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HPLC Separation and NMR Structural Elucidation of *sn*-1,2-, 2,3-, and 1,3-Diacylglycerols from Olive Oil as Naphthylethylurethane Derivatives

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In this study, *sn*-1,2-, *sn*-2,3-, and *sn*-1,3-diacylglycerols were isolated from olive oil, and their urethane derivatives (urethanes) were prepared. Normal-phase high-performance liquid chromatography (NP-HPLC) separation of the urethane isomers was performed and the separate classes were studied by nuclear magnetic resonance (NMR). The use of ^{1}H NMR and homo- and heteronuclear 2D techniques provided a great amount of information in a very short time, particularly when a high-field NMR instrument (700 MHz) was used. Particularly diagnostic for this kind of compound was the glyceridic moiety that presents typical chemical shifts both for carbon and hydrogen. These studies show the usefulness of NMR spectroscopy to recognize clearly the *sn*-1,3- and, moreover, *sn*-1,2- with respect to *sn*-2,3-diacylglycerols, although very minor differences occur between them.

KEYWORDS: Diacylglycerol enantiomers; olive oil; NP-HPLC; NMR

INTRODUCTION

Virgin olive oil, according to the trade standard of International Olive Oil Council for olive oils and olive-pomace oils, is the oil obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions, particularly thermal, that do not lead to alterations in the oil, which has not undergone any treatment other than washing, decanting, centrifuging, and filtration (*1*). Research efforts in the field of olive oil chemistry over the years have been concerned with the establishment of analytical methodologies for detecting quality, adulteration, and geographical origin of olive oils. Spectroscopic methods combined with computer-aided statistical and mathematical procedures are the emerging analytical techniques in the field of olive oil chemistry. A great deal of experimental work is in progress to check the availability of these new techniques for detecting adulteration and determining the geographical origin of olive oils, because they improve on the time-consuming procedures based on chemical and physical data (*2*). To assess the quality of a virgin olive oil, both the amount and type of diacylglycerols are important. As *sn*-1,2- and *sn*-2,3-diacylglycerols present in freshly olive oils tend to isomerize to the more stable *sn*-1,3-diacylglycerols by transposition of an acyl group, the relative quantities of *sn*-1,2-, *sn*-2,3-, and *sn*-1,3-diacylglycerols are characteristic of the state (age and storage conditions) of an oil sample (*3*−*6*). While *sn*-1,2-diacylglycerols are natural intermediates of glyceride biosynthesis, *sn*-2,3-diacylglycerols can only result from nonstereospecific enzymatic hydrolysis of triglycerides or from nonenzymatic racemization of *sn*-1,2-diacylglycerols. In accordance with this assumption, the occurrence of *sn*-2,3-diacylglycerols could suggest that the original composition has been modified. From this point of view, it is very relevant to separate and characterize *sn*-1,3-, 1,2-, and 2,3-diacylglycerols.

In recent years, many methods have been developed to perform the chromatographic separation of enantiomeric diacylglycerols; chiral-phase high-performance liquid chromatography (HPLC) of enantiomeric 1,2- and 2,3-diacylglycerols on chiral stationary phase has been carried out exclusively for their ultraviolet (UV) detectable derivatives, such as 3,5-dinitrophenylurethanes (*7*−*9*) and 1,2(2,3)-di-*O*-benzoyl-3-*tert*-butyldimethylsilyl-glycerols (*10*), using HPLC and gas chromatography (*11*) or HPLC coupled with mass spectrometry (*12*). On the other hand, normal-phase HPLC separation of enantiomeric diacylglycerols on chiral silica gel columns has been carried out for their diastereomers prepared with either chiral UV or fluorescent labeling reagents, such as (*S*)-(+)- or (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate (*13*, *14*), (*R*)-(+)-1-phenylethyliso-

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cyanate (*15*), (*S*)-(+)-2-*tert*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid (*16*), and fluoro-(1-naphthyl)acetic acid (*17*). This study was designed to separate enantiomeric 1,2- and 2,3-diacylglycerols and regioisomeric 1,3-diacylglycerols as their naphthylethylurethanes by normal-phase HPLC with UV detection and then to give their NMR structural elucidation and to assess the importance of spectroscopic methods in the study of these lipid classes. Very few spectroscopic data on these lipids and in particular on diacylglycerol isomers and enantiomers are reported in the literature (*18*). To the best of our knowledge, this is the first study that reports complete mono- and two-dimensional spectroscopic data on the three individual classes of diacylglycerol derivatives.

MATERIALS AND METHODS

Commercial extra virgin olive oil was purchased from a local market.

Silica gel 60 F254 plates (Merck, Darmstadt, Germany) were used for analytical and preparative thin-layer chromatography (TLC). All organic solvents (analytical and HPLC grade), 4-pyrrolidinopyridine, and (*S*)-(+)-1-(1-naphthyl)ethyl-isocyanate were purchased from Sigma-Aldrich (St. Louis, MO). Commercial NMR-grade $CDCl_3$ was also purchased from Sigma-Aldrich.

Diacylglycerol Extraction from Olive Oil Samples. The olive oil sample (about 150 mg) was dissolved in *n*-hexane and was applied to a silica plate (20 × 20 cm, 0.25-mm thickness) previously treated with a 5% boric acid solution. The TLC plate was eluted using an *n*-hexane/diethyl ether solution (50/50, v/v). The *sn*-1,3-diacylglycerol and *sn*-1,2(2,3)-diacylglycerol fractions were revealed by iodine vapor and were extracted from silica with anhydrous diethyl ether (4 × 3 mL).

Synthesis of (*S*)-(+)-1-(1-Naphthyl)ethyl-urethane Derivatives. *sn*-1,2(2,3)- and *sn*-1,3-diacylglycerols, obtained from TLC, as previously described, were gently dried under a nitrogen stream and were dissolved in 5 mL of anhydrous toluene, to which was added 4 mg of pyrrolidinopyridine and 12.5 μL of (*S*)-(+)-1-(1-naphthyl)ethyl-isocyanate (*14*). The mixture was stirred for 16 h at 50 °C. After removing the solvent under a gentle stream of nitrogen, 6 mL of a methanol/water (95/5, v/v) solution was added. The crude mixture containing the derivatized diacylglycerols was filtered through a 500-mg SPE ODS column (Baker Analyzed, Phillipsburg, NJ), previously conditioned with 10 mL of the above methanol/water solution; the column was washed with 15 mL of the same solvent mixture, and then the fraction containing the diacylglycerols was recovered by elution with 10 mL of acetone. This fraction was dried under a light stream of nitrogen, and the sample was diluted with 100 μL of a mixture composed of *n*-hexane and 2-propanol containing 2% H_2O, (99.6/0.4; v/v).

HPLC Separation of Diacylglycerol Urethane Derivatives. Twenty microliters of the sample was injected to perform the HPLC separation and was eluted with the above *n*-hexane/2-propanol mixture at 1 mL/min (*14*), in isocratic conditions, using the following equipment: Rheodyne 7725i HPLC injector, Shimadzu LC 10A-D HPLC pump; two 250 × 4.6 mm i.d., 3 μm, Hypersil columns in series (HiChrom Ltd, Berkshire, United Kingdom) enclosed in a plexiglass cylinder thermostated by a cooler at −15 °C; and Shimadzu SPD-10A UV detector, operated at 280 nm. The data were acquired through Shimadzu Class VP 4.3 software on a PC IBM station.

NMR Experiments. ^{1}H NMR and ^{13}C NMR spectra were recorded using $CDCl_3$ as deuterated solvent on a Brucker Avance DPX 400 spectrometer at frequencies of 400.13 and 100.62 MHz, respectively, or on a Varian UnityInova spectrometer at a frequencies of 700 and 175 MHz, respectively; δ values are given in ppm, and the coupling constants *J* are expressed in Hz. ^{13}C signals were assigned with the aid of J-modulated spin echo (C, H) spectroscopy (JMOD) and ^{1}H−^{13}C correlation experiments (heteronuclear multiple quantum correlation spectroscopy (HMQC), heteronuclear multiple bond correlation spectroscopy (HMBC)). ^{1}H signals were assigned with the aid of correlation spectroscopy (COSY) ^{1}H−^{1}H.

sn-1,2-Diacyl-3-[(S)-(+)-1-(1-naphthyl)ethyl-urethan]glycerol. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (t, 6, J = 7 Hz, 2 × CH_3), 1.30 (m, ∼48, $(CH_2)_n$), 1.59 (bs, 4, 2 × C$H_2$$CH_2$CO), 1.69 (d, 3, J = 7 Hz, CH_3), 2.03 (m, 8, 4 × CH_2C=), 2.31 (m, 4, 2 × CH_2CO), 4.15 (dd, 1, J = 12, 6 Hz, HC_1HO), 4.20 (dd, 1, J = 12, 6 Hz, HC_3HO), 4.31 (m, 2, HC$_1$HO + HC$_3$HO), 5.08 (d, 1, J = 7.4 Hz, NH), 5.28 (m, 1, CHO), 5.37 (m, 4, olefinics), 5.68 (m, 1, NHC$H$$CH_3$), 7.53 (m, 4, ArH), 7.81 (d, 1, J = 7.5 Hz, ArH), 8.12 (d, 1, J = 7.9 Hz, ArH), 8.13 (d, 1, J = 8 Hz, ArH).

^{13}C NMR (100 MHz, $CDCl_3$): δ 14.4, 21.9, 23.0, 25.1, 27.46, 27.51, 29.3, 29.41, 29.45, 29.6, 29.8, 29.99, 30.05, 32.2, 34.3, 34.5, 47.0, 62.4, 63.2, 69.5, 122.5, 123.4, 125.5, 126.1, 126.8, 128.3, 128.6, 129.2, 130.0, 130.5, 134.2, 138.7, 155.2, 173.2, 173.6.

sn-1-[(S)-(+)-1-(1-Naphthyl)ethyl-urethan]-2,3-diacyl-glycerol. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (t, 6, J = 7 Hz, 2 × CH_3), 1.29 (m, ∼48, $(CH_2)_n$), 1.60 (bs, 4, 2 × C$H_2$$CH_2$CO), 1.69 (d, 3, J = 7 Hz, CH_3), 2.03 (m, 8, 4 × CH_2C=), 2.31 (m, 4, 2 × CH_2CO), 4.15 (dd, 1, J = 12, 6 Hz, HC_3HO), 4.23 (dd, 1, J = 11, 6 Hz, HC_1HO), 4.29 (m, 2, HC$_1$HO + HC$_3$HO), 5.09 (d, 1, J = 7.5 Hz, NH), 5.26 (m, 1, CHO), 5.37 (m, 4, olefinics), 5.67 (m, 1, NHC$H$$CH_3$), 7.53 (m, 4, ArH), 7.82 (d, 1, J = 7.9 Hz, ArH), 7.90 (d, 1, J = 7.5 Hz, ArH), 8.11 (d, 1, J = 7.9 Hz, ArH).

^{13}C NMR (100 MHz, $CDCl_3$): δ 14.4, 21.9, 23.0, 25.1, 27.48, 27.52, 29.3, 29.4, 29.5, 29.6, 29.8, 30.0, 30.1, 32.2, 34.3, 34.5, 47.0, 62.4, 63.1, 69.4, 122.6, 123.4, 125.6, 126.1, 126.8, 128.3, 128.6, 129.2, 130.0, 130.3, 131.1, 134.2, 138.7, 155.2, 173.2, 173.6.

sn-1,3-Diacyl-2-[(S)-(+)-1-(1-naphthyl)ethyl-urethan]glycerol. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.90 (t, 6, J = 7 Hz, 2 × CH_3), 1.28 (m, ∼48, $(CH_2)_n$), 1.59 (m, 4, 2 × C$H_2$$CH_2$CO), 1.69 (d, 3, J = 6.7 Hz, CH_3), 2.02 (m, 8, 4 × CH_2C=), 2.23 (t, 2, J = 7.3 Hz, CH_2CO), 2.36 (t, 2, J = 7.3 Hz, CH_2CO), 4.17 (dd, 1, J = 12, 6 Hz, HCHO), 4.19 (dd, 1, J = 12, 6 Hz, HCHO), 4.22 (dd, 1, J = 12, 5 Hz, HCHO), 4.28 (dd, 1, J = 12, 5 Hz, HCHO), 5.10 (d, 1, J = 8 Hz, NH), 5.22 (m, 1, CHO), 5.37 (m, 4, olefinics), 5.68 (m, 1, NHC$H$$CH_3$), 7.53 (m, 4, ArH), 7.82 (d, 1, J = 8 Hz, ArH), 7.90 (d, 1, J = 8 Hz, ArH), 8.14 (d, 1, J = 8.3 Hz, ArH).

^{13}C NMR (100 MHz, $CDCl_3$): δ 14.4, 21.9, 23.0, 25.0, 25.1, 27.45, 27.50, 29.4, 29.6, 29.8, 30.0, 30.1, 32.2, 34.2, 34.4, 47.1, 62.6, 70.0, 122.5, 123.4, 125.5, 126.1, 128.6, 129.2, 129.6, 130.0, 130.3, 134.2, 138.7, 154.7, 173.5.

RESULTS AND DISCUSSION

The in-depth characterization of *sn*-1,2-, 2,3-, and 1,3-diacylglycerols was developed for two reasons: to obtain pure samples of diacylglycerol isomeric classes and to determine significant differences in the ^{1}H NMR spectra between the three (*S*)-(+)-1-(1-naphthyl)ethyl-urethane diacylglycerols derivatives.

In **Figure 1**, the HPLC separation performed on diacylglycerol derivatives is shown; it was possible to obtain pure samples of three classes of diacylglycerol derivatives from the HPLC collected fractions.

While the characterization of *sn*-1,3-diacylglycerols is rather easy, because it exists as single isomer, the characterization of the *sn*-1,2- and *sn*-2,3-diacylglycerols can be particularly demanding. For the characterization of diacylglycerols by NMR, the glyceridic moiety is particularly diagnostic presenting typical chemical shifts both for carbon and hydrogen; in particular, the spectroscopic differences were more significant when the NMR experiments were carried out with a 700 MHz spectrometer. In a typical ^{1}H NMR experiment, it is possible to recognize practically all protons of a diacylglycerol, but particularly evident are the protons from fatty acid residues, showing several signals in the aliphatic range (0.9−2.3 ppm) and in the olefinic range (∼5.3 ppm). Usually, the glyceridic moiety falls in the typical range of alcoholic protons and appears as partially overlapped multiplets. Olive oil diacylglycerols are esterified mainly by oleic acid (>70%) besides other minor fatty acids of similar chain length; this is not a real problem because usually the long-chain fatty acids do not affect the NMR chemical shifts

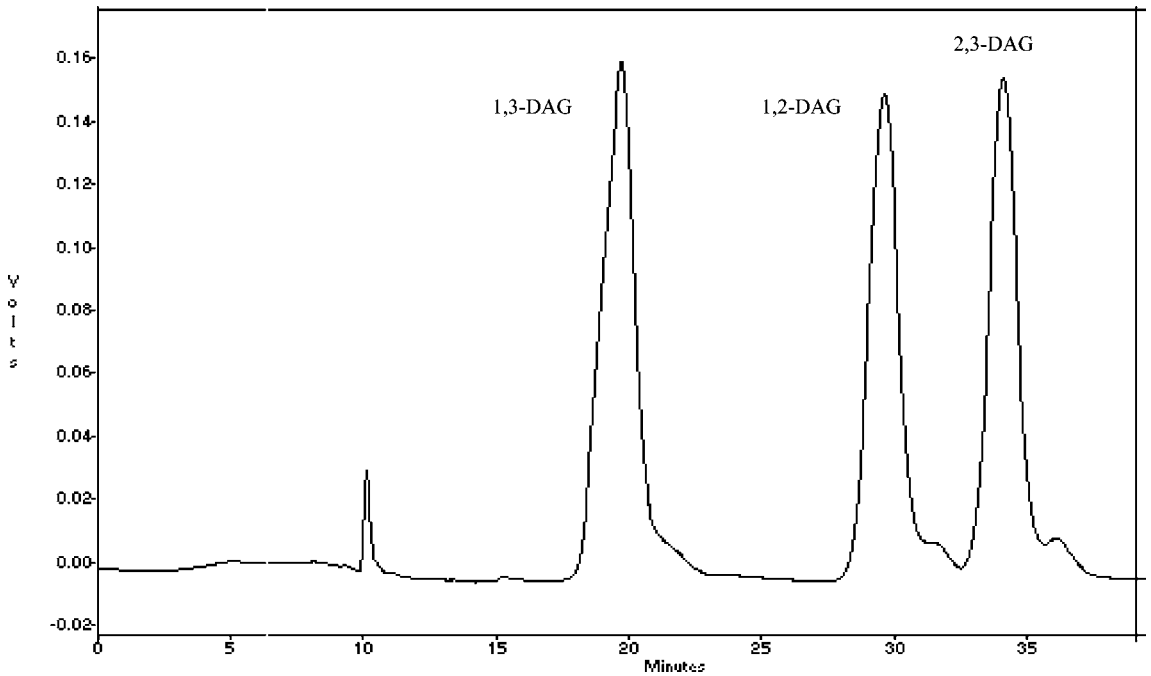


Figure 1. HPLC separation of the three classes of diacylglycerol urethane derivatives.

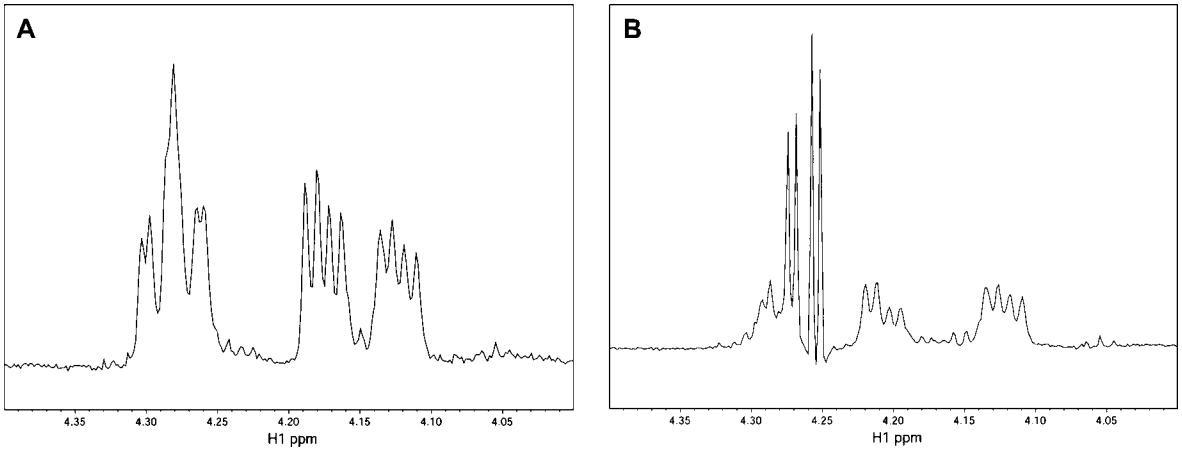


Figure 2. Expanded ^{1}H NMR area of glyceridic moiety recorded at 700 MHz of (**A**) *sn*-1,2-diacylglycerol and (**B**) s*n*-2,3-diacylglycerols.

Table 1. Chemical Shifts, Multiplicity, and Coupling Constants of Protons Bonded to C-1 and C-3 of Glyceridic Moiety of (A) *sn*-1,2-DAG and (B) *sn*-2,3-DAG

	proton	δ (ppm)	mult.	J_1 (Hz)	J_2 (Hz)
A	C_1–H_a	4.12	dd	6	12
	C_1–H_b	4.26	dd	6	12
	C_3–H_a	4.17	dd	4	
	C_3–H_b	4.29	dd	4	
B	C_1–H_a	4.20	dd	5	12
	C_1–H_b	4.26	dd	4	12
	C_3–H_a	4.12	dd	6	12
	C_3–H_b	4.28	m		

of the glyceridic moiety. For these reasons, the NMR results can be considered relative to homogeneous diacylglycerol classes.

Another problem that sometime occurs in ^{1}H NMR experiments is the overlapping of important signals that make difficult the assignment of a particular chemical shift. For instance, the ^{1}H NMR signals related to the proton on C-2 of the glyceridic moiety of *sn*-1,2- and *sn*-2,3-diacylglycerols derivatized as Mosher esters (*18*) lie in the same chemical shifts area as the protons bound to C-1 and C-3, preventing the chemical shift assignment of protons of the glyceridic moiety. During our attempt to separate and characterize the diacylglycerol (*S*)-(+)-1-(1-naphthyl)ethyl-urethane derivatives, we found that the glyceridic moiety in ^{1}H NMR experiments resulted in a more clear pattern of signals using deuterated chloroform as solvent. In a typical ^{1}H NMR experiment, it is possible to observe the chemical shift of the proton on C-2 around 5.2 ppm, while the signals of the protons bound to C-1 and C-3 fall in a typical range of 4.0−4.5 ppm. The value of 5.2 ppm is not anomalous for the proton on C-2, in fact, it is typical for the glyceridic moiety in triacylglycerols (*19*). This aids in the ^{1}H NMR spectra elucidation so it is easier to observe a different distribution of the signals in the chemical shifts area of protons on C-1 and C-3. In this case, the multiplicity of the protons on C-1 and C-3 appear as four doublets of doublets, two of which are partially overlapped. The importance of these results is that *sn*-1,2- and 2,3-diacylglycerols have significant differences in the range 4.0−4.5 ppm which can be used to achieve the assignment of the protons on C-1 and C-3. These preliminary results encouraged us to exploit other NMR techniques for an in-depth

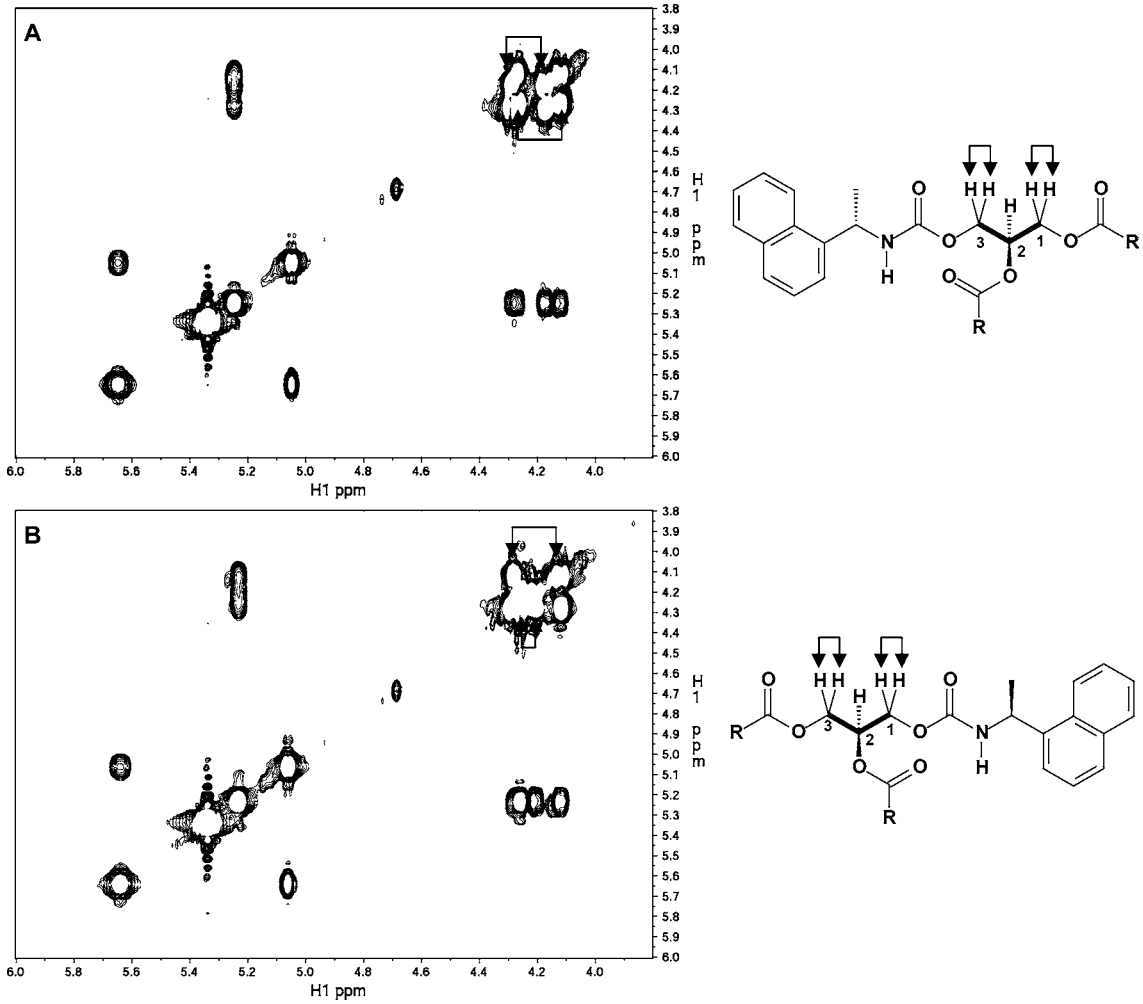
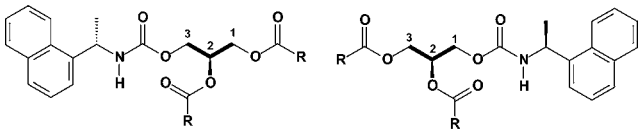


Figure 3. Expanded COSY area of glyceridic moiety recorded at 700 MHz of (**A**) s*n*-1,2-diacylglycerol derivatives and (**B**) s*n*-2,3-diacylglycerol derivatives.

Table 2. Correlations C–H of the Glyceridic Moiety



	C_1–H	C_2–H	C_3–H	C_1–H	C_2–H	C_3–H
^{13}C (ppm)	62.4	69.5	63.2	63.1	69.4	62.4
^{1}H (ppm)	4.12 4.26	5.22	4.17 4.29	4.20 4.26	5.26	4.12 4.28

investigation to define a better characterization of the spectroscopic differences between *sn*-1,2 and 2,3-diacylglycerols.

Panels **A** and **B** of **Figure 2** show a typical pattern of ^{1}H NMR signals of glyceridic moiety of *sn*-1,2- and 2,3-diacylglycerols, respectively. In **Table 1** is reported the final assignment of chemical shifts of protons bonded to C-1 and C-3. The complete ^{1}H-chemical shifts data set assignments are reported under Materials and Methods for each diacylglycerol. Because of the similar geminal coupling constants ($J \cong 12$ Hz), the assignment of the two protons on C-1 and the two protons on C-3, of the glyceridic moiety, cannot be done solely on the basis of a ^{1}H NMR experiment but a ^{1}H−^{1}H two-dimensional experiment (COSY) can be used for this purpose. Correlation spectroscopy (*20*) (COSY) is a homonuclear 2D technique that is used to correlate the chemical shifts of ^{1}H nuclei which are *J*-coupled to one another.

As shown in **Figure 3A**,**B**, these experiments permitted recognition of which protons are coupled with the corresponding geminal constant in the 4.0−4.4 ppm range of the two diacylglycerols.

The transformation of diacylglycerols into 1-(1-naphthyl)-ethyl-urethane derivatives influenced the chemical shifts in ^{1}H NMR spectra, simplifying the assignment of protons on the glyceridic moiety. In contrast, the influence on ^{13}C NMR spectra was less remarkable.

The large spectral width of ^{13}C resonance frequency permits a good resolution of the signals for each carbon atom of the glycerol moiety of the diacylglycerol derivatives, but by itself does not introduce further information on these derivatives. Instead, a JMOD experiment can supply more information. The advantage of these ^{13}C experiments is such that CH_3 and CH carbons are phased opposite to CH_2 and quaternary carbons. As reported in **Table 2**, the carbon chemical shift values of *sn*-1,2-, *sn*-2,3, and *sn*-1,3-diacylglycerol derivatives fall in a range that is reasonably similar to other DAG-derivatives (*18*). However, of particular interest were the two-dimensional experiments of C−H correlation HMQC and HMBC, affording important information about the glyceridic moiety.

Heteronuclear multiple quantum correlation spectroscopy (*21*, *22*) (HMQC) is an inverse chemical shift correlation experiment

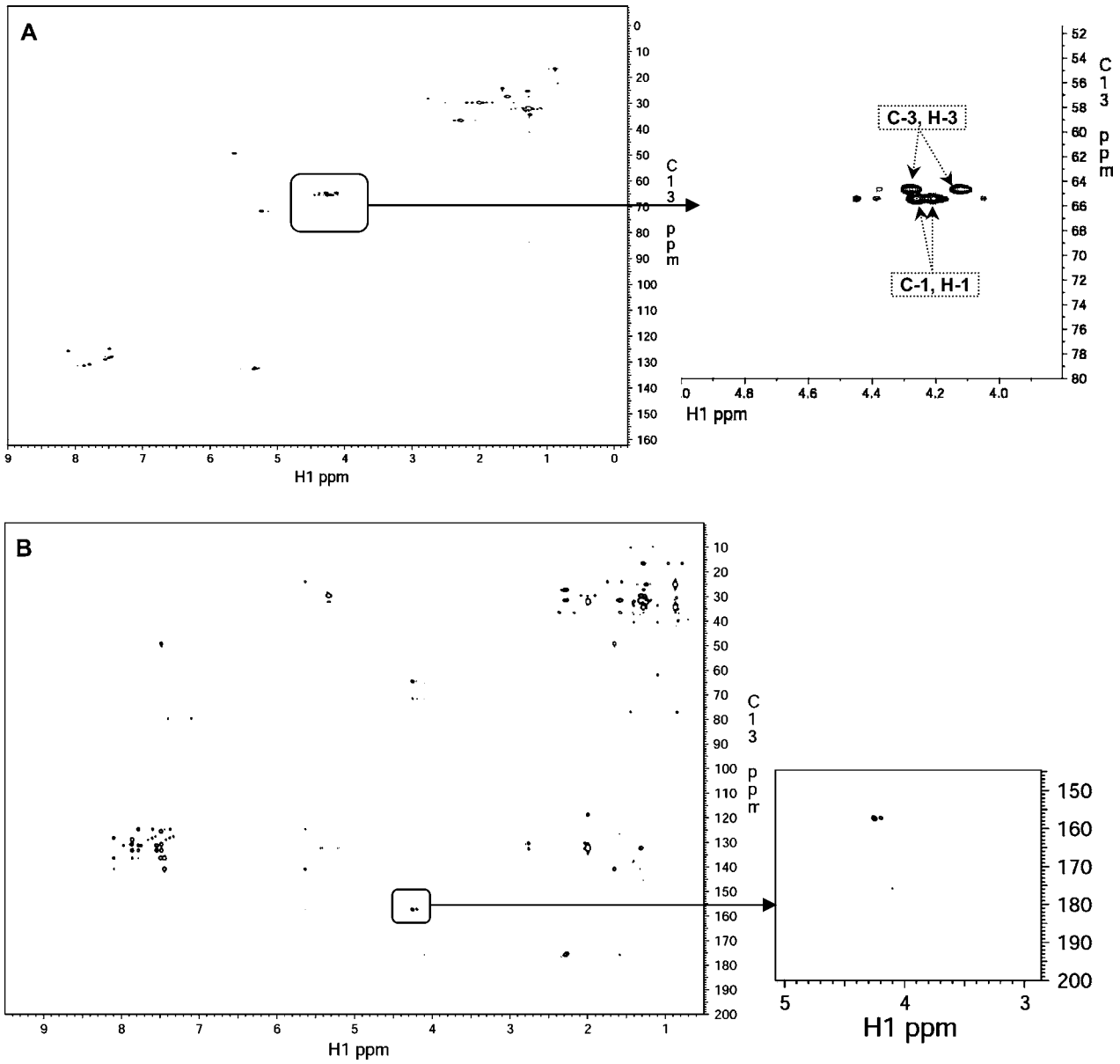


Figure 4. (**A**) HMQC spectrum of s*n*-2,3-diacylglycerol derivatives and enlargement of the area showing the coupling between H-1 and C-1 and between C-3 and H-3; (**B**) HMBC spectrum of s*n*-2,3-diacylglycerol derivatives and enlargement of the area showing the coupling between H-1 and carbamic carbon.

that, like heteronuclear (X, H) shift correlation spectroscopy (XHCORR), is used to determine which ^{1}H of a molecule is bonded to which ^{13}C nuclei (or other X nuclei). The advantage of HMQC over XHCORR is that in HMQC the nucleus with the highest γ (^{1}H) is detected, and so it is possible to obtain the highest sensitivity. This type of experiment was particularly useful to assignments previously made by COSY experiments for the glyceridic moiety of *sn*-1,2- and 2,3-diacylglycerols. As shown in **Figure 4A** for *sn*-2,3-diacylglycerols, it is possible to see the correlation of the protons at 4.12 and 4.28 ppm with the carbon at 62.4 ppm and the protons at 4.21 and 4.26 ppm with the carbon at 63.1 ppm. This information resolved doubts of the attribution of protons at 4.26 and 4.28 ppm because of the partial overlapping of these protons. The same experiment was made on *sn*-1,2-diacylglycerols and even in this case it is possible to observe a correlation between the CH_2 carbons and the corresponding protons of the glyceridic moiety. In **Table 2** are summarized the values for these kind of correlations.

Although a comparison with the data already reported in the literature could be used to establish which carbon of the glyceridic moiety was the carbon number 1 (*18, 19*), we tried to perform a C−H long-range correlation, such as HMBC, for this purpose. Heteronuclear multiple bond correlation spectroscopy (*21*−*24*) (HMBC) is a modified version of HMQC suitable for determining long-range $^{1}H-^{13}C$ connectivity. This is useful in determining the structure and ^{1}H and ^{13}C assignments of molecules. Since it is a long-range chemical shift correlation experiment, HMBC provides basically the same information as COLOC; however, since it is also an inverse experiment, HMBC has a higher sensitivity than COLOC.

Unfortunately, we were able to obtain this kind of information only for the *sn*-2,3-diacylglycerol sample (**Figure 4B**).

The assignment of carbon number 1 of the 2,3-diacylglycerols was possible because a correlation between carbamic carbon and the protons at 4.21 and 4.26 ppm exists. These protons were previously correlated with the carbon at 63.1 ppm in the HMQC

experiments. This means that the carbon at 63.1 ppm is the carbon number 1 of the glyceridic moiety of the 2,3-diacylglycerols.

However, although we did not see a similar correlation in the HMBC experiment of *sn*-1,2- diacylglycerols, the assignment of carbon 1 could be done by the consideration that the CH_2-OH of glyceridic moiety esterified with carbamic group should have a similar chemical shift. By this consideration, the carbon number 1 of *sn*-1,2-diacylglycerols should be at 62.4 ppm.

From these findings, we can conclude that the three classes of diacylglycerol isomers can be recognized and characterized by means of NMR spectroscopy. In particular, by performing a set of mono- and two-dimensional experiments (^{1}H NMR, COSY, HMQC, HMBC), we were able to establish the complete assignment of signals regarding the glyceridic moiety of the three isomeric classes when they are derivatized as (*S*)-(+)-1-(1-naphthyl)ethyl-urethanes. In this way, we can recognize clearly the *sn*-1,3-diacylglycerols and, moreover, *sn*-1,2- with respect to *sn*-2,3-diacylglycerols.

Although the NMR technique by itself is not enough to completely characterize the diacylglycerol isomers in olive oil samples, it becomes of considerable significance when it is applied to diacylglycerl isomers isolated and derivatized as naphthylethyl-urethanes. In this case, the reported chemical shift differences can be useful for the identification and characterization of the diacylglycerol isomeric classes without the need to use reference compounds. On the basis of the explained considerations, this work for the first time reports the spectroscopic data of diacylglycerol urethanes, useful tools for comparison purpose.

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