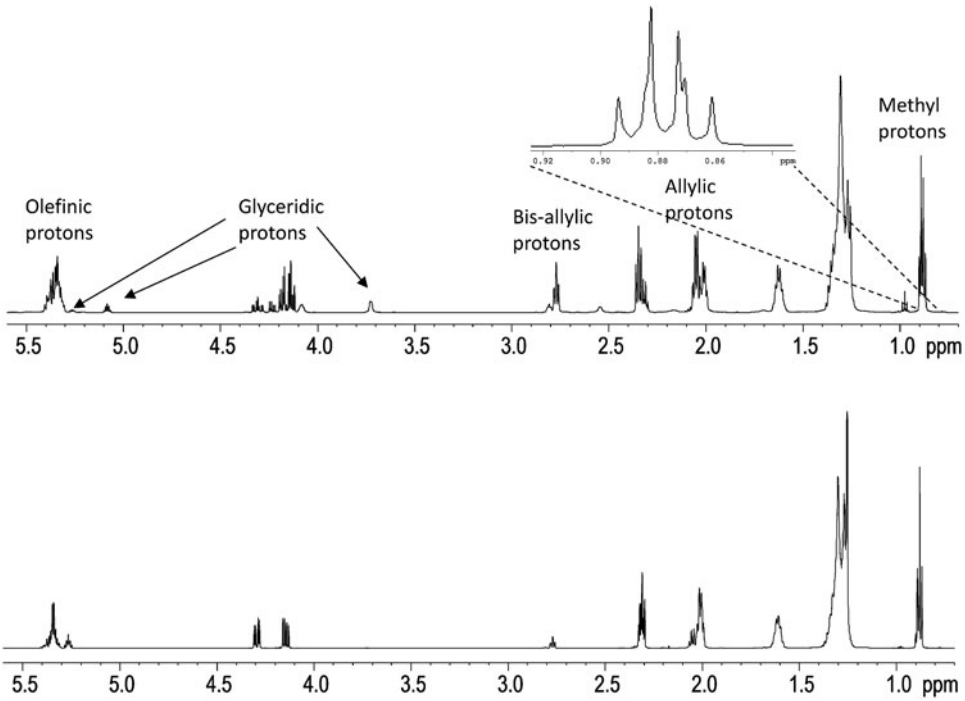

Fig. 1 Comparison of the 600 MHz ^{1}H-NMR spectra of DAG oil (*top*) and olive oil (*bottom*) in $CDCl_3$ solutions. The *inset* shows the methyl proton signals of oleic and linoleic acids recorded at higher spectral resolution (see *text*)

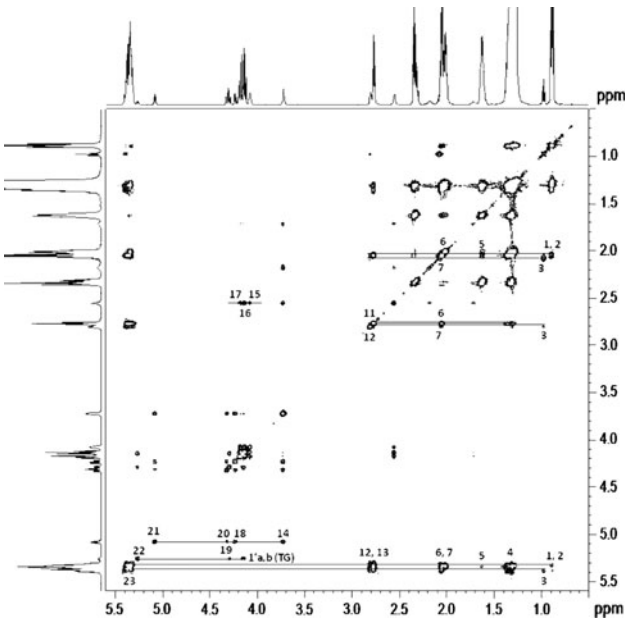

Fig. 2 600 MHz TOCSY spectrum of DAG oil in $CDCl_3$ solution. The numbering system in Table 1 was adopted to denote the various proton connectivities

of the saturated and unsaturated acyl chains. This puzzling spectroscopic feature was resolved by recording either a proton spectrum with the highest possible resolution, or by applying a window function for resolution enhancement. This experiment revealed that the assumingly quartet was actually two triplets at δ 0.88 and δ 0.87 with *J*-coupling $\sim$7 Hz, in agreement with signal intensities corresponding to six protons (inset in Fig. 1). The high frequency signal was attributed to the methyl protons of the linoleyl and saturated chains, whereas that at low frequency signal was ascribed to the methyl protons of the oleyl chain. The presence of a second double bond in the linoleyl chain produces a measurable deshielding effect on protons up to five or six bonds away from the functional group [24]. The weak signal a δ 3.59 (dd, $J = 11.11$ and 5.99 Hz) belongs to the *sn*-1 protons of glycerol in 1-MAG. The assignment of this signal was based solely on the monoolein model compound, since the remaining protons of minor compounds 1- and 2-MAG was hidden underneath the much stronger signals of DAG and TAG.

The volatile compounds in vegetable oils have been studied earlier by high field NMR spectroscopy [7, 21]. Accordingly, the singlet observed in the proton spectrum of DAG oil at δ 9.74 (not shown) was accredited to the aldehydic protons of the saturated aldehydes, such as hexanal, and heptanal, whereas the aldehydic proton of *trans*-hexen-2-al was found to resonate at δ 9.48 (d, $J = 7.98$ Hz). The singlet at δ 7.96 was assigned tentatively to the olefinic proton next to the aldehyde group of *trans*-exen-2-al. Other minor unassigned signals appeared in the region 7.5–5.5 ppm. As volatiles are considered the secondary products of the unsaturated chains oxidation by the atmospheric oxygen and result from the degradation of the hydroperoxidienes, which constitutes the primary oxidation products. Both primary and secondary oxidation products may be used as quality attributes reflecting the degree of the oxidative alteration of DAG oil.

Table 1 ^{1}H-NMR chemical shifts of mono- di-, and tri-acylglycerols of DAG oil in $CDCl_3$ solutions

Signal	δ (ppm)	Proton	Compound
1	0.87	$CH_2\underline{CH_3}$ (t) $J_{18,17} = 6.95$ Hz	Oleyl chains
2	0.88	$CH_2\underline{CH_3}$ (t) $J_{18,17} = 7.02$ Hz	Linoleyl chains
3	0.97	$CH_2\underline{CH_3}$ (t) $J_{18,17} = 7.53$ Hz	Linolenyl chains
4	1.30	$-(\underline{CH_2})_n$- (envelope)	All acyl chains
5	1.62	$OCOCH_2\underline{CH_2}$ (m)	All acyl chains
6	2.00	$\underline{CH_2}CH=CH$ (m)	Oleyl chains
7	2.04	$\underline{CH_2}CH=CH$ (m)	Linoleyl and linolenyl chains
8	2.30	$OCO\underline{CH_2}CH_2$ (t) $J_{2,3} = 7.59$ Hz	All acyl chains of TAG
9	2.32	$OCO\underline{CH_2}CH_2$ (t) $J_{2,3} = 7.53$ Hz	All acyl chains of 1,2-DAG
10	2.34	$OCO\underline{CH_2}CH_2$ (t) $J_{2,3} = 7.53$ Hz	All acyl chains of MAG and 1,3-DAG
11	2.76	$CH=CH\underline{CH_2}CH=CH$ (m)	Linoleyl chains
12	2.80	$CH=CH\underline{CH_2}CH=CH$ (m)	Linolenyl chains
13	3.59	3′a-$\underline{CH_2}OCO$[A] (dd) $J_{3'a,3'b} = 11.40$ Hz $J_{3'a,2'} = 6.02$ Hz	Glycerol of 1-MAG
14	3.72	3′a, 3′b-$\underline{CH_2}OCO$ (br)	Glycerol of 1,2-DAG
15	4.07	2′-$\underline{CH}OH$ (br)	Glycerol of 1,3-DAG
16	4.13	1′a, 3′a,-$\underline{CH_2}OCO$ (dd) $J_{1'a,1'b} = 11.35$ Hz $J_{1'a,2'} = 5.97$ Hz	Glycerol of 1,3-DAG
17	4.18	1′b, 3′b-$\underline{CH_2}OCO$ (dd) $J_{1'a,1'b} = 11.35$ Hz $J_{1'a,2'} = 4.35$ Hz	Glycerol of 1,3-DAG
18	4.23	1′a-$\underline{CH_2}OCO$ (dd) $J_{1'a,1'b} = 11.94$ Hz $J_{3'a,2'} = 5.70$ Hz	Glycerol of 1,2-DAG
19	4.29	1′a,b-$\underline{CH_2}OCO$ (dd) $J_{3'a,3'b} = 11.91$ Hz $J_{3'a,2'} = 4.35$ Hz	Glycerol of TAG
20	4.31	1′b-$\underline{CH_2}OCO$ (dd) $J_{1'a,1'b} = 11.94$ Hz $J_{1'a,2'} = 4.50$ Hz	Glycerol of 1,2-DAG
21	5.08	2′-$\underline{CH}OCO$	Glycerol of 1,2-DAG
22	5.26	2′-$\underline{CH}OCO$ $J_{1'a,2'} = 5.88$ Hz	Glycerol of TAG
23	5.28–5.40	$\underline{CH}=\underline{CH}$ (m)	all acyl chains

The meaning of the symbols are as follows: *1-MAG* 1-monoacylglycerol, *1,2-DAG* 1,2-diacylglycerols, *1,3-DAG* 1,3-diacylglycerols

[A] Primed and unprimed letters denote glycerol and acyl chains carbons, respectively

^{13}C-NMR Spectra

The assignment of carbon resonances of the glycerol moiety and those of fatty acids is of crucial importance in the analytical characterization of DAG oil. The ^{13}C-NMR spectrum of DAG oil (Fig. 3) shows a large number of signals spread over a wide range of chemical shifts. This made the spectrum appear complicated but nevertheless was much more informative than the ^{1}H-NMR spectrum, which extends to a narrow region of a few ppm (see above). The resonances of the glycerol backbone carbons of mono-, di-, and triacylglycerols as well as those of the fatty acids

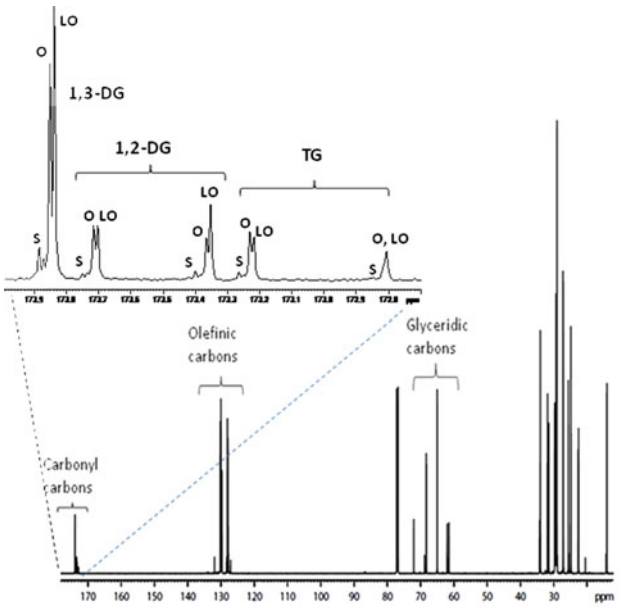

Fig. 3 600 MHz ^{13}C-NMR spectrum of DAG oil in $CDCl_3$ solution. The *inset* shows the carbonyl carbon signals of the acylglycerols recorded at higher spectral resolution (see *text*)

have been assigned in previous studies by several investigators starting from the pioneer work of Gunstone [25, 26] and Wollenberg [27] to more recent results employing advanced NMR techniques and using the high resolving power of modern NMR spectrometers [8, 9, 28–32]. The majority of these studies utilized triacylglycerol model compounds to assign the carbon signals of the acyl chains of vegetable oils. A few different spectral features are expected for DAG oil due to the fact that this oil contains significant amounts of mono- and diacylglycerols relative to vegetable oils and is characterized by a diverse fatty acids composition. Furthermore, this study attempts, whenever possible, to provide solid proof of several earlier and current carbon signal assignments using 2D-NMR spectroscopy. Table 2 summarizes the chemical shifts of 57 carbon signals observed in the in the ^{13}C-NMR spectrum of DAG oil.

The satisfactory resolution of the proton resonances of the glycerol backbone protons allows the direct assignment of the respective carbon signals through a 2D-NMR experiment that combines the usual one C–H bond correlation (*g*HSQC) together with carbon multiplicity selection similar to that obtained using the DEPT-135 experiment. The *g*HSQC-DEPT spectrum is shown in Fig. 4. Correlations phased negatively (unframed) and positively (framed) represent the methylene and methine protons, respectively. This assignment is in accord with literature data [7–9, 23, 25, 28]. It is interesting to note that this experiment discloses the position of the glycerol backbone proton H1′a,b

Table 2 ^{13}C-NMR chemical shifts of mono- di-, and tri-acylglycerols of DAG oil in $CDCl_3$ solutions

Signal	Chemical shift	Carbons
1′	72.09	C2′[a] (1,2-DAG)
3′	68.85	C2′ (TAG)[b]
4′	68.34	C2′ (1,3-DAG)
5′	65.14	C3′ (1-MAG)
6′	65.01	C1′ (3′) (1,3-DAG)
7′	63.30	C1′ (1-MAG)
8′	62.07	C1′ (TAG)
9′	61.98	C1′ (1,2-DAG)
10′	61.49	C3′ (1,2-DAG)
1	173.88	C1 (1,3-DAG) SFA
2	173.85	C1 (1,3-DAG) OL
3	173.83	C1 (1,3-DAG) LO
4	173.75	*sn*-1 C1 (1,2-DAG) SFA
5	173.71	*sn*-1 C1 (1,2-DAG) OL
6	173.69	*sn*-1 C1 (1,2-DAG) LO
7	173.40	*sn*-2 C1 (1,2-DAG) SFA
8	173.36	*sn*-2 C1 (1,2-DAG) OL
9	173.35	*sn*-2 C1 (1,2-DAG) LO
10	173.27	*sn*-1 C1 (TAG) SFA
11	173.23	*sn*-1 C1 (TAG) OL
12	173.22	*sn*-1 C1 (TAG) LO
13	172.81	*sn*-2 C1 (TAG) SFA, OL, LO
14	131.92	C16 (DAG, TAG) LN
15	130.19	C13 (DAG, TAG) (LO)
		C9 (DAG, TAG) (LN)
16	129.98	C10 (all acylglycerols) OL
		C9 (all acylglycerols) LO
17	129.68	C9 (all acylglycerols) OL
18	128.26	C12 (DAG, TAG) LN
19	128.20	C13 (DAG, TAG) LN
20	128.04	C10 (DAG, TAG) LO
21	127.86	C12 (DAG,TAG) LO
22	127.72	C10 (DAG, TAG) LN
23	127.08	C15 (DAG, TAG) LN
24	34.23	C2 (MAG, 1,2-DAG) OL, LO, LN
25	34.16	C2 (all acylglycerols) LN
26	34.05	C2 (1,3-DAG) all acyl chains
27	33.99	C2 (TAG) (OL)
28	31.89	C16 (all acylglycerols) SFA
29	31.87	C16 (all acylglycerols) OL
30	31.49	C16 (all acylglycerols) LO, LN
31	29.73	C12 (all acylglycerols) OL
32	29.68	C7 (all acylglycerols) OL
33	29.66	C7 (all acylglycerols) LO
34	29.63	C7 (all acylglycerols) LN
35	29.56	C14 (MAG, DAG, TAG) OL
36	29.49	C6 (all acylglycerols) OL, SFA

Table 2 continued

Signal	Chemical shift	Carbons
37	29.31	C13, C15 (all acylglycerols) OL
38	29.29	C6, C15 (all acylglycerols) LO, LN
39	29.16	C5 (1,2- DAG, TAG) all acyl chains
40	29.12	C5 (1-MAG, 1,3-DAG) all acyl chains
41	29.08	C4 (all acylglycerols) SFA
42	29.06	C4 (all acylglycerols) OL, LO, LN
43	27.19	C8 (all acylglycerols) LN
44	27.16	C8, C11 (all acylglycerols) OL
45	27.14	C8 (all acylglycerols) LO
46	27.12	C14 (MAG, DAG, TAG) (LO)
47	25.60	C11 (all acylglycerols) OL, LO, LN
48	25.50	C14 (all acylglycerols) LN
49	24.88	C3 (all acylglycerols) SFA
50	24.84	C3 (all acylglycerols) LO,OL
51	24.80	C3 (all acylglycerols) LN
52	22.65	C17 (all acylglycerols) OL, SFA
53	22.54	C17 (all acylglycerols) LO
54	20.52	C17 (all acylglycerols) LN
55	14.24	C18 (all acylglycerols) LN
56	14.08	C18 (all acylglycerols) OL
57	14.04	C18 (all acylglycerols) LO

[a] Primed and unprimed letters denote glycerol and acyl chains carbons, respectively

[b] The meaning of the symbols is as follows: *All acylglycerols* MAG, DAG, TAG; *MAG* monoacylglycerol; *1,2-DAG* 1,2-diacylglycerols; *1,3-DAG* 1,3-diacylglycerols; *SFA* saturated fatty acids; *OL* oleic acid; *LO* linoleic acid; *LN* linolenic acid; *sn-2* 2-position and *sn-1* (*3*) 1 (3)-position on the glycerol backbone, respectively

of the TAG species through the cross-peak observed between the corresponding carbon signal at δ 62.07 and this proton, which is overlapped by the strong signals of the 1,3-DAG glycerol protons. The same experiment was able to assign the carbon resonances of the methyl, allylic and bis-allylic carbons of linolenic acid, as well those of linoleic acid. The relevant chemical shifts are depicted in Table 2. The NMR signals of the carbonyl carbons have been assigned by using the gradient selected HMBC experiment, which connects carbon and proton resonances that are separated by more than one bond. This experiment optimized for three bonds C–H coupling was able to assign the C1 carboxyl carbon resonances of the various acyl chains in the region δ 174–δ 172. The portion of the *g*HMBC spectrum of DAG oil in Fig. 5a illustrates for the first time cross-peaks which correlate the protons of the glycerol segment with the carboxyl carbons of the attached fatty acid chains. Earlier assignments of these carbon signals for vegetable oils were explained by taking into consideration the double bonds inductive effect on the carbonyl groups [8, 33]. The carbonyl signals are grouped

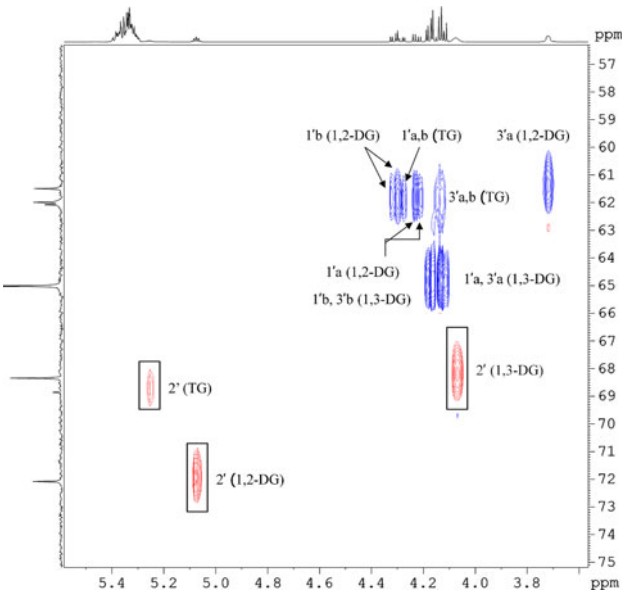

Fig. 4 600 MHz HSQC-DEPT spectrum of DAG olive oil in $CDCl_3$ solution, showing one bond correlations between the glycerol backbone protons and carbons; negative (*unframed*) signals for the CH_2 carbons and positive signals (*framed*) for the CH carbons

into three clusters corresponding to 1,3-DAG (δ 173.92–δ 173.82), 1,2-DAG (δ 173.84–δ 173.30), and TAG (δ 172.91–δ 172.84). The second and third cluster involves two sets of signals at low and high frequencies (Fig. 5a). The low frequency set of carbonyl signals corresponds to chains esterified at the *sn*-2 position of glycerol as indicated by the cross peaks connecting these carbon signals with the H2′ glycerol protons. The cross-peaks between the high frequency set of carbonyl signals and the H1′ and H3′ signals of glycerol in DAG and TAG identify the chain esterification at the *sn*-1(3) glycerol position. The shift difference observed for the carbonyl carbons at *sn*-2 and *sn*-1(3) positions was attributed to the two γ-gauche interactions experienced by the CO group at *sn*-2 position against just one interaction for the carboxyl carbon of the 1(3)-chains [8]. According to earlier ^{13}C-NMR assignment of plant oils, the unsaturated chains esterify preferentially the glycerol moiety at *sn*-2 position, whereas saturated chains are present at the *sn*-1(3) positions [8, 9, 29]. These findings are in very good agreement with the most widely accepted 1,3-random-2-random theory [34] of fatty acids distribution amongst the glycerol positions, although recent results [35] support the conclusion that the unsaturated fatty acids deviate from 2-random distribution in TAG. At any rate, this theory seems to be inappropriate for DAG oil, inasmuch the various acylglycerols entities in this oil were not the product of natural biosynthesis as in vegetable oils. DAG oil was formed enzymatically by lipase-catalysed esterification of glycerol with fatty acids using lipases of various specificities, including non-specific ones [3].

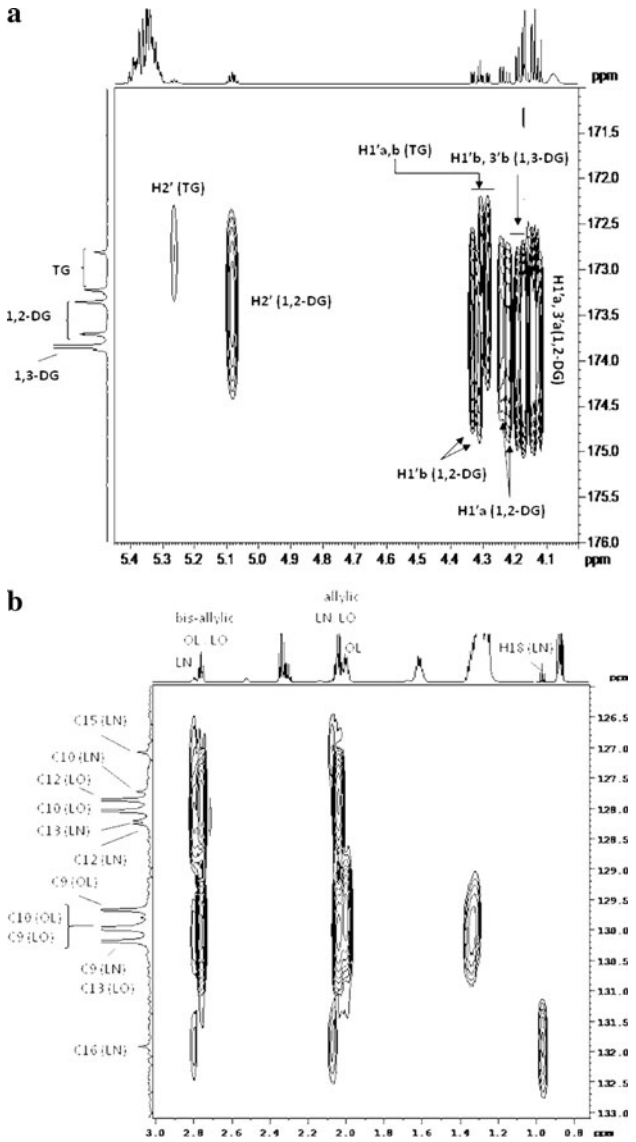

Fig. 5 600 MHz *g*HMBC spectrum of DAG oil in $CDCl_3$ solutions, showing correlations **a** between the carbonyl carbons and the glycerol backbone protons, and **b** between the olefinic carbons and the allylic and bis-allylic protons of the oleyl, linoleyl and linolenyl chains

Furthermore, within each set of signals the saturated, oleyl, and linoleyl chains appear from higher to lower frequency as deduced from triacylglycerol model compounds [8, 9, 29]. Also, signals from linolenyl chains usually overlap with those of the linoleyl chains. Thus, the 1,3-DAG cluster comprises three resonances at δ 173.88, δ 173.85, and δ 173.83 attributed to saturated, oleate and linoleate chains, respectively. Saturated chains (δ 173.75) oleyl (δ 173.71) and linoleyl (δ 173.69) at *sn*-1(3) positions of 1,2-DAG resonate at higher frequency from chains at *sn*-2 position, namely, saturated chains (δ 173.40), oleyl (δ 173.35) and linoleyl (δ 173.34) chains. Following the same reasoning as in 1,2-DAG, the assignment of the carboxyl signals in the triacylglycerol cluster is straightforward and depicted in Fig. 5a. The inset of Fig. 3 shows an expansion of the carbonyl region of DAG oil for easier inspection. The presence of saturated chains at *sn*-2 glycerol position supports our earlier hypothesis that saturated acyl chains are distributed in both *sn*-1(3) and *sn*-2 positions of glycerol segment, and this feature may differentiates the DAG oil from vegetable oils. The cross-peaks in the optimized for two bonds coupling *g*HMBC spectrum (not shown) between the carboxyl carbons and the neighboring methylene protons of the acyl chains confirms the origin of the triplets covering the range δ 2.35–δ 2.29. These resonances correspond to the methylene protons of the 1,3-DAG, 1,2-DAG and TAG acyl chains from a higher to a lower frequency in that order. The assignment of the olefinic signals of vegetable oils has been performed in earlier studies [7–9, 28–31] and the locations of these signals are well known. In the *g*HMBC spectra of DAG oil, cross-peaks were observed between the olefinic carbons and the corresponding allylic and bis-allylic protons of oleyl, linoleyl, and linolenyl chains determined previously (Fig. 5b). Unambiguous assignment was offered for the C16 carbon (δ 131.92) of linolenic acid, which correlates with the methyl protons (δ 0.97). No other carbon signals of the acyl chains could be identified in the *g*HMBC spectra due to the severe overlap of proton signals already mentioned.

To verify previous assignments and possibly to establish new ones, we performed an HSQC-TOCSY experiment. This experiment is useful when overlap in the proton spectrum prevents analysis, while the corresponding carbons are well resolved, or alternatively, the origin of proton signals is known and that of the carbon signals is sought. Cross-peaks are seen between the *J*-coupled protons in a spin network and each carbon involved in this network. The HSQC-TOCSY method has so far been used to analyze the positional distribution of unsaturated chains in triacylglycerol model compounds [32] and to correlate carbon signals with the adjacent allylic protons of unsaturated fatty acids [36, 37]. As an example, Fig. 6 illustrates the *g*HSQC-TOCSY spectrum of DAG oil in the region between 0 and 36 ppm, where the allylic and bis-allylic carbons of the unsaturated chains resonate. Unambiguous assignments of carbon-proton pairs not only connected over two bonds, but also in more remote positions could be easily achieved. The adopted mixing time furnished the assignment of several carbon atoms provided that the resolution in the carbon dimension was sufficient. Accordingly, the assignment of allylic and bis-allylic carbons of the various acyl chains was established. For instance, starting from the pairs H7, C17 of linolenic acid, direct assignment of the allylic carbons C17, C16, C14, C11, and C8 of three acyl chains (OL, LO, LN) could be achieved. By the same token, the pairs of the bis-allylic protons allowed the assignment of the bis-allylic carbon nuclei, C11 and C14 of the unsaturated acyl chains.

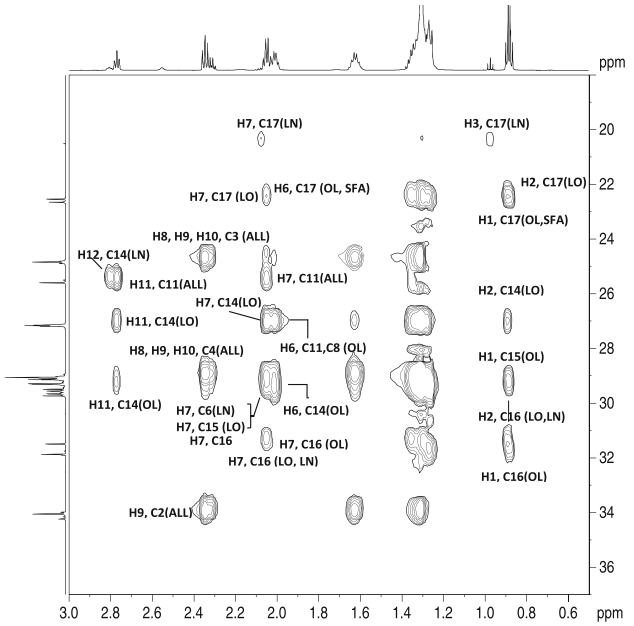

Fig. 6 600 MHz gHSQC-TOCSY spectrum of DAG oil in $CDCl_3$ solution, showing consecutive connectivities between carbons and protons along a common coupling pathway. The numbering system in Table 1 for proton resonances was adopted

^{31}P-NMR Spectra

Figure 7 illustrates the ^{31}P-NMR spectrum of DAG oil after its phosphitilation with the phosphorus reagent **1**. The chemical shifts of the phosphitylated hydroxyl groups of mono-, and diacylglycerols are well established [10, 20]. The signal of the phosphitylated hydroxyl group of tocopherols appears at δ 144.87 (Fig. 7). Integration of these signals in combination with the internal standard cyclohexanol allowed an easy quantification of the partially esterified glycerols and tocopherols, the latter compounds being determined as a whole.

Quntitative Analysis of DAG Oil Samples

^{1}H, ^{13}C, and ^{31}P-NMR spectroscopy has been used widely in quantitative analysis provided some precaution to be taken into consideration, e.g., differences in the longitudinal relaxation of the various nuclei in the molecule, NOE effects on signal intensities, pulse imperfections, etc. All these factors as well as their impact on quantitative measurements have been described in detail, and remedies have been suggested [11, 38, 39]. The carbon and phosphorus nuclei appear to be more sensitive to these factors since they usually convey a different number of protons in the molecule. Earlier experiments [10, 12] using quantitative ^{31}P NMR showed that the inverse gated decoupling technique in combination with the paramagnetic chemical shifts reagent $Cr(acac)_3$ and a long relaxation delay, about five times the longest relaxation time $NT_1 = 4.6$ s of the internal standard, is sufficient for obtaining quantitative results.

Earlier T_1 measurements in acylglycerols have shown [8, 23, 27, 29, 30, 40] that the NT_1s (N is the number of the directly attached protons) of the glycerol carbons ranged between 0.35 and 0.55 s, whereas those of the protonated acyl chain carbons was dependent on the degree of unsaturation, but they usually increased progressively from carbon C2 to the terminal carbon due to the cumulative C–C internal rotations. An increase in the NT_1s was observed for the unsaturated carbons bearing single hydrogens, and because the internal motions slow down at this site of the acyl chain [41]; their NT_1s ranged from 2.1 to 3.2 s. The highest NT_1s values for an acyl chain of 18 carbon atoms were observed for the terminal methyl carbons (5.3–5.7 s), and for the carbonyl carbons bearing no hydrogens (3.4–5.6 s). Our relaxation measurements using the null method were focused on those carbon nuclei that would be used to quantify the acylglycerols and fatty acid.

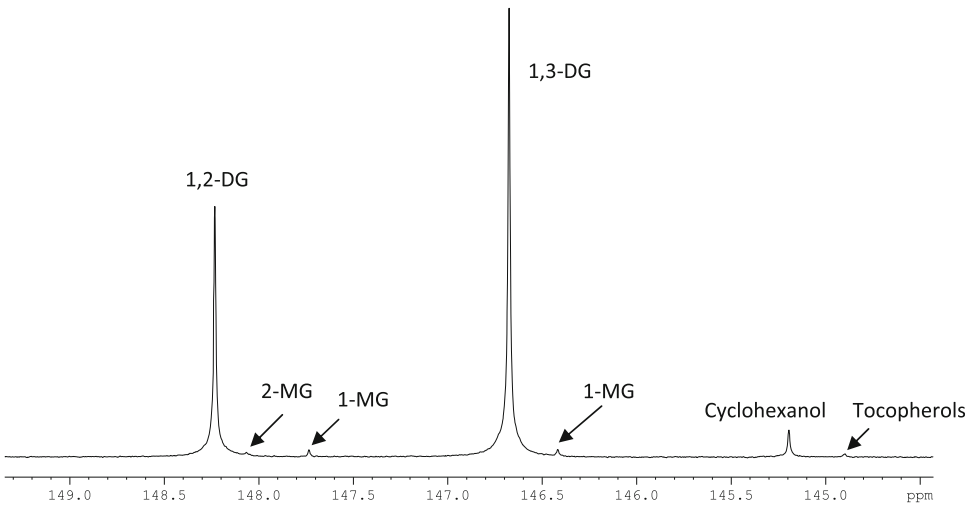

Fig. 7 202.2 MHz ^{31}P-NMR spectrum of the phosphitylated DAG oil in pyridine-chloroform solutions

Our measured NT_1s values were within the time scales mentioned above, namely 0.26 and 0.55 s for the glycerol methylene and methane carbons, respectively, 2.85 to 3.10 s for the olefinic carbons and 4.23 to 4.96 s for the carbonyl carbons. Apart from the quaternary carbonyl carbons, the measured NOE factors were found 3.0 ± 1.0 for all protonated carbons, indicating that the dominant relaxation process is the dipole–dipole ^{1}H-^{13}C interactions. Moreover, the measured NOE factors (1.70–1.80) demonstrated that the carbonyl carbons of different hydrocarbon chains in different acylglycerols were affected by proton decoupling to the same extent in agreement with earlier observations. As a result, the ^{13}C spectra can be acquired under full NOE conditions with the benefit of working with higher signal-to-noise ratios in shorter experiment times. Table 3 contains the quantitative results for acylglycerols and tocopherols of six samples of DAG oil obtained by the three NMR methods. Inspection of this table reveals a number of useful characteristics: (a) all three spectroscopic techniques show comparable results regarding the measured concentrations of the acylglycerol entities. (b) The concentration range of the various acylglycerols is within the range expected for DAG oil [1–3]. (c) ^{31}P NMR is the sole NMR technique that gives information about both MAG isomers and tocopherols, although this methodology was unable to measure the TAG concentration. (d) ^{1}H NMR is the faster of all, whereas derivatization is required to obtain ^{31}P-NMR spectra. (e) Contrary to ^{31}P-NMR techniques, ^{1}H and ^{13}C NMR needs no internal standard for the calculation of the absolute concentrations of the DAG oil constituents.

Table 3 Acylglycerols and tocopherols composition of six DAG oil samples determined by multinuclear (^{1}H, ^{13}C, ^{31}P) NMR spectroscopy

Components/samples	1	2	3	4	5	6
^{1}H NMR						
1-MAG	0.41	0.55	0.52	0.58	0.52	0.55
1,2-DAG	29.50	27.80	29.42	28.40	28.68	28.98
1,3-DAG	56.75	61.27	62.10	60.84	59.67	60.29
TAG	12.35	11.38	10.96	10.18	11.12	10.18
^{31}P NMR						
1-MAG	0.43	0.57	0.55	0.60	0.55	0.56
2-MAG	0.38	0.52	0.37	0.48	0.46	0.38
1,2-DAG	30.71	27.80	28.32	29.68	29.20	29.28
1,3-DAG	57.05	60.22	61.42	60.32	59.40	60.19
Tocopherols	0.24	0.25	0.30	0.26	0.27	0.27
^{13}C NMR						
Glycerol carbons						
1,2-DAG	32.46	29.03	29.83	29.48	29.27	28.98
1,3-DAG	57.14	62.58	61.61	62.17	60.46	61.58
TAG	10.39	10.46	9.96	10.35	10.13	9.93
Carbonyl carbon						
1,2-DAG	29.73	29.76	31.01	31.79	30.02	29.19
1,3-DAG	57.85	60.03	59.76	59.79	60.47	60.23
TAG	12.42	10.71	10.23	9.85	10.51	10.58

1-MAG 1-monoacylglycerol, *2-MAG* 2-monoacylglycerol, *1,2-DAG* 1,2-diacyl glycerols, *1,3-DAG* 1,3-diacylglycerols, *TAG* triacylglycerols

Two NMR techniques were to be compared regarding the quantification of fatty acids. Acids concentration using ^{31}C NMR could be obtained by integration of the appropriate carbonyl and olefinic signals (Table 2) [8, 29, 33, 35, 40]. In ^{1}H NMR, most of the signals are assigned to non-equivalent groups of protons that are common to fatty acyl chains. Therefore, the concentration of fatty acids in DAG oil can be calculated only by combination of various signal intensities in the ^{1}H-NMR spectrum. An exception is the signal *E* at δ 0.97, which corresponds to the methyl protons of linolenic acid (LN). Therefore, the concentration of this acid is obtained from the relationship:

$$\mathrm{LN} = \mathrm{E}/(\mathrm{E} + \mathrm{F}) \quad (1)$$

F is the signal intensity of the methyl protons of all acids (δ 0.87–0.88) except linolenic acid. The remaining acids, oleic (OL), linoleic (LO), and total saturated fatty acids (mainly palmitic and stearic acids) (SFA) are calculated by the following relationships:

$$\mathrm{OL} = 3/4C - 3/2A1 - 3/4A2 \quad (2)$$

$$\mathrm{LO} = 3/2\mathrm{A1}/(\mathrm{E} + \mathrm{F}) \quad (3)$$

$$\mathrm{SFA} = 1 - (\mathrm{LN} + \mathrm{LO} + \mathrm{OL}) \quad (4)$$

A1, *A2*, and *C* are the integrals of bis-allylic protons of LO (δ 2.78), LN (δ 2.80) and allylic protons (δ 2.00) of all acids, but LN, respectively. The arithmetic coefficients in Eqs. 2 and 3 were used to normalize the different number of protons associated with each group. The results are summarized in Table 4.

The ^{1}H-NMR spectrum furnishes the total concentration of the acyl chains of the various acylglycerols of DAG oil, but it is unable to give concrete information about their positional distribution on the glycerol backbone. Notwithstanding, the superior resolution observed in ^{1}H-NMR spectra allowed the separate determination of linoleyl and linolenyl chains. The positional and compositional distribution of acyl chains was achieved by ^{13}C NMR using the carbonyl resonances extended in the high frequency region of the spectrum according to the degree of glycerol esterification and acyl chain positional distribution (Fig. 3). The concentration of the saturated fatty acids esterified at 1,3 glycerol positions was fairly constant amongst the DAG and TAG; average values (for six samples) 5.30% for 1,3-DAG, 5.42% for 1,2-DAG and 4.62% for TAG, and decreased to 3.63% for the saturated chains at the

Table 4 Fatty acids composition of six DAG oil samples as determined by ^{1}H and ^{31}C-NMR spectroscopy

Components/samples	1	2	3	4	5	6
^{1}H NMR						
SFA	3.11	2.48	3.31	2.91	2.09	2.11
OL	37.09	39.79	41.22	40.39	42.50	41.87
LO	53.00	51.22	49.28	50.20	50.00	50.04
LN	6.80	6.51	6.19	6.50	5.41	5.98
LO + LN	59.80	57.73	55.47	56.70	55.41	56.03
^{31}C NMR						
Carbonyl carbons						
1,3-diacylglycerols						
SFA	5.38	4.20	5.80	6.13	5.13	5.24
OL	40.86	40.41	40.06	39.87	40.40	38.42
LO + LN	53.76	55.39	54.14	53.9	54.47	56.34
1,2-diacylglycerols						
(1-position)						
SFA	4.78	5.23	6.12	6.89	4.48	5.01
OL	40.85	39.84	38.84	41.83	40.12	39.63
LO + LN	54.37	54.93	55.04	51.28	55.4	55.36
(2-position)						
SFA	4.51	3.68	4.01	2.53	3.23	4.83
OL	33.35	35.84	37.30	35.8	36.85	34.02
LO + LN	61.23	60.48	58.69	61.63	59.92	61.15
Triacylglycerols						
(1,3-position)						
SFA	3.35	5.86	4.43	4.85	4.56	4.72
OL	48.73	44.87	45.21	46.32	45.01	45.23
LO + LN	47.91	49.27	50.36	48.82	50.43	49.95
(2-position)						
SFA	6.96	6.23	7.21	5.98	7.45	7.92
Unsaturated	93.04	93.77	92.79	93.41	92.55	92.08
Olefinic carbons						
SFA	8.21	9.20	7.52	9.38	6.40	6.35
OL	37.80	38.01	38.77	36.21	39.46	39.80
LO	48.00	46.79	48.01	48.90	49.14	48.25
LN	6.00	6.00	5.70	5.51	5.00	5.06

SFA saturated fatty acids, *OL* oleic acid, *LO* linoleic acid, *LN* linolenic acid

2-position, indicating that even in DAG oil the saturated chains populate preferentially the primary hydroxyl groups of the glycerol segment. The percentages of the monounsaturated oleyl chain at the 1,3-position decreased from TAG (45.90%) to 1,3-DAG (40.00%) and 1,2-DAG (40.18%), whereas the opposite order was observed for the sum total of the polyunsaturated linoleyl and linolenyl chains at the same glycerol, namely TAG (49.46%), 1,3-DAG (54.70%) and 1,2-DAG (54.40%). On the other hand, the quantity of the unsaturated chains at the 2-position was significantly different than those measured at the 1,3-position (compare average values in Table 4); it was about ~11% lower for oleyl chains, and ~10% higher for linoleyl and linolenyl chains. This pattern demonstrates that the largest variation between the 1,3- and 2-positions occurred for the unsaturated chains.

The repeatability for each NMR methodology was checked by running six consecutive spectra of the same DAG oil sample adopting the same experimental protocol. The resonance areas of the separate signals of the glycerol backbone carbons and the phosphitylated hydroxyls for DAG in ^{13}C-NMR and ^{31}P-NMR spectra showed coefficients of variation <2%, whereas the coefficients of variation for the most overcrowded signals in ^{1}H-NMR spectra were found to be around 2.5%. This value exceeded 3% (close to 3.5%) for signals characterized by lower S/N ratio, such the monoacylglycerols signals in ^{31}P-NMR spectra and the carbonyl carbons in ^{13}C-NMR spectra, making the signal integration less accurate.

A final point that should be clarified is that values in Tables 3 and 4 represent relative concentrations for ^{1}H and ^{13}C NMR, but absolute concentrations for ^{31}P NMR. However, relative concentrations can be easily converted to absolute ones with a very good approximation, since the components determined by NMR consist of >99% of DAG oil total concentration.

Conclusions

^{1}H-, ^{13}C- and ^{31}P-NMR spectroscopy was proved to be a powerful multinuclear technique able to determine in qualitative and quantitative manner the full pattern of the complex mixture of DAG oil samples. The acylglycerols could be determined by the three nuclei with very similar results and within the concentration range expected for DAG oil. The quantitative analysis performed by integration of the appropriate signals preceded by a secure assignment of the corresponding signals making use of the modern 2D pulse sequences, such as TOCSY, HSQC-DEPT, etc. Regarding the effectiveness of the three NMR techniques, ^{31}P NMR appears to be more appropriate to determine the concentration of the partially esterified glycerol entities and other minor compounds bearing free hydroxyl groups despite the fact that the phosphitylation reaction destroys the sample and lengthens somewhat the duration of the experiment. This drawback is compensated by the high accuracy involved in the integration of single signals spread out at a large scale of chemical shifts, and hence lowering the cost of the analysis by purchasing cheaper NMR spectrometers operating at low magnetic field strength. Finally, the compositions of the two different pools of fatty acids esterifying the 1,3- and 2-positions were also determined. The best nucleus for this purpose

was ^{13}C and the shift range of the carbonyl carbons (172–189 ppm) was used.

Acknowledgments The 600 MHz NMR spectrometer used in this study was purchased with the CHE-0821552 grant from the NSF.

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