Prediction of Isocratic Nonaqueous Reversed-Phase High-Performance Liquid Chromatography Retention Parameters and Response Factors of Triacylglycerols Detected by an Ultraviolet-Diode Array–Evaporative Light-Scattering On-Line System

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Abstract

Nonaqueous reversed-phase high-performance liquid chromatography of 10 homogeneous triacylglycerol molecular species (TAG), both saturated and unsaturated, is carried out. The eluate from the column is detected by an ultraviolet diode array detector (DAD) on-line with an evaporative light-scattering detector (ELSD). The retention parameters (as selectivities, α) for 220 TAGs are determined, and the obtained values are related to the following structural parameters: total carbon number; mono-, di-, and triunsaturated fatty acid residues number/molecule; and monounsaturated fatty acid carbon number. Multiple regression analysis is carried out to obtain a relationship for the prediction of α values of any TAG when the same experimental conditions are used. In regard to the quantitative analysis of the separated TAG species, the dependence of response of the two on-line detectors on the aforementioned structural parameters is studied. Three different wavelengths (205, 210, and 215 nm) are considered for TAG detection by DAD; in each case, the obtained multiple regression model shows a good correlation between the dependent variable and predictive values of the TAG species (response factors and considered structural parameters, respectively). The ELSD gives responses exponentially related to injected amounts. Also, in this case, an attempt to relate the response factors of each considered detector to some structural parameters of TAG species is carried out. The results of this study are used to analyze the TAG fraction from an olive oil.

Introduction

Triacylglycerol (TAG) fraction, the most abundant class in natural lipids, is a complex mixture of molecular species, n^3 being their potential number when n fatty acids are present. In recent decades, the analysis of TAG by high-performance liquid chromatography (HPLC) became increasingly popular (1). Because of TAG insolubility in aqueous solvents, and because all the TAG species have the same polar group but differ in their aliphatic moieties, the best conditions for HPLC separations are nonaqueous (NA) mobile phases and reversed-phase (RP)-HPLC conditions (2). Nowadays, the availability of efficient chromatographic columns has made it possible to separate TAG mixtures into defined groups of molecular species whose retention parameters depend substantially on the total chain length of the constitutive acyls and their double-bond number (3). Moreover, many different types of detectors are available (refractive index, infrared, ultraviolet absorption, light scattering and mass spectrometry), even if their characteristics must be considered for a correct use (4). In regard to the most popular ultraviolet (UV) detector, a limitation comes from the difficulties concerning the use of solvents that absorb UV radiation and from the lack of strong chromophores in TAG molecules. With the availability of multiwavelength UV detectors (diode array detectors, DAD), the recording of the complete UV spectrum of each component in the effluent from HPLC is allowed, so that more information can be obtained with a single chromatographic run (5). Among the other available detectors, the evaporative light-scattering detector (ELSD) has gained popularity in the field of lipid analysis because of its flexibility in the selection of chromatographic conditions (6). Nevertheless, the lipid analyst has to consider the problems of the choice of the best separation conditions and the identification of specific TAG molecular species (usually not available as standards).

The problem of the identification of TAG molecular species has been considered in several papers; parameters such as equivalent chain length (ECL) (7), partition number (PN) (8), and equivalent carbon number (ECN) (9) have been introduced and often utilized by lipid analysts. On the basis of a previous paper (10)

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where a procedure for the prediction of retention parameters "selectivities" (α) and response factors of TAG with high-resolution gas chromatography–flame-ionization detection analysis was reported, a multiple regression method was applied in the present study to correlate the α value of different TAG molecular species analyzed by NARP-HPLC to some of their structural parameters. The detection system was a DAD on-line with an ELSD. In regard to the quantitative analysis of the separated TAG species, the dependence of the response of the two detection systems on the aforementioned structural parameters was studied. Concerning UV detection, only the TAG chromatographic profiles recorded at three different wavelengths (205, 210, and 215 nm) were considered. In order to use the obtained chemometric models to value the qualitative and quantitative composition of both natural and variously modified TAG matrixes, the TAG fraction from an extra-virgin olive oil was analyzed in this research as an example.

Experimental

Homogeneous reference TAG molecular species, all of purity about 99%, were purchased from Sigma (Sigma Chemical Co., St. Louis, MO): trilaurin (LaLaLa), trimyristin (MMM), trimyristolein (MoMoMo), tripalmitin (PPP), tripalmitolein (PoPoPo), tristearin (SSS), triolein (OOO), trilinolein (LLL), trilinolenin (LnLnLn), and trigadolein (EEE). A natural extra-virgin olive oil sample was also used to qualify and quantitate the isolated TAG fraction by analyzing the TAG with the NARP-HPLC and on-line DAD–ELSD system; for this aim, the regression models were obtained by studying the influence of the following structural parameters of reference TAG both on the selectivity values (α) and on the response factors by weight relative to triolein (*rrfw*) of any TAG species: total carbon number (TCN); mono-, di-, and triunsaturated fatty acid residues number/molecule (MAN, DAN, and TAN, respectively); and monounsaturated fatty acid carbon number (MCN).

The NARP-HPLC analyses of TAG were carried out using a Gilson (Middletown, WI) model 307 HPLC equipped with a Rheodyne (Cotati, CA) 7125 sample injection valve with a 10-µL loop. The separations were performed by a Superspher 100 RP-18 (244 × 4.0 mm, 5-µm particle diameter) endcapped column (Merck, Darmstadt, Germany). The temperature was maintained at 24°C during the analysis. The eluent was the mixture acetonitrile–2-propanol–hexane (66.7:21.9:11.4, v/v/v), and the flow rate was 0.8 mL/min. The HPLC apparatus was also equipped with a UV-DAD Unicam (York Street, Cambridge, U.K.) Mod. Crystal 250 operating in the acquisition field 200–365 nm with a resolution of 1.2 nm. The Unicam UICS and Unicam 4880 software were used for the acquisition and successive data elaboration and for integration, respectively. This UV-DAD was connected on-line with a DDL 21 ELSD (Eurosep Instruments, Cergy – Pontoise Cedex, France) set up with the following operating conditions: evaporator temperature, 40°C; nitrogen inlet pressure, 14 psi; photomultiplier voltage, 550 V. Additionally, a Hewlett-Packard (Palo Alto, CA) HP 3394 integrator was used to plot and integrate the ELSD signals.

For NARP-HPLC, both the reference TAG and those obtained from an extra-virgin olive oil sample by thin-layer chromatography (11) were dissolved in the mixture of chloroform–2-propanol (1:1, v/v) to give a concentration of 0.25% (w/v).

The HPLC analyses were carried out in triplicate, and the reported results represent the mean values; the coefficient of variation (%CV = percent standard deviations) were never higher than 10.

Results and Discussion

The α and *rrfw* values for both detectors were determined according to the procedure reported in a previous paper (10). In this work, the preparation of the models is based on the relation between the free energy of the separation process in chromatography and the structure of the chromatographed molecule reported by Martin (12) as well as on the hypothesis of Goiffon (13) on the linear addition of the Gibb's solubilization free energies and that of Perry and Naudet (14) who assume that each residue in a TAG molecule contribute separately to the selectivity values of the total molecule.

The α value of the XXX TAG is defined here as follows:

$$\alpha_{XXX} = 100 \cdot \log (10 \cdot t_{XXX} / t_{OOO}) \qquad \text{Eq. 1}$$

where t_{XXX} and t_{OOO} represent the retention times of XXX and OOO (an undefined homogeneous TAG molecular species and triolein, respectively).

In this work, the 10 homogeneous TAGs were analyzed by NARP-HPLC, and the α values of 220 different molecular species were calculated (10). The *rrfw* values relative to the triolein were determined for the same 220 TAG species in a similar way.

The best model for calculating the α values was obtained by considering a new parameter (MCN) besides the TCN, MAN, DAN, and TAN already used to calculate the HRGC α values (10). Considering the α values calculated from the retention parameters of the reference TAG measured in the chromatograms acquired by the DAD at 205 nm, the following equation was then obtained:

$$\alpha_{XYZ} = -176.8640 + 5.9776 \cdot TCN - 7.8504 \cdot MAN - 28.3449 \cdot DAN - 39.0213 \cdot TAN - 0.4132 \cdot MCN \qquad \text{Eq. 2}$$

where α_{XYZ} is the selectivity of a generic mixed XYZ TAG molecular species.

The correlation coefficient of Equation 2, $r^2 = 0.9994$, shows that the model well explains the dependence of the retention parameter of each TAG species on the structural parameters of the same species; in particular, the different influence on the α value of double bonds located in a mono-, di-, or triunsaturated acylic residue is shown by the values of MAN, DAN, and TAN coefficients. The fact that the double bonds in polyunsaturated chains cannot be considered independent from each other was also reported by Stolyhwo et al. (15). The meaning of influence of the new parameter MCN on the α value is not immediate, even if it seems able to state precisely the significance of the MAN parameter,

because the latter doesn't consider the chain length of the monounsaturated acylic residues. A similar equation not reported in this paper was obtained using the selectivity values for the multiple regression analysis calculated from the retention parameters of the reference TAG measured in the chromatogram acquired by the ELSD. It should be pointed out that the delay observed in the detection of each TAG species by the two detectors was due to the length of transfer line (1 s/cm); in fact, as already reported (10), the parameters of each regression model must be considered valid only if the experimental conditions used to build it are precisely reproduced.

In regard to the quantitative aspect of the NARP-HPLC analysis of TAG, the same approach was carried out to verify the possibility of obtaining regression models useful for the prediction (on the basis of structural parameters) of the *rrfw* (relative to triolein) of any TAG molecular species. Obviously, any TAG mixture should be analyzed under the same experimental conditions used to build the model. In this respect, necessary considerations are relative to the detection system used in this study, the on-line DAD–ELSD. In the two cases, the detection of the TAG molecular species is based on different physical principles; in fact, the detections by DAD and ELSD are obviously separately considered in this study.

For the DAD, the linearity of the response in the considered concentration range (1–40 µg) was verified for each reference TAG; because of the high absorbance value of the polyunsaturated species, for trilinolenin, the linearity range was 1–10 µg.

The response factors (relative to triolein) of the 220 TAG molecular species appeared well correlated to the structural parameters TCN, MAN, DAN, and TAN; the coefficients of the regression equation

$$rrfw_{XYZ} = a + b \cdot TCN + c \cdot MAN + d \cdot DAN + e \cdot TAN \qquad \text{Eq. 3}$$

for the detection at the three wavelengths are reported in Table I together with the relative correlation coefficients (r^2). As expected, the $rrfw_{XYZ}$ values are dependent on the wavelength of analysis; for example, the ratio between the $rrfw_{XYZ}$ of a triunsaturated residue in respect to that of a monounsaturated one changes from 9.7:1 to 24:1 until 80:1 in dependence of the detection wavelength (205, 210, and 215 nm, respectively). Moreover, the values of the coefficients *c*, *d*, and *e* show that $rrfw_{XYZ}$ strongly depends on the total number of double bonds ($e > d > c$) and also on the distribution of the double bonds among the acylic residues of a TAG molecular species ($d > 2 \cdot c$ and $e > 3 \cdot c$). The increase of the r^2 value with increasing detection wavelength should be pointed out; this occurrence is probably due to the better stability of the baseline at higher wavelengths (slower noise or higher signal-to-noise ratio), with the consequent repeatibility of the analytical results.

Concerning the ELSD, exponential responses in relation to injected amounts of the reference TAG were observed as already reported by other authors (4,16,17). The obtained models were as follows:

$$\ln Y = A_0 + A_1 \cdot \ln X \qquad \text{Eq. 4}$$

where *Y* represents the chromatographic peak area, *X* is the TAG amount (as injected weight), and A_0 and A_1 are constant coefficients. The values of A_0 and A_1 and the relative correlation coefficients (r^2) are reported in Table II for the reference TAG. For any mixed TAG molecular species, the values of A_0 and A_1 coefficients were obtained from the values of the reference TAG according to the following relation:

$$A_{0(1)XYZ} = \tfrac{1}{3}A_{0(1)XXX} + \tfrac{1}{3}A_{0(1)YYY} + \tfrac{1}{3}A_{0(1)ZZZ} \qquad \text{Eq. 5}$$

The attempt to correlate the A_0 and A_1 coefficients of any mixed TAG molecular species to some structural parameters showed a

Table I. Coefficients of Regression Equation 3 and Relative r^2 for the Detection of TAG by DAD at Different Wavelengths

λ (nm)	*a*	*b*	*c*	*d*	*e*	r^2
205	0.3033	–0.0052	0.3156	2.1553	2.9253	0.9997
210	0.4929	–0.0077	0.3075	3.6919	7.3227	0.9999
215	1.7348	–0.0194	0.1003	2.5596	8.0174	1.0000

Table II. A_0 and A_1 Values of Equation 4 and Relative r^2 for the Reference TAG

TAG	A_0	A_1	r^2	DL* (ng)
LaLaLa	5.435	1.568	0.9966	30
MMM	5.372	1.515	0.9939	40
MoMoMo	5.243	1.640	0.9980	30
PPP	4.843	1.500	0.9978	220
PoPoPo	3.800	1.659	0.9996	50
SSS	4.980	1.414	0.9971	400
OOO	4.537	1.594	0.9965	150
LLL	4.765	1.610	0.9963	40
LnLnLn	6.184	1.523	0.9971	20
EEE	5.180	1.503	0.9992	300

* DL = detection limit (established as explained in the text).

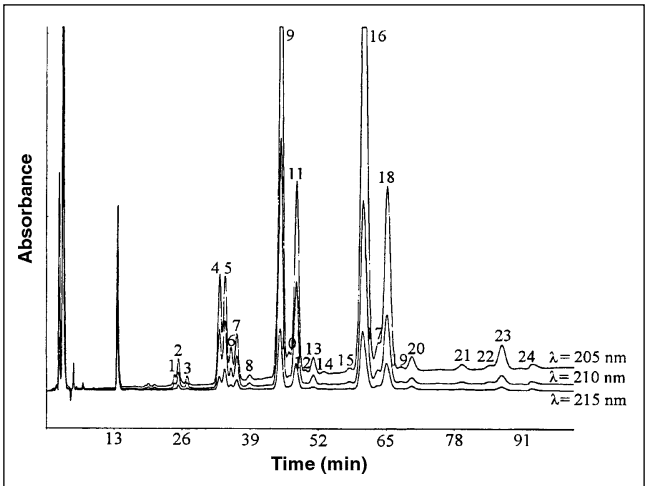

Figure 1. Chromatogram of a NARP-HPLC separation of olive oil TAG acquired by DAD (peak identification is reported in Table III).

nonlinear dependence. As already reported (4,16,17), the response of ELSD depends on factors relative to the analyte, among which are the melting point and the molecular structure, and on instrumental factors, among which are light-beam wavelength, evaporator design, temperature of the evaporation chamber in relation to the evaporation temperature of the eluent, voltage of the photomultiplier, and driving gas flow rate. Moreover, the results obtained in this study showed that the A_0 and A_1 coefficients are not correlated with the TAG retention time, even if it was possible to observe a slight decrease in both coefficients with the increase in retention time. These results don't agree with those reported by other authors (17); in particular, opposite results were obtained for the A_1 coefficient.

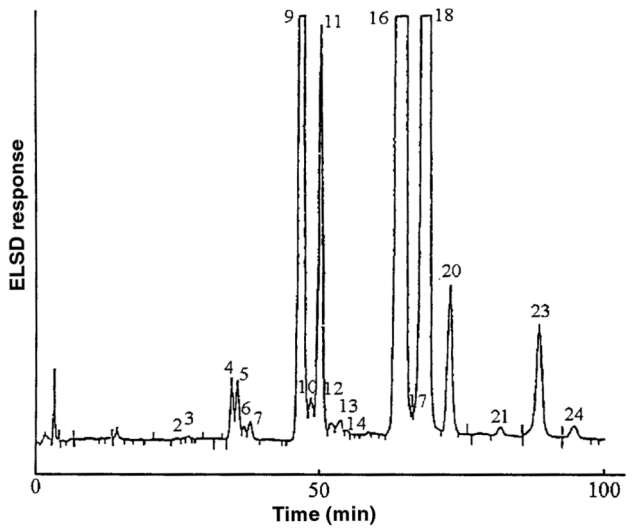

Figure 2. Chromatogram of a NARP-HPLC separation of olive oil TAG acquired by ELSD (peak identification is reported in Table III).

Table III. Comparison of Percent Compositions of TAG Olive Oil

TAG species	DAD* 205 nm	DAD* 210 nm	DAD* 215 nm	ELSD	1,3R-2R distribution
1. LLL	0.1	0.0	0.0	0.0	0.0
2. OLLn	0.2	0.2	0.2	0.2	0.2
3. PLLn	0.1	0.1	0.1	0.1	0.0
4. OLL	1.0	0.9	0.9	0.9	0.9
5. OOLn	1.4	1.2	1.1	0.8	1.1
6. PLL	0.4	0.3	0.3	0.4	0.2
7. POLn	0.7	0.6	0.5	0.4	0.3
8. PPLn	0.1	0.1	0.1	0.0	0.0
9. OOL	10.2	9.8	10.1	10.7	11.9
10. OOPo	1.1	1.0	0.9	1.1	1.2
11. PLO	5.0	4.7	4.8	4.5	4.0
12. OPPo	0.4	0.4	0.4	0.5	0.3
13. PLP	0.5	0.5	0.5	0.5	0.3
14. SPLn	0.1	0.0	0.0	0.3	0.0
15. LOE	0.2	0.1	0.1	0.0	0.1
16. OOO	43.7	44.9	46.7	47.6	48.6
17. SOL	0.6	0.6	0.6	0.2	0.7
18. POO	24.8	24.6	23.3	25.3	22.3
19. SLP	0.2	0.1	0.1	0.0	0.1
20. POP	3.6	3.8	3.1	2.6	2.7
21. OOE	0.5	0.5	0.4	0.4	0.6
22. POE	0.5	0.8	0.4	0.0	0.1
23. SOO	3.8	3.7	3.7	2.9	3.7
24. POS	1.0	1.1	1.5	0.6	0.8

* The percent abundance of each species was obtained by correcting the area with the rrf relative to triolein (Equation 3 and Table I).
† The percent abundance of each species was obtained by Equation 4 after the calculation of A_0 and A_1 constants by Equation 5.
‡ The TAG percent composition was obtained according to the 1,3-random-2-random distribution theory (18).

In Table II, the detection limits (expressed in nanograms of TAG required to give a peak height three times the height of the baseline noise) for each reference TAG are also shown; apart from some TAG for which the solubility is a probable cause of reduced sensitivity, the detection limits for the considered reference TAG were even less than 100 ng.

The results obtained using NARP-HPLC analysis with DAD–ELSD of the reference TAG were applied to the qualitative and quantitative analysis of the TAG fraction isolated from an extra-virgin olive oil sample. In Figures 1 and 2, the NARP-HPLC chromatograms of the olive oil TAG (obtained by DAD and ELSD, respectively) are shown. For DAD, the overlapped chromatograms registered at 205, 210, and 215 nm are reported.

In Table III, the TAG percent composition of the considered olive oil sample is reported. The comparison shows the data of the NARP-HPLC analysis of TAG with DAD at the considered wavelengths and with ELSD. The results in all cases were obtained by applying the parameters of the models built with the reference TAG. In the same Table III, the results of the indirect determination of the olive oil TAG percent composition according to the 1,3-random-2-random distribution theory (18) is reported. It is possible to observe a good accordance of the results, so as to validate the approach described in this paper for the TAG qualitative and quantitative analysis by the NARP-HPLC with DAD–ELSD on-line system.

Conclusion

In the present paper, an approach useful for obtaining qualitative and quantitative results for every TAG molecular species (with the restriction of working in determined experimental conditions) in the NARP-HPLC analysis with on-line DAD and ELSD is reported. The results for olive oil samples validate such a procedure.

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