Improved Quantitative ^{13}C Nuclear Magnetic Resonance Criteria for Determination of Grades of Virgin Olive Oils. The Normal Ranges for Diglycerides in Olive Oil

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ABSTRACT: A quantitative method was established to determine the presence and composition of mono-, di-, and triglycerides of olive oils of superior grade *via* ^{13}C NMR. The total diglyceride content and the ratio of *sn*-1,2- and *sn*-1,3-diglycerides in extra virgin oils extracted from different olive cultivars were correlated with maturity. The correlation can be applied to identify the oils by variety. No monoglycerides were detected in the oils examined.
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KEY WORDS: Glycerides, quantitative ^{13}C NMR, virgin olive oil.

Interest in superior grades of olive oil has been steadily increasing in recent years. This trend can be attributed not only to their superior flavor, but also to reports of possible potential health benefits. Superior grade virgin olive oil is obtained from healthy olive fruits by mechanical means under conditions that avoid any oil alteration. Thus, only crushing and washing at temperatures near room temperature, kneading, decantation, centrifugation, and filtration are permitted. If the processing was illegal or the drupes were not of the best quality, a virgin oil of inferior quality is obtained that has a chemical composition indicative of any ill-performed extraction of poor-quality fruits. We were interested in studying the chemical changes in acyl glycerol composition brought about by lipolysis on the original natural olive oil. The purpose of the present work is to develop a rapid and precise method that is usable for quantitating the monoacylglycerols (MAG), diacylglycerols (DAG), and triacylglycerols (TAG) constituting olive oil.

The content and the chemical structures of MAG and DAG should constitute simple criteria that would allow one to set ranges for identifying the various olive oil grades and to discriminate, even within extra virgin olive oils, according to olive cultivars, quality of the fruits extracted, and storage conditions. Most of the analytical methods for this purpose were developed based on gas chromatography, or thin-layer chromatography. Although some studies showed that high-performance liquid chromatography (HPLC) is also capable of separating the three classes of glycerides, none of these methods permitted a total separation and identification of all compounds making up olive oil. Consequently, quantitative determination of these compounds could not be achieved in a single run. Therefore, reliable methods for determining the precise chemical composition of olive oil products were required.

Consideration of these factors led us to focus on a nuclear magnetic resonance (NMR) method to develop a screening procedure to both quantitate and determine the chemical structure of partial glycerides in extra virgin olive oil. Information on DAG content influenced by the maturity of the fruits was also gained. The NMR analysis failed to detect monoglycerides in the oils studied.

EXPERIMENTAL PROCEDURES

The ^{13}C NMR spectra were for solutions in deuterated chloroform (250 mg olive oil/0.5 mL $CDCl_3$) at 25°C and performed on a Bruker AC 300 Spectrometer (Bruker Instruments, Inc., Karlsruhe, Germany) with a 5 mm proton/carbon dual probe. Chemical shifts were relative to the signal of Me_4Si (tetramethylsilane).

The broadband proton decoupled (Waltz-16) ^{13}C spectra were run with 64K data points at a spectral width of 13,000 Hz, acquisition time of 2.5 s. To avoid saturation and assure integral accuracy for quantitative analysis, 70° pulses and a pulse delay of 1 s were used on account of the T1 longitudinal relaxation times of 1,3-CH_2- and 2-CH-carbons, which were determined to be 0.30 and 0.29 s, respectively (1). The spectrum resolution was enhanced by a Gaussian multiplication. The number of scans was standardized as an overnight experiment period in order to detect the diglyceride resonances, which were present below the 2% level; standard MAG, DAG, and TAG were purchased from Sigma Chimica (Milano, Italy).

Line of regression and variance analyses (ANOVA) were obtained with Microsoft Excel ver. 5.

RESULTS AND DISCUSSION

^{13}C NMR chemical shifts of glycerol carbons of mono-, di-, and triglycerides. We ran the spectra of four kinds of glycerides, 1-MAG, 1,2-DAG, 1,3-DAG, and TAG in which the acyl chains were those of palmitic, oleic, and linoleic acid. The latter was present only in the corresponding triglyceride.

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The symmetrical 1,3-DAG and TAG gave two signals for the glycerol moiety with intensity ratios 1:2. The asymmetrical 1-monoacyl and 1,2-diacyl glycerides gave three signals with intensities in a 1:1:1 ratio.

Assignment of resonances for symmetrical glycerides was made on the basis of signal intensities. Distortionless Enhancement by Polarization Transfer experiments were run for the correct assignment of CH carbons in asymmetric glycerol esters. A summary of the data obtained is reported in Table 1. The C2 signals were found to resonate always downfield compared to C1 and C3 resonances.

The 1,3-glycerol carbon resonances, CH_2OH and CH_2OCOR, in asymmetric glycerol esters, were not definitively assigned and may be interchanged (2). One observation worthy of note from the data in Table 1 is that the length and degree of unsaturation of the acyl chain do not influence the chemical shifts of the glycerol carbons bearing the substituents (1).

Quantitative ^{13}C NMR analysis of model glyceride mixtures. A set of samples of mono-, di-, and trioleoylglycerols at different concentrations were prepared to verify the reliability of the NMR quantitative technique.

The concentrations of mono- and diglyceride standards used were in the range 1.5–25.0 mg/0.5mL $CDCl_3$, with triolein added to reach a final glyceride total weight of 250 mg. This procedure was adopted to reproduce the concentration of the olive oil samples used for NMR analysis as closely as possible.

Linear relationships were found between glyceride concentrations and the corresponding areas of glycerol C2 signals. The best straight lines were calculated by the method of least squares for mono- and diglycerides (Fig. 1) and triglycerides (Fig. 2). Table 2 summarizes all calculations performed on the data.

Analysis of virgin olive oil. The oils investigated were from the olive varieties Grossa di Cassano, Nebbio, Coratina, Leccino, Dritta, and Caroleo, grown in the Institute olive groves. Olive fruits were harvested in early December 1994 and were processed within two days of storage. The continuous three-phase process was adopted for oil extraction (3,4).

Table 2 reports the concentrations of 1,2-, 1,3-DAG and TAG of the virgin olive oils under study as determined by our NMR procedure. As stated previously, monoglycerides were absent or under level of detection of 1.34 mg/0.5 mL $CDCl_3$.

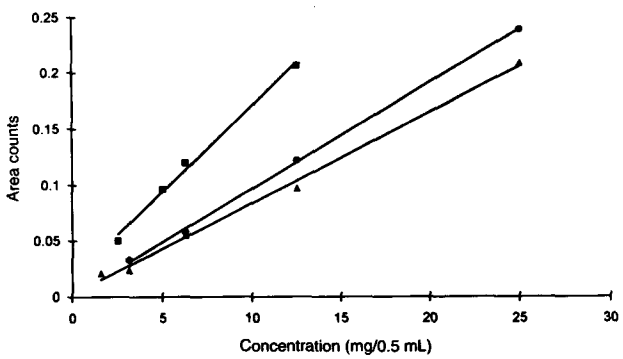

FIG. 1. Correlation plot of nuclear magnetic resonance signal integration areas and concentration of 1-monooleoyl-(■), 1,2-dioleoyl- (▲), and 1,3-dioleoylglycerol (●).

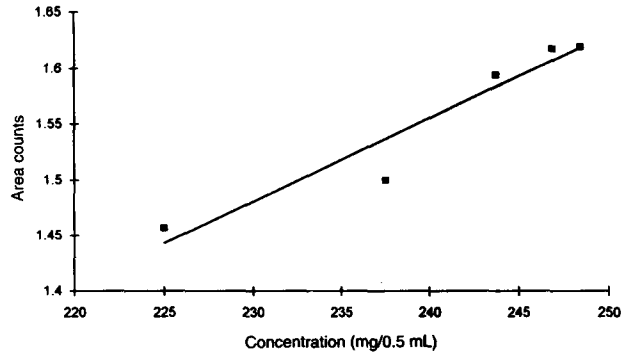

FIG. 2. Correlation plot of nuclear magnetic resonance signal integration areas and concentration of 1,2,3-trioleoylglycerol.

The weight percentages of the three classes of glycerides were calculated through the line of regression of integrated NMR signal areas on known concentrations of oleoylglycerols. The assumption was made that the olive oil glycerides were those of oleic acid esterified with glycerol.

According to the Kennedy pathway (5), plant tissues synthesize the bulk of their TAG by acylation of DAG at position *sn*-3. It is therefore most important to have a precise picture of the content of 1,2-DAG in an olive oil just after its expression from fresh and healthy fruits. Eventually we hope to prove that the 1,3-DAG, sometimes found to occur in commercial oil samples, may not be the result of artifacts due to a

TABLE 1
^{13}C NMR Chemical Shifts of Glycerol Carbon Atoms[a]

Glycerol ester	$1CH_2$	2CH	$3CH_2$
1-Monopalmitoyl	63.34	70.26	65.11
1-Monooleoyl	63.35	70.26	65.13
1,2-Dipalmitoyl	61.54	72.11	62.02
1,2-Dioleoyl	61.56	72.12	62.00
1,3-Dipalmitoyl	65.04	68.38	65.04
1,3-Dioleoyl	65.03	68.34	65.03
1,2,3-Tripalmitin	62.10	68.87	62.10
1,2,3-Trilinolein	62.10	68.88	62.10
1,2,3-Triolein	62.08	68.87	62.08

[a]Chemical shifts are referenced to TMS = 0 ppm. NMR, nuclear magnetic resonance.

TABLE 2
Calculations of Regression Lines of Mono-, Di-, and Trioleoylglycerols

Glycerides	Slope	Intercept	*n*	*r*	L.o.d.[a]
1-Monooleoyl	$1.53 \cdot 10^{-2}$	$1.74 \cdot 10^{-2}$	4	0.996	1.34
1,2-Dioleoyl	$8.10 \cdot 10^{-3}$	$2.58 \cdot 10^{-3}$	5	0.999	2.21
1,3-Dioleoyl	$9.46 \cdot 10^{-3}$	$1.91 \cdot 10^{-3}$	4	0.999	0.87
Triolein	$7.48 \cdot 10^{-3}$	$-2.39 \cdot 10^{-1}$	5	0.959	31.96

[a]Limit of detection (mg/0.5 mL).

number of interacting factors, such as olive storage, extraction and purification methods, use of organic solvents, and factors that do not comply with the official procedures proposed by the International Olive Oil Council (IOOC) (6). In fact, a possibility exists that part of 1,3-DAG may form in the fruits during the maturation stage as suggested in a literature report (7).

We first considered that the total DAG content may be related to the cultivars and maturation stage of the olives extracted. This assumption was used to represent a valid parameter to discriminate between late and early varieties, that is olives that reach full maturation in the early harvest season and olives that mature mostly toward the end of the season. According to sparse data in the literature, the late maturing olives produce oil with lower total DAG levels than the early ripening olive fruits (7). The data shown in Table 3 may shed some light on this argument.

The total DAG values were analyzed with one-way analysis of variance (ANOVA). Since the calculated value of F was greater than the critical F (P = 0.05), the null hypothesis was rejected and the oil sample means did differ significantly.

The least significant difference (LSD) test was used to determine where the differences existed. Comparing the LSD difference value of 0.18 (P = 0.05) with the differences between the means suggested that total DAG means of Nebbio and Coratina cultivars differ significantly from each other and from the other cultivars, which, however, do not differ significantly from each other.

These findings let us conclude that the DAG contents of Nebbio and Coratina were significantly lower than those of the other varieties, thus enabling a possible classification of Nebbio and Coratina as late cultivars, unlike Dritta, Caroleo, and Cassano as early maturing fruits. The observation that the ratios DAG 1,2/DAG 1,3 shown in Table 3 may vary considerably, however not exceeding the range 0.5–1.6, is interesting. Significant (P = 0.05) differences were observed between varieties, and we consider these findings a further indication that the 1,2- and 1,3-DAG in oils obtained from healthy and sound olives are determined by the cultivars and consequently by their seasonal maturation process. However, how far one can go with these simple genetics speculations remains to be established.

In conclusion our work confirmed the validity of the NMR procedure as a superior reliable technique for a quantitative analysis of glycerides. Besides, in perspective, it provided, on the basis of total diglyceride content and DAG 1,2/DAG 1,3 ratio, a complete compositional glyceride picture for a selection of Italian olive cultivars and useful information regarding the varietal origin of the oil and the maturation stage of the olives processed. Further, our results may represent also preliminary new parameters to be used for the assessment of olive oil of different grade.

TABLE 3
^{13}C NMR Quantitative Profiles of Glycerides in Some Elemental Olive Oils

Olive varieties	DAG[a] 1,2	DAG 1,3	TAG[a] 1,2,3	DAG Tot.	1,2/1,3 DAG
Cassano	1.04	0.79	98.50	1.83	1.32
Nebbio	0.77	0.73	98.90	1.50	1.05
Coratina	0.75	0.48	98.89	1.23	1.56
Leccino	0.61	1.06	99.10	1.67	0.58
Dritta	0.58	1.19	99.14	1.77	0.49
Caroleo	0.89	0.99	98.68	1.89	0.90
LSD[b]	—	—	—	0.18	0.14

[a]All values are expressed as percentage weight. Each value represents a mean of three repetitions. DAG, diacylglycerols; TAG, triacylglycerols. See Table 1 for other abbreviation.
[b]Least significant difference ($P = 0.05$).

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