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Regiospecific Analysis of Natural Mixtures of Triglycerides Using Quantitative ^{13}C Nuclear Magnetic Resonance of Acyl Chain Carbonyl Carbons

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ABSTRACT: High-resolution ^{13}C NMR spectroscopy of the carbonyl carbons of triglyceride acyl chains was used as a quantitative method for the regiospecific analysis of the triglyceride fraction of vegetable oils of different botanical origins. The carbonyl carbon resonances of saturated, oleyl and linoleyl chains were baseline resolved. Moreover, the chains attached at the 1,3-position were shifted by 0.4 ppm at higher frequencies than those of the corresponding chains attached at the 2-position. This quantitative NMR method was adopted after demonstrating that proton decoupling affected the carbonyl intensities to the same extent, no significant differences being found among the NOE factors of different acyl chains. The proton-decoupled spectra were measured for quantitative purposes with full NOE enhancement. As a result, the spectrum signal-to-noise ratios were significantly improved in shorter experiment times. The ^{13}C NMR method was used to determine the acyl chain composition of vegetable oil triglycerides, the composition of the two acyl chain pools which entered the glycerol 1,3- and 2-positions and the specificity of each chain for the 2-position. © 1998 John Wiley & Sons Ltd.

KEYWORDS: ^{13}C NMR; triglycerides; regiospecific analysis

INTRODUCTION

A significant effort has been made in the past few years to develop a quantitative analytical method for the stereospecific analysis of triglycerides in order to define the acyl chain compositions of the *sn*-1-, *sn*-2- and *sn*-3-positions of the glycerol molecule (*sn* indicates stereospecific numbering). All the methodologies which have been adopted for this purpose resort to high-performance liquid chromatography to detect diastereoisomers derived from enantiomeric triglycerides through reaction with a chiral reagent[1] or to resolve the enantiomeric triglycerides on a chiral stationary phase.[2]

Regiospecific analysis provides limited information on triglyceride structure because this methodology only defines the fatty acid composition of the *sn*-1(3)-positions as a whole and *sn*-2-positions. Both regiospecific and stereospecific analyses of triacylglycerols are performed by means of chromatographic techniques after chemical degradation of triglycerides into mono- and diglycerides using a Grignard reagent. Both of the above-mentioned methods have problems in obtaining intermediate partial glycerides which are representative of the original triglycerides.[1,3,4]

^{13}C NMR spectroscopy has been successfully used to carry out the regiospecific analysis of natural mixtures of triglycerides. High-resolution ^{13}C NMR spectra of different vegetable oils showed that the carbonyl and olefinic carbon resonances were resolved on the basis of the degree of chain unsaturation and their attachment position on the glycerol backbone, i.e. *sn*-1(3)- and *sn*-2-positions.[5–8] The pattern of triglyceride unsaturated chains, i.e. C18:1–C18:2–C18:3, was determined by means of the resonances in the C=C shift range, but the full chain composition can only be achieved through the carbonyl shift region where also carbonyl carbons of saturated chains resonate.

The carbonyl carbon resonances have been extensively used to perform the regiospecific analysis of seed oil triglycerides esterified with acyl chains containing cyclopropenic[9] and double–triple bond functional groups.[10] The carbonyl signals have also been employed to determine the quantitative acyl chain composition of monovarietal olive oil triglycerides to establish a relationship between the *sn*-2-positional specificity of each chain and the varietal and geographical origins of olive oils.[11]

Proton decoupled carbon-13 spectra have been measured using the inverse-gated sequence to suppress nuclear Overhauser enhancements.[12] The NOE can affect signal intensities to different extents; as a result, they are distorted and the relative chain ratios are no longer representative of the true chain composition. Although this methodology improves the possibility of measuring quantitative signal intensities, it reduces the signal-to-noise ratio and thus the integration accuracy.[13,14]

The primary role that the carbonyl region of ^{13}C NMR spectra plays in the study of the triglyceride acyl

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chain composition suggested the need to check whether ^{13}C NMR methodology based on carbonyl resonance detection could be substantially improved in order to obtain a rigorously quantitative method with reasonable experiment times.

EXPERIMENTAL

^{13}C NMR spectroscopy

The T_1 longitudinal relaxation times and nuclear Overhauser enhancement factors of carbonyl carbons were determined using standard solutions of triglycerides made up of 40 mg of tripalmitin, 80 mg of triolein and 80 mg of trilinolein diluted with 0.5 ml of $CDCl_3$, to reproduce the oil/solvent ratio (200 mg/0.5 ml $CDCl_3$) that was used in vegetable oil spectroscopy.

The spectra were obtained using a Bruker 300 MHz spectrometer equipped with a 5 mm probe operating at 25 °C. The spectra were acquired with 128 scans. Standard Bruker software was used.

Proton-decoupled ^{13}C NMR spectra with full NOE. The proton-decoupled ^{13}C NMR spectra were measured using a spectral width of 2200 Hz, where the acyl chain carbonyl carbons resonate, 64K data points and a 90° pulse length with a 14.7 s acquisition time. The decoupler was turned on before and during acquisition to produce full NOE and proton decoupling, respectively. Since the decoupler power required to generate heteronuclear NOE is lower than the power needed to decouple protons, two decoupling powers were used; the power was lower before acquisition.[15] The relaxation delay was set at 15 s.

The spectra were zero-filled to 128K and the resolution enhancement function which uses the Lorentzian-to-Gaussian conversion was applied by entering the parameters − 0.1 Hz Lorentzian narrowing and 0.12 Hz Gaussian broadening.

Proton-decoupled ^{13}C NMR spectra with suppressed NOE. The proton-decoupled spectra with suppressed NOE were measured by means of the inverse-gated sequence,[12] which requires the decoupler to be turned on only during the acquisition time. The pulse delay was set at 30 s.

Measurement of spin lattice relaxation time, T_1. The T_1 longitudinal relaxation times of carbonyl carbons were determined by means of the 180°–τ–90° inversion–recovery pulse sequence.

Statistical analysis

The spectroscopic data related to NOE factors were analysed statistically with a one-way analysis of variance (ANOVA). This statistical technique is used to separate and estimate the variations that occur in measurements resulting from random error and experimental factors. ANOVA applies the F-test to compare the variances between samples. In particular, if the calculated F value is lower than the critical F value, the sample means differ significantly. The least significant difference test was used to compare the means and verify whether they differ significantly.

The standard deviation and coefficient of variation for each carbonyl carbon signal were also calculated to check the repeatability of the method, i.e. the within-run precision.

RESULTS AND DISCUSSION

Assignment of carbonyl carbon resonances

Carbonyl carbon signals were used to carry out the regiospecific analysis of vegetable oil triglycerides. The olefinic carbons of triglyceride acyl chains spread over a wider shift range where all the resonances of C:18 unconjugated mono-, di- and triunsaturated chains were resolved on the basis of chains and glycerol positions. However, olefinic carbons can only provide ancillary data because of the saturated chain resonances being lost.

The signals of carbonyl resonances were assigned according to the chemical shifts of standard triacylglycerols. The saturated (173.10 ppm), oleyl (173.07 ppm) and linoleyl (173.06 ppm) chains esterified at *sn*-1(3)-positions of glycerol resonated at frequencies higher than those of the same chains attached to the glycerol *sn*-2-positions; the chemical shifts were 172.70, 172.67 and 172.66 ppm, respectively. A high-resolution ^{13}C NMR spectrum is reported in Fig. 1. The carbonyl carbons of all the *sn*-1(3)-chains were shifted by 0.4 ppm from the *sn*-2-chains at higher frequency because of their different γ-*gauche* interactions.[16]

T_1 and NOE of carbonyl carbons

Routine ^{13}C NMR spectra were measured under proton decoupling to remove all the ^{13}C–^{1}H spin–spin coup-

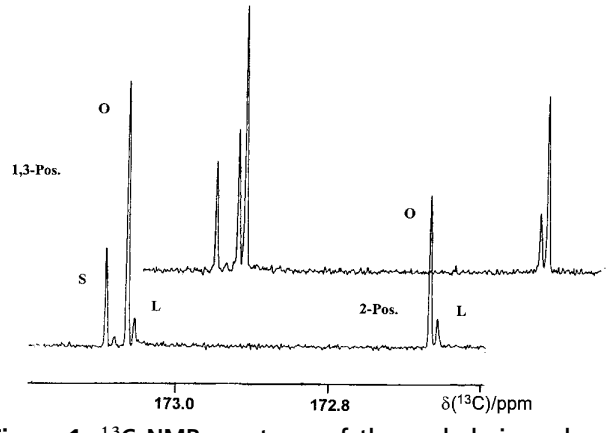

Figure 1. ^{13}C NMR spectrum of the acyl chain carbonyl resonances of triglycerides of olive oil (bottom trace) and corn oil (upper trace): S, saturated chain; O, oleyl chain; L, linoleyl chain.

lings and to facilitate interpretation. The intensities of ^{13}C resonances in proton-decoupled spectra are rigorously quantitative provided that they have the same 'intrinsic' intensities, i.e. ^{13}C nuclei are affected by the nuclear Overhauser effect (NOE) to the same extent.

Generally, whenever quantitative spectra are required, the NOE effect can be eliminated by using inverse-gated decoupling. The drawback of this strategy is that the experiment times which are necessary to achieve signal-to-noise ratios high enough to guarantee integration accuracy become extremely long.[13,14]

The alternative approach would be to correct the NOE effect by determining the enhancement factor for each resonance. The carbonyl carbons of acyl chains of palm oil triglycerides were expected to have the same NOE on the basis of the 2:1 ratio of the intensities of carbonyl signals at the glycerol *sn*-1(3)- and *sn*-2-positions, respectively.[17] The conclusion was drawn that quantitative ^{13}C NMR spectra of carbonyl carbons could be run under proton decoupling without NOE suppression.[6]

The $^{13}C\{^1H\}$ NOE enhancement factors of carboxyl carbons of formic and acetic acid and their methyl esters have been experimentally determined[18] but no data were available for fatty acids in triglyceride molecules. A standard mixture of triglycerides, tripalmitin, triolein and trilinolein, prepared as described in the Experimental section, was used to determine the NOE factors of carbonyl carbons of saturated, oleoyl and linoleoyl chains. The T_1 longitudinal relaxation times were calculated in order to set up correctly the D_1 delays used throughout NOE experiments and quantitative analyses. The T_1 relaxation times, reported in Table 1, are the means resulting from three independent experiments. The carbonyl carbons of the chains attached to glycerol 1,3-positions in accordance with their increased mobility as compared with chains attached to the 2-position exhibited longer relaxation times.

The $^{13}C\{^1H\}$ NOE enhancement factors $(1 + \eta)$ were determined from the ratio of the integrated intensities of the ^{13}C spectra measured with full NOE and with suppressed NOE. The experiment to measure spectra with full NOE required a total decoupling time of 30 s, i.e. 15 s relaxation delay plus 14.7 s acquisition time. There was sufficient time to produce full NOE and fulfil the requirement $5T_1$ to avoid signal saturation. The relaxation delay D_1 was increased to 30 s in the inverse-gated experiment in agreement with the statement that $D_1 \geqslant 5T_1$ is suitable to quench the NOE that can be induced during the acquisition time.[5]

Five replicate measurements of carbonyl carbon intensities of the standard triglyceride mixture were carried out under full NOE (I) and suppressed NOE (I_0) conditions. The carbonyl carbon resonances of palmitoyl, oleyl and linoleyl chains were integrated and the NOE values $(1 + \eta = I/I_0)$ were calculated for each chain, at both the 1,3- and 2-positions of the glycerol molecule. One-way ANOVA was used to test whether the differences between the means of NOE factors, calculated for each chain at the 1,3- and 2-glycerol positions, were too large to be explained by a random error. The experimental statistic $F = 1.346$ (probability $P = 0.05$) was found to be lower than the critical $F = 2.621$, thus proving that the NOE factors measured at the carbonyl carbons of palmitoyl, oleyl and linoleyl chains on the glycerol 1,3- and 2-positions were not significantly different.

Table 1. NOE $(1 + \eta)$ and T_1 values for carbonyl carbons of triglyceride acyl chains

Carbon atom	δ (ppm)[a]	NOE $(1 + \eta)$	T_1 (s)[b]
C_1 *sn*-1(3)-			
Tripalmitin	173.10	1.77 ± 0.14	5.6 ± 0.36
Triolein	173.07	1.78 ± 0.09	5.6 ± 0.10
Trilinolein	173.06	1.73 ± 0.07	5.4 ± 0.21
C_1 *sn*-2-			
Tripalmitin	172.70	1.73 ± 0.08	3.9 ± 0.15
Triolein	172.67	1.67 ± 0.07	4.5 ± 0.38
Trilinolein	172.66	1.68 ± 0.07	4.5 ± 0.26

[a] Chemical shifts are referenced to TMS = 0 ppm.
[b] Accuracies of NOE factor and T_1 are quoted as standard deviation of the mean.

Regiospecific analysis of natural mixtures of triglycerides

The NOE enhancement factors demonstrated that the carbonyl carbon resonances of different chains were affected by proton decoupling to the same extent. On the basis of these findings, the carbonyl carbon spectral region of triglyceride acyl chains can be acquired for quantitative purposes under full NOE conditions with the benefit of higher signal-to-noise ratios in shorter experiment times.

The repeatability of the ^{13}C NMR methodology was verified by eight replicate measurements of proton-decoupled spectra with full NOE using an olive oil sample. The resonances were integrated by the spectrometer software and the areas were checked for standard deviation and coefficient of variation. The results are reported in Table 2.

Table 2. Investigation of the repeatability of the ^{13}C NMR method for the regiospecific analysis of triglyceride acyl chains of vegetable oils

Carbon signal	Mean (peak area)	Standard deviation	Coefficient of variation (%)
C_1 *sn*-1(3)-			
C*n*:0	12.15	0.22	1.83
C18:1	33.06	0.65	1.97
C18:2	3.53	0.12	3.37
C_1 *sn*-2-			
C18:1	19.96	0.34	1.71
C18:2	4.07	0.12	3.05

Table 3. Regiospecific analysis of triglycerides of different vegetable oils (%)

	Composition 1,2,3			Composition 1,3			Composition 2		Specificity 2	
Oil sample	Cn:0[a]	C18:1	C18:2	Cn:0	C18:1	C18:2	C18:1	C18:2	C18:1	C18:2
Sunflower	11.8	29.7	58.5	17.5	29.7	52.7	29.7	70.3	32.7	39.3
Peanut	21.7	50.1	28.2	32.4	49.3	18.4	51.7	48.3	34.0	56.4
Grapestone	11.0	21.7	67.2	16.5	18.8	64.6	27.5	72.5	42.0	35.9
Corn	13.9	28.7	57.4	20.6	29.2	50.3	27.7	72.3	31.1	40.6
Hazel	9.2	80.2	10.6	13.7	78.5	7.8	83.6	16.4	34.1	50.5
Olive fruit[b]	16.7	72.9	10.4	24.9	67.8	7.2	82.9	17.1	37.5	53.9
L.s.d.[c]	0.48	1.80	2.11	0.81	1.35	1.80	2.93	2.93	1.39	1.93

[a] n = Number of chain carbon atoms.
[b] Dritta cultivar olive oil extracted by pressure from olive fruits.
[c] L.s.d. is the least significant difference between the means (all the values are the averages of two determinations on the same oil sample).

The resonance areas of the most abundant chains of olive oil, i.e. saturated and oleyl chains, showed coefficients of variation within 2%. This value exceeded 3% for the linoleyl chain, as expected for a minor chain which produced a line with a lower signal-to-noise ratio and made signal integration less accurate. Nevertheless, the accuracy of the methodology was demonstrated by coefficients of variation $\leqslant 3.5\%$, and the reliability of ^{13}C NMR as a quantitative technique was confirmed in studies of vegetable oils.[19,20]

The method was used to determine the acyl chain compositions of the triglycerides of different vegetable oils which are reported in Table 3 under the heading 'Composition 1,2,3.' The analysis of the chains in the 1,3-positions and of the acyl groups attached to the 2-positions are under the headings 'Composition 1,3' and 'Composition 2,' respectively. The unsaturated chain, i.e. oleyl and linoleyl, specificities for the 2-position were also calculated and reported under the heading 'Specificity 2.'

Significant differences ($P < 0.05$) were observed among oils with reference to their chain composition, unlike the linoleyl chain contents of hazel and olive oils where the differences were lower than the value calculated for the least significant difference (2.11). Sunflower, peanut, grapestone and corn oils appeared to be 'high linoleic, low oleic acid' oils, whereas olive and hazel oils proved to be 'high oleic, low linoleic acid' oils. The chain distribution between the 1(3)- and 2-positions of the glycerol backbone showed that two different pools of fatty acids namely saturated, oleic and linoleic acids, entered the 1,3- and 2-positions of glycerol; saturated acids were found to be esterified only at the 1,3-positions of glycerol.

Significant differences ($P < 0.05$) were observed among the oleyl and linoleyl specificities for the glycerol 2-position in all the oils studied. The values obtained for the 2-position specificity of the oleyl chain were close to 33%, proving that the fatty acid was randomly distributed among the glycerol positions. However, the 2-position specificities of the linoleyl chain, which were constantly higher than 40%, confirmed the deviation of this chain from a random distribution.

These findings demonstrate that ^{13}C NMR spectroscopy can be used as a quantitative method to study the triglyceride compositions of vegetable oils. This methodology has the advantages of facilitating sample preparation and ensuring repeatibility with a coefficient of variation lower than 3.5%. Moreover, the results obtained confirmed that high-resolution ^{13}C NMR of the carbonyl carbon region can be regarded as a major methodology for carrying out the regiospecific analysis of natural mixtures of triglycerides.

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