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MALDI-TOF Mass Spectrometry Profiling of Polar and Nonpolar Fractions in Heated Vegetable Oils

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Triacylglycerol oxidation of thermally stressed (6 h at 180 °C, simulating deep-frying conditions) edible vegetable oil (sunflower and olive) was studied using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Chromatographic separation of the nonpolar and polar components from the heated oil performed on silica gel prior to MS analysis significantly enhanced the detection of oxidized components. The spectra contained signals that were assigned to triacylglycerols (TAG), diacylglycerols (DAG), triacylglycerol oxidative dimers, oxidized TAG, and TAG fragments arising from the homolytic β-scission of linoleyl, peroxy, and alkoxy radicals. Enrichment of the polar compounds prevented mass spectrometric ion suppression, thus allowing the detection of minor species originating from thermal oxidation. In addition, this allowed the monitoring of polar compounds in vegetable oils undergoing mild thermal treatment. As such, chromatographic separation coupled with MALDI-TOF MS analysis provided a rapid, sensitive, and specific tool to assess the thermal oxidation of vegetable oils.

KEYWORDS: Sunflower oil; virgin olive oil; thermo-oxidation; polar and nonpolar fractions; MALDI-TOF MS

INTRODUCTION

Thermal stress strongly oxidizes unsaturated vegetable oils, which has been determined to cause losses in nutrients and sensory attributes (*1*). The most important reaction in overheated/fried oils is the radical-mediated autoxidation of unsaturated/polyunsaturated triacylglycerols (TAG) that primarily leads to the formation of conjugated fatty acid hydroperoxides (**Figure 1**). As hydroperoxides, the primary products of lipid oxidation, are unstable at frying temperatures, peroxy and alkoxy radicals are formed from the major unsaturated alkyl chains, such as linolenoyl (18:3 *n*-3), linoleoyl (18:2 *n*-6), and oleoyl (18:1 *n*-9). These radical compounds readily decompose to form a wide variety of secondary oxidation products (**Figure 1b**). Some additional decomposition TAG reactions (e.g., hydrolysis, polymerization, cyclization, and cracking) could occur to form β-scission products, cyclic fatty acids, or monomeric and oligomeric oxidized TAG (*1*). Both nonvolatile and volatile compounds are formed during the frying process at temperatures in the 170−200 °C range (*2*). For the most part, volatile fried-flavors and off-flavors escape from the frying medium. The nonvolatile decomposition products, including nonpolar nonoxygenated and polar oxygenated fractions, gradually accumulate in the oil, are absorbed by the fried foods, and are finally ingested. The level of oxidized products present and the kinetics of oxidation depend on the chemical structure of the TAG and on a series of other factors including the fat blend, heating temperature, frying time, food dryness, oxygen accessibility, and presence of transition metal ions and antioxidants. In addition to the off-flavor (*3*), the oxidized compounds of fried oils may contribute to adverse health effects (*4*). Among the chemical and physical indices, total polar compounds (TPC) is considered to be one of the most objective indicators for the evaluation of the deterioration of oils and fats during deep-frying (*5*, *6*). TPC of oils increase with frying time, and generally ~25% by weight is considered to be the safe upper limit (*7*).

Although accurate, the standard method based on silica gel column chromatography is relatively expensive and time-consuming (*8*). To characterize the oxidized and polymerized TAG, the complex mixture resulting from fried oils has been fractionated by solid phase extraction (SPE) on silica gel followed by high-performance size exclusion chromatography (HPSEC) (*6*, *9*, *10*). Alternative strategies, such as near-infrared spectroscopy (NIR), dielectric capacitance measurement (*11*), pressurized differential scanning calorimetry (*3*) combined with NMR, or ultrasonic-based technologies (*12*), have also been utilized. Although a rapid test to determine the level of TPC has been developed (*13*), a universally accepted test procedure for monitoring TAG oxidation is still lacking. Furthermore, there is a need to validate straightforward and high-throughput procedures capable of providing precise information about the chemical nature of the compounds formed during frying.

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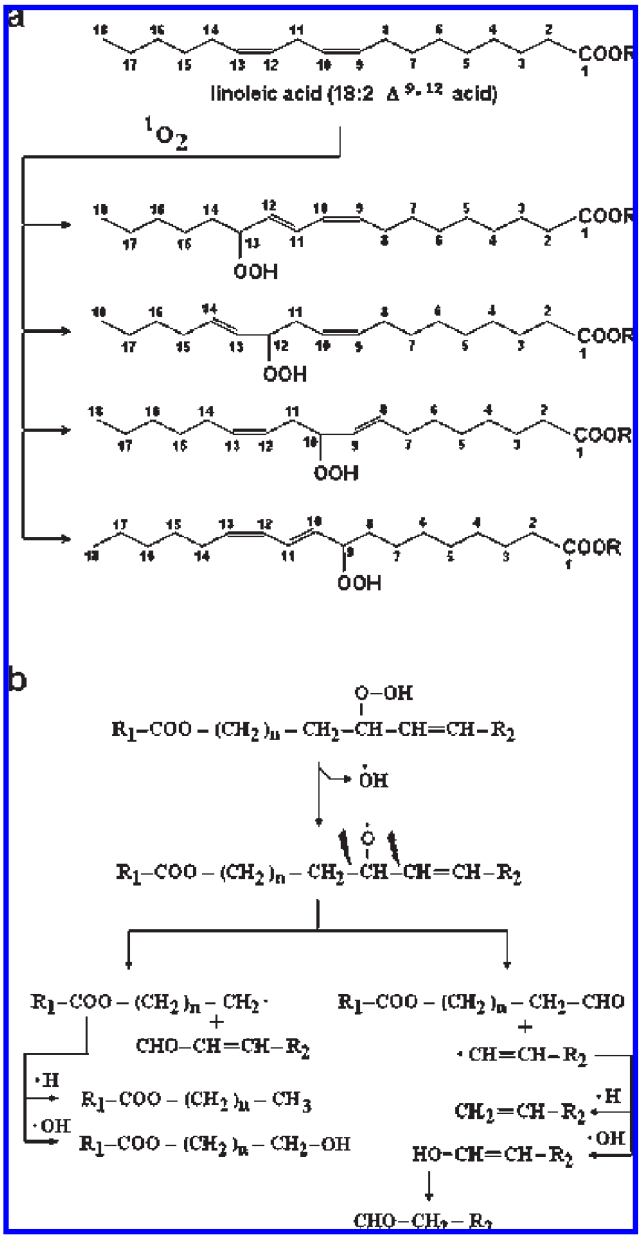

Figure 1. (**a**) Formation of allyl hydroperoxides from linoleic acid. (**b**) Main routes for β-scission of allyl hydroperoxides leading to short-chain glycerol-bound compounds, "core aldehydes", and aliphatic hydrocarbons.

In the past, a number of papers have been dedicated to the assessment of the oxidative stability of natural vegetable oils by mass spectrometric (MS) techniques. Application of soft ionization mass spectrometric sources such as electrospray (ESI) and atmospheric pressure chemical ionization (APCI) enabled the structural characterization of oxidized TAG (*14*, *15*). APCI-MS and ESI-MS were demonstrated to give complementary information for a complete characterization of TAG, also including the positional distribution of fatty acids (*16*). Dimeric and higher oligomeric TAG (up to tetramers) in heated model TAG were also characterized by ESI-MS (*15*). It was demonstrated that HPLC chromatographic separation prior to ESI-MS analysis, also in the direct HPLC/ESI-MS analysis, was necessary for the characterization of oligomers due to their low abundance in complex mixtures (*15*, *16*). On the other hand, quantitative analysis through ESI-MS lacks accuracy because the spectrometric response of TAG progressively increases with the degree of unsaturation (*16*).

A growing body of evidence is demonstrating that MALDI-TOF MS is a powerful method to analyze lipid mixtures because of the following advantages: (i) sample preparation is simple and extremely fast; (ii) no derivatization is required; (iii) buffer or salt contaminants are tolerated to some extent; (iv) the low degree of fragmentation of TAG−metal ion adducts produces unique molecular weights (*17*−*20*), allowing rapid profiling of the complex TAG mixtures (*21*).

Although the structural characterization of the autoxidized TAG is out of its capability, MALDI MS analysis makes possible (i) the reproducible and specific detection of the molecular species, with high sensitivity; and (ii) TAG evaluation, as signal intensities are well correlated to the TAG amounts (*22*). In addition, by determining the molecular weight with high accuracy it is possible to infer structural details, as mechanisms underlying oxidative modifications of lipids are, for many aspects, well-known.

Thermal oxidative degradation of vegetable oils has already been monitored either by MALDI-TOF MS (*23*, *24*) or by laser-based ionization method without matrix (LDI-TOF) (*25*). However, both MALDI (*26*) and ESI (*15*) suffer from strong ion suppression effects in the analysis of complex lipid mixtures, in which the components cover a high dynamic range. Thus, in heated oils, the coexistence of large amounts of unmodified TAG can affect the ion production and the detection of oxidized or dimeric TAG species. In this study, we exploit MALDI-TOF MS for the investigation of thermo-oxidative TAG deterioration during frying of sunflower and virgin olive oils, in conditions of real-scale and subreal-scale thermal stress, respectively, by coupling a simple chromatographic fractionation of polar and nonpolar species to the mass spectrometric analysis.

MATERIALS AND METHODS

Hexane, diethyl ether, chloroform, ethanol, and sodium chloride were acquired from Carlo Erba (Milan, Italy). Sodium chloride, 2,5-dihydroxybenzoic acid (DHB), trifluoroacetic acid (TFA), iodine, phosphomolybdic acid, and standard TGA, including triolein, tripalmitin, trilaurin, and tricaprine, were purchased from Sigma (Milan, Italy). Acetic acid glacial and methanol were from J. T. Baker (Deventer, The Netherlands).

All solvents and chemicals were of the highest purity grade commercially available and were used without any further purification. Silica gel 60 and thin-layer chromatography (TLC) plates (no. 11845; 20636, 20 cm) were from Merck (Darmstadt, Germany).

Sampling. Refined sunflower oil and extra-virgin olive oil samples were obtained from the local market and continually heated at 180 °C for 6 h, using a thermostatic household frying bath (Tefal, Milan, Italy). Oil samples were collected in duplicate at different times during the heating, 0 (initial time), 60, 120, 240, and 360 min, and stored in glass bottles at −20 °C until analysis. Visible impurities were removed from heated oils by paper filtration after homogenization.

Polar and nonpolar fractions were obtained by chromatography on silica gel according to the official IUPAC procedure (*5*). Briefly, 500 mg of oil dissolved in 2 mL of hexane/diethyl ether 90:10 (v/v) was loaded onto a hand-packed silica gel column containing 15 g of stationary phase. The nonpolar fraction containing the unoxidized TAG was eluted with hexane/diethyl ether 90:10 (v/v), and the solvent was evaporated. The polar compounds were then eluted by diethyl ether and finally dried. The efficacy of separation was checked by TLC by using precoated silica gel plates eluted with hexane/diethyl ether/acetic acid 80:20:1 (v/v/v) and visualized by exposure to iodine vapors. The plates were then sprayed with a 10% (w/v) phosphomolybdic acid solution in ethanol. A clear separation between the two fractions was achieved.

MALDI-TOF MS Analysis. MALDI-TOF MS experiments were carried out on a PerSeptive BioSystems (Framingham, MA) Voyager DE-Pro instrument, equipped with an N_2 laser (337 nm, 3 ns pulse width, 20 Hz repetition rate). Mass spectra were acquired in both the linear and reflector ion modes using Delayed Extraction technology with a delay time

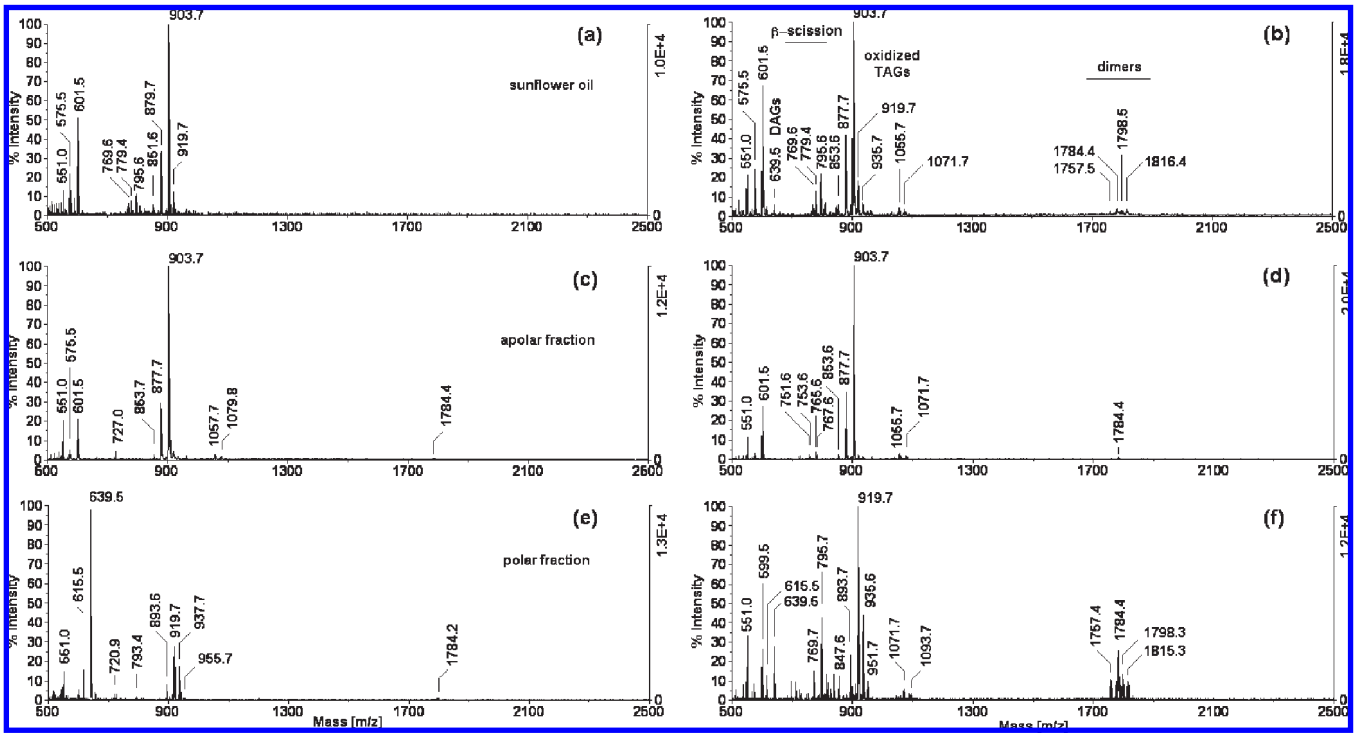

Figure 2. MALDI-TOF MS analysis of unfractionated (**a**) commercial and (**b**) thermally stressed (6 h at 180 °C) sunflower oil, nonpolar fractions of (**c**) commercial and (**d**) thermally stressed sunflower oil, and polar fractions of (**e**) commercial and (**f**) thermally stressed sunflower oil.

of 150 ns applied between laser pulses with the high voltage activated. The *m*/*z* 400−4000 range was explored. The instrument operated with an accelerating voltage of 20 kV. The matrix solution was prepared by dissolving 10 mg of crystalline DHB in 1 mL of methanol containing 0.1% TFA. Native and heated oils as well as the fractionated samples were dissolved in $CHCl_3$ at a concentration of 10 μL/mL. Chloroform solution was then vigorously shaken with 1 mL of aqueous 1 M NaCl as cationization agent, and then the aqueous layer was removed. An aliquot of the $CHCl_3$ layer (10 μL) was mixed with the matrix (1:1, v/v), and 1 μL of the resulting solution was deposited directly onto the sample plate and air-dried. Typically, 250 laser pulses were acquired for each mass spectrum. To minimize source fragmentation, the laser power was kept at a value not higher than 10% above threshold. External mass calibration was performed with a separate acquisition using a mixture of standard TAG and low mass standard peptides. To check the repeatability, samples were analyzed in triplicate. Mass spectra were elaborated using the Data Explorer 4.0 software (PerSeptive BioSystems).

RESULTS AND DISCUSSION

MALDI-TOF Analysis of Thermally Stressed Sunflower Oil. Whole and fractionated sunflower oil, before and after 6 h of heating at 180 °C, gave the MALDI-TOF MS spectra shown in **Figure 2**. Because TAG and DAG of vegetable oils exclusively contain medium-chain and long-chain fatty acids, spectra for sunflower and olive oil were acquired with a low-mass gate fixed at *m*/*z* 400. This prevents saturation of the detector by ions resulting from both DHB matrix and TAG fragmentation, without information loss. In MALDI-TOF MS TAG are detected as alkaline metal ion adducts in the positive ion mode (*27*). MALDI spectra of the commercial sunflower oil sample (**Figure 2a**) exhibited a somewhat complex profile indicating C_{54} as the most abundant within the TAG families. Although TAG oligomers having mass higher than *m*/*z* 2500 formed in thermally stressed unsaturated oils (*15*, *24*), the use of MALDI in the reflector ion mode was unable to detect oxidized components greater than dimer TAG. Components up to tetramers were detected as low-abundance broad peaks operating in only the linear ion mode, covering the *m*/*z* 400−4000 range (data not shown). However, in agreement with previous findings (*24*), in the linear mode information about the degree of unsaturation and isotopic distribution within all of the peak clusters was virtually lost. Carbon-linked trimers and tetramers in heated triolein have been identified using RP-HPLC online coupled to an ion trap mass spectrometer (*15*). Notwithstanding this, ESI-MS and APCI-MS were not sufficiently sensitive to detect higher molecular weight oxidized TAG without prior HPLC isolation (*16*).

The ubiquitous presence of alkaline metal ions could justify the presence in the spectra of minor amounts of K^+−TAG adducts (+16 Da with respect to the Na^+ adduct). Saturation of TAG chloroform solution with sodium chloride exclusively addresses the sodium adduct formation (*21*) so that the minor signals (+16 Da) occurring between two consecutive TAG clusters, rather than adducts, correspond to mono-oxidized TAG. In the low mass range, there were a few defined signals, for example, *m*/*z* 601.5 and 575.5, formed as a consequence of the $RCOO^-Na^+$ loss by in source fragmentation of TAG (*17*, *23*).

MALDI-TOF MS analysis of whole oil samples withdrawn at increasing heating times, that is, 1, 2, 3, 4, and 5 h, demonstrated that signal intensity of mono-oxidized TAG progressively increased (not shown), although the signals of TAG remained the most intense. At intermediate heating times of oil samples, the signals of TAG at higher degree of oxidation progressively intensified in the polar fraction (for instance, at *m*/*z* 935.8 and 951.7; cf. assignment in **Table 1**). The signal intensity of mono-oxidized TAG increased at a higher rate with respect to the signal of DAG, thus becoming the most represented among the polar compounds (**Figure 2f**). Whereas the polar fraction was found to be highly time-dependent, spectra of apolar fractions were not significantly affected by heating times, except for some minor signals of the nonoxygenated β-scission products. In the sunflower oil sample heated for 6 h at 180 °C, other mass signal clusters, missing or in trace amounts in the commercial sample, were clearly formed or markedly intensified in the *m*/*z* 1750−1850 mass range (**Figure 2b**).

Table 1. Assignment of the Primary Mass Signals Detected in the MALDI-TOF MS Spectra of Commercial and Heated Sunflower Oil[a]

measured MW	identification	prevalence in the apolar (a) or polar (p) fraction
	Diacylglycerols	
551.0	DHB matrix	
575.5	PL^+	(fragment) a, p
577.5	PO^+	(fragment) a, p
597.5	LLn^+	(fragment) a, p
599.5	LL^+	(fragment) a, p
601.5	LO^+	(fragment) a, p
603.5	OO^+	(fragment) a, p
613.5	PLn or oxidized fragment LLn^+	a, p
615.5	PL or oxidized fragment LL^+	a, p
617.5	PO or oxidized fragment LO^+	a, p
639.5	LL	p
641.5	LO	p
643.5	OO	p
	β-Scission Fragments	
751.6	LL-heptanoate ($C_{7:0}$)	a
753.6	LO-heptanoate ($C_{7:0}$)	a
767.7	$C_{52:5} - C_9H_{16} + {}^{16}O$	p
769.7	$C_{52:4} - C_9H_{16} + {}^{16}O$	p
771.7	$C_{52:3} - C_9H_{16} + {}^{16}O$	p
779.7	$C_{54:6} - C_{10}H_{18} + {}^{16}O$	p
781.7	$C_{54:5} - C_{10}H_{18} + {}^{16}O$	p
793.7	$C_{54:6} - C_9H_{16} + {}^{16}O$	p
795.7	$C_{54:5} - C_9H_{16} + {}^{16}O$	p
797.7	$C_{54:4} - C_9H_{16} + {}^{16}O$	p
805.7	$C_{54:6} - C_8H_{16} + {}^{16}O$	p
807.7	$C_{54:5} - C_8H_{16} + {}^{16}O$	p
809.7	$C_{54:4} - C_8H_{16} + {}^{16}O$	p
821.7	$C_{54:5} - C_7H_{14} + {}^{16}O$	p
823.7	$C_{54:4} - C_7H_{14} + {}^{16}O$	p
833.7	$C_{54:6} - C_6H_{12} + {}^{16}O$	p
835.7	$C_{54:5} - C_6H_{12} + {}^{16}O$	p
837.7	$C_{54:4} - C_6H_{12} + {}^{16}O$	p
845.7	$C_{54:7} - C_5H_{10} + {}^{16}O$	p
847.6	$C_{54:6} - C_5H_{10} + {}^{16}O$	p
	TAG/Oxidized TAG	
851.7	$C_{50:3}$ (PPLn)	a
853.7	$C_{50:2}$ (PPL)	a
877.7	$C_{52:4}$ (PLL)	a
879.7	$C_{52:3}$ (PLO)	a
893.7	$C_{52:4}$ (PLL)+ ^{16}O	p
895.7	$C_{52:3}$ (PLO) + ^{16}O	p
901.7	$C_{54:6}$ (LLL)	a
903.7	$C_{54:5}$ (LLO)	a
905.7	$C_{54:4}$ (LOO)	a
907.7	$C_{54:3}$ (OOO)	a
917.7	$C_{54:6}$ (LLL) + ^{16}O	p
919.7	$C_{54:5}$ (LLO) + ^{16}O	p
921.7	$C_{54:4}$ (LOO) + ^{16}O	p
923.7	$C_{54:3}$ (OOO) + ^{16}O	p
933.7	$C_{54:6}$ (LLL) + 2 ^{16}O	p
935.8	$C_{54:5}$ (LLO) + $2^{16}O$	p
937.8	$C_{54:4}$ (LOO) + $2^{16}O$	p
939.8	$C_{54:3}$ (OOO) + $2^{16}O$	p
949.7	$C_{54:6}$ (LLL) + $3^{16}O$	p
951.7	$C_{54:5}$ (LLO) + $3^{16}O$	p
953.7	$C_{54:4}$ (LOO) + $3^{16}O$	p
955.7	$C_{54:3}$ (OOO) + $3^{16}O$	p
	TAG Coupled to β-Scission Fragments	
1045.7	$C_{54:4} + C_9H_{18}O$ (nonanal)	p
1055.7	$C_{54:6} + C_{11}H_{20}O$ (undecene) $C_{54:6} + {}^{16}O + C_9H_{16}O$ (nonenal)	p
1057.7	$C_{54:6} + {}^{16}O + C_9H_{18}O$ (nonenal)	p
1059.7	$C_{54:5} + {}^{16}O + C_9H_{18}O$ (nonanal)	p
1069.7	$C_{54:6} + C_{11}H_{22}O$ (undecanal) or $C_{54:5} + C_{11}H_{20}O$ (undecenal) or $C_{54:6}{}^{16}O$ + undecene	p
1071.7	$C_{54:5} + C_{11}H_{22}O$ (undecanal) or $C_{54:5} + {}^{16}O$ + undecene	p
1073.7	$C_{54:4} + C_{11}H_{22}O$ (undecanal)	p
1079.8	LLL+ $C_{12}H_{20}O$ (dodecenedienale)	a
1085.7	$C_{54:5} + C_{12}H_{24}O$ (dodecanal) or $C_{54:5} + {}^{16}O$ + dodecene	p
1087.7	$C_{54:4} + C_{12}H_{24}O$ (dodecanal)	p
1093.7	$C_{54:6} + C_{13}H_{22}O$	p
1095.7	$C_{54:5} + C_{13}H_{22}O$	p
1097.7	$C_{54:4} + C_{13}H_{22}O$ or $C_{54:5} + {}^{16}O + C_{12}H_{20}O$	p
	Dimers	
1754.4	$C_{54} - C_{52}$; $n = 10$[b]	p
1756.4	$C_{54} - C_{52}$; $n = 9$[b]	p
1758.4	$C_{54} - C_{52}$; $n = 8$[b]	p
1760.4	$C_{54} - C_{52}$; $n = 7$[b]	p
1762.4	$C_{54} - C_{52}$; $n = 6$[b]	p
1774.4	$C_{54} - C_{52}$; $n = 8 + {}^{16}O$[b]	p
1776.4	$C_{54} - C_{52}$; $n = 7 + {}^{16}O$[b]	p
1778.4	$C_{54} - C_{54}$; $n = 12$[b]	p
1780.4	$C_{54} - C_{54}$; $n = 11$[b]	p
1782.4	$C_{54} - C_{54}$; $n = 10$[b]	p
1784.4	$C_{54} - C_{54}$; $n = 9$[b]	p
1786.4	$C_{54} - C_{54}$; $n = 8$[b]	p
1788.4	$C_{54} - C_{54}$; $n = 7$[b]	p
1786.4	$C_{54} - C_{54}$; $n = 8$[b]	p
1788.4	$C_{54} - C_{54}$; $n = 7$[b]	p
1796.4	$C_{54} - C_{54}$; $n = 11 + {}^{16}O$[b]	p
1798.4	$C_{54} - C_{54}$; $n = 10 + {}^{16}O$[b]	p
1800.4	$C_{54} - C_{54}$; $n = 9 + {}^{16}O$[b]	p
1802.4	$C_{54} - C_{54}$; $n = 8 + {}^{16}O$[b]	p
1804.2	$C_{54} - C_{54}$; $n = 7 + {}^{16}O$[b]	p
1812.4	$C_{54} - C_{54}$; $n = 11 + 2{}^{16}O$[b]	p
1814.4	$C_{54} - C_{54}$; $n = 10 + 2{}^{16}O$[b]	p
1816.4	$C_{54} - C_{54}$; $n = 9 + 2{}^{16}O$[b]	p
1818.4	$C_{54} - C_{54}$; $n = 8 + 2{}^{16}O$[b]	p
1820.4	$C_{54} - C_{54}$; $n = 7 + 2{}^{16}O$[b]	p
1830.5	$C_{54} - C_{54}$; $n = 10 + 3{}^{16}O$[b]	p
1832.5	$C_{54} - C_{54}$; $n = 10 + 3{}^{16}O$[b]	p

[a] In the assignment of TAG, the possible and most represented species are reported, but occurrence of isobaric species should also be considered. Except for the indicated fragments, DAG, TAG, and their derivatives are detected as Na^+ adducts. Measured MW are referred to monoisotopic mass values (see text). [b] *n* denotes the total number of double bonds within the dimeric species. In the computation of molecular mass, a loss of two H, due to the formation of a C−C between two different TAG molecules, has been taken into account.

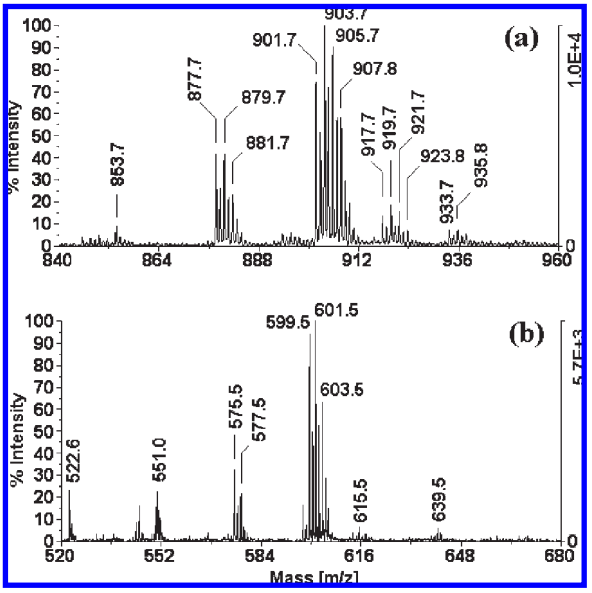

Figure 3. Magnified views of the MALDI-TOF spectral range of unfractionated, commercial sunflower oil: (**a**) region of TAG and oxidized TAG; (**b**) region of the source produced fragments.

The list of signals and their tentative assignment to TAG in the MALDI-TOF spectra are reported in **Table 1**. Mass signals were assigned by considering the previous studies on hydroperoxide formation of unsaturated TAG and subsequent thermal decomposition (*1*, *23*, *24*). In addition to this, the nonpolar and polar fractions from commercial and thermally stressed oil samples each gave a simplified MALDI-TOF spectrum. Here, the mass signals were assigned according to the indications by Frankel (*1*) for native TAG. Similarly, DAG, TAG dimers, oxidized TAG, and TAG fragments arising from the homolytic β-scission of linoleyl, peroxy, and alkoxy radicals were assigned. The different classes of such compounds are discussed in the following paragraphs.

Triacylglycerols of Sunflower Oil. In previous studies, the signal at m/z 903.7 was assigned to the Na^+ adduct of a $C_{54:5}$ TAG that could correspond to the isobaric LLO, OOLn, or SLLn (S = stearic, O = oleic, L = linoleic, and Ln = linolenic acid). Considering that LLO is the main TAG of sunflower oil, the major signals at m/z 901.7, 903.7, 905.7, and 907.7 would correspond to the multiple unsaturated LLL, LLO (isobaric with LOL), OOL (isobaric with OLO), and OOO, respectively. Similarly, signals at m/z 877.7, 879.7, and 881.7 have to be assigned to PLL, PLO, and POO, respectively, although each form is indistinguishable from their isobaric positional isomers. By analogy, the low-intensity signal at m/z 853.7 can be assigned to PPO and/or POP. The cluster signals corresponding to the most abundant TAG in the commercial sunflower oil sample are shown in the expanded view of the spectrum in **Figure 3a**, and signals are assigned in **Table 1**. Although the signals of the oxidized species intensified, the TAG pattern of the heated oil remained unchanged.

Oxidized Triacylglycerols. Oxidized TAG have been detected and quantified in fried or thermally stressed vegetable oils by separating the polar fraction (*6*, *9*, *10*). MALDI spectra of the polar oxygenated compounds, which were isolated by silica gel column chromatography, confirmed the assignment of the +16 Da signals to oxidized TAG (**Figure 2a**). Although the chemical structure of these compounds cannot be deduced from the MALDI spectra (the oxygen atom could be present as a hydroxy, keto, or epoxy group), the +16 Da can be considered diagnostic for the presence of extra oxygen in at least one of the fatty acyl groups of the TAG molecules. The "oxygenated TAG" are here collectively designated "oxidized TAG". TAG signal clusters, centered at m/z 919.7 (mono-oxidized TAG) and m/z 935.8 (dioxidized TAG), were already detected in low amount in commercial sunflower oil, consistently with the prior studies (*25*). This has to be ascribed to both the thermal stress, which sunflower oil undergoes during refining process, and the susceptibility of the polyunsaturated fatty acids to activated (singlet) oxygen. Oxidized species at progressive increments of 16 Da, for example, m/z 919.7, 935.7, and 951.7, arise due to the mono- and polyoxidized LLO, respectively. The oxidized PLL (m/z 893.7) was found to be highly concentrated in the polar fraction of commercial (**Figure 2e**) sunflower oil and, especially, heated (**Figure 2f**) sunflower oil. The most abundant polar compound in commercial sunflower oil (**Figure 2e**) was found to be the DAG LL (m/z 639.5). By heating (**Figure 2f**), a series of new polar compounds was formed. Among these, the mono-oxidized TAG became dominant. The oxidized TAG, having lost $RCOO^-Na^+$ in the MALDI ionization, produced the oxidized species seen at m/z 617.5 and 615.5, with the native fragments present at m/z 601.5 and 599.5, respectively (see above). This demonstrates that the chromatographic depletion of TAG allows the straightforward characterization of the oxidized species, including the newly created polar forms. The spectral range of the fragments of TAG/oxidized TAG produced in the MALDI source for unfractionated commercial sunflower oil is magnified in **Figure 3b**.

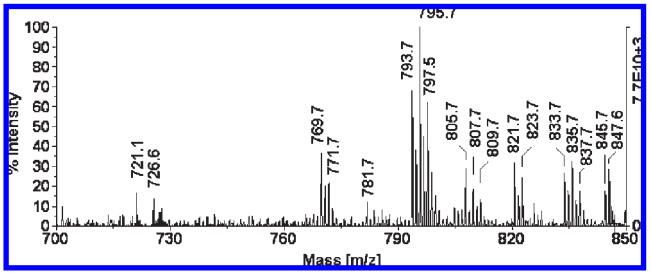

Figure 4. Magnified view of the MALDI-TOF spectral range for the polar fraction of thermally stressed sunflower oil in the region of the β-scission products.

Triacylglycerol Fragments Arising from the Homolytic β-Scission of Hydroperoxides. The main mechanism leading to the formation of aldehydes and nonvolatile oxidized compounds, including short-chain glycerol-bound compounds, aldehydes, acids, ketones, and alcohols from lipid hydroperoxides, is the homolytic β-scission of C−C bonds on either side of the alkoxy radicals from allylic hydroperoxides (**Figure 1**). The term "core aldehydes" has been used as a generic name for the β-scission-derived, TAG-containing aldehydic esters (*28*). These compounds are mainly concentrated in the chromatographic polar fraction. The nonvolatile TAG degradation products, which remain in the frying oil, are ingested together with the food and are potentially harmful. For instance, it has been reported that short-chain glycerol-bound compounds, such as core aldehydes, are easily absorbed in the intestinal tract after hydrolysis by pancreatic lipase and might induce lipid peroxidation and affect hepatic metabolism (*29*). The mass spectral range encompassing the signals of the β-scission products, arising from the polar fraction of thermally stressed sunflower oil, is shown in **Figure 4**. The most abundant β-scission products are represented by the cluster centered at m/z 795.7, due to the core aldehyde, TAG-containing 9-oxononanoic acid, which is generated by the breakdown of oleic or linoleic acids. This observation is consistent with the previous GC-MS determination indicating that 9-oxononanoic acid is the most abundant fatty acid produced by thermally

induced cleavage of both oleic and linoleic acid (*30*−*32*). By analogy, the signal clusters centered at *m*/*z* 807.5, 821.7, and 835.7 can be assigned to 10-oxo-8-decenoate, 11-oxo-9-undecenoate, and 12-oxo-9-dodecenoate containing TAG, respectively.

The tentative assignment of the spectral signals to β-scission species identified by the alkyl chain loss from native TAG is shown in **Table 1**.

Consistent with previous reports (*30*), the TAG-containing short-chain acyl groups constitute a minor fraction of the total oxidized TAG in thermally stressed oil.

Homolytic β-scission of the alkoxy radical, from the 8- or 9-hydroperoxide of oleic acid, involves carbon−carbon cleavage on either side of the carbon-bearing oxygen. The resulting radical could generate saturated short-chain fatty acyl $C_{7:0}$ or $C_{8:0}$ groups attached to the acylglycerol backbone by $H^{\bullet}$ radical addition. Thus, the short-chain fatty acid containing TAG, which do not exhibit the aldehyde function, preserves their nonpolar property. The expected volatile aldehydes and short-chain fatty acid bound compounds are those coming from the major hydroperoxides of oleic and linoleic acid, and the relative proportions depend on the fatty acid composition of the frying oil (*33*). Based on the previous observation (*33*), it is reasonable to detect low-abundance LL-heptanoate ($C_{7:0}$) (*m*/*z* 751.6) or LO-heptanoate ($C_{7:0}$) (*m*/*z* 753.6, generated exclusively from oleic acid) in the nonpolar fraction of the heated sunflower oil (**Figure 2d**). Slightly higher amounts of LL-octanoate ($C_{8:0}$) (*m*/*z* 765.6) or LO-octanoate ($C_{8:0}$) (*m*/*z* 767.6) were produced (**Figure 2**d).

Diacylglycerols. As indicated above, the loss of $RCOO^{-}Na^{+}$ from TAG produced the clusters of intense signals centered at *m*/*z* 575.5 and 601.5 (**Figure 3b**). Similarly, oxidized TAG gave rise to the fragment signal clusters centered at *m*/*z* 599.5 and 615.5. Even though these signals can also be attributed to diacylglycerols, they have to be considered, at least in part, artifacts arising from the ionization event. Real LL and related species, such as LO and OO, are actually identified by their molecular mass (*m*/*z* 639.5, 641.5, and 643.5, respectively) and are recovered in the polar fraction due to their alcohol functional groups (**Figure 2e**).

The component at *m*/*z* 615.5 could be due to the contribution of both the "native" LP as Na^{+} adduct and the isobaric oxidized LL^{+} fragment source-produced from the oxidized TAG LLL or LLO. Therefore, molecular mass did not allow distinction of these two isobaric signals. Interestingly, the signal at *m*/*z* 615.5 is more intense in the commercial sunflower oil than in the heated sample. This suggests that LP actually occurs in the commercial sunflower oil, where DAG naturally accounts for 1−3% (*34*).

Although the level of DAG should increase during heating because of the hydrolysis of acyl bonds, especially in the presence of moisture, TAG oxidation is the prevalent reaction at the frying temperature. Thus, signals of the oxidized TAG progressively increased (**Figure 2f**), becoming dominant over those of the DAG, after only 3 h of heating (not shown).

Triacylglycerol Dimers. The signal clusters in the *m*/*z* 1730−1850 range most likely correspond to the triacylglycerol dimers. Although low levels were detected in commercial oil (**Figure 2a**) as a result of autoxidation or refining, TAG dimers drastically increased after 6 h at 180 °C (**Figure 2b**). TAG dimers were specifically enriched in the polar chromatographic fraction (**Figure 2f**), and the components, which are singled out in the expanded MALDI spectrum (**Figure 5a**), are tentatively identified as reported in **Table 1**. The main signals matched the mass values expected for nonoxygenated dimers or "thermal dimers". These arrangements arise from the interactions of major polyunsaturated TAG (LLL, LLO, PLL). In this interaction, the linoleoyl

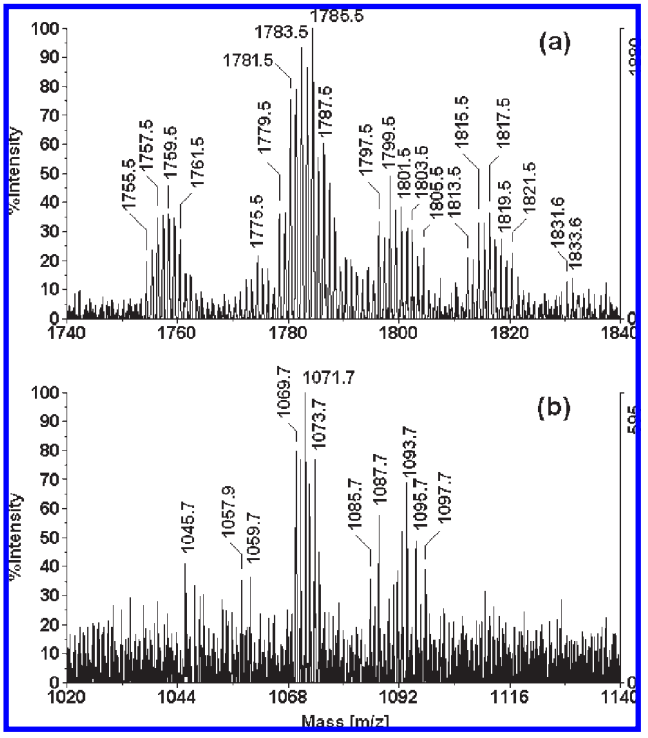

Figure 5. MALDI-TOF MS analysis of the polar fraction for thermally stressed sunflower oil: mass region of (**a**) TAG dimers and (**b**) TAG short-chain condensation products.

radicals dimerize, forming a covalent C−C bond (*16*). Assuming that the concentration is roughly proportional to the signal intensity, the nonpolar dimers at *m*/*z* 1781.5 ($C_{54:6}$−$C_{54:5}$), 1783.5 ($C_{54:5}$−$C_{54:5}$), and 1785.5 ($C_{54:4}$−$C_{54:5}$) result in higher amounts than the polar dimers (or "oxidative dimers"). The signal clusters centered at *m*/*z* 1799.5 (1783.5 + 16) and at 1815.4 (1783.5 + 32) are the mono-oxygenated or dioxygenated "oxidative dimers", respectively. The signals have been assigned by matching the measured *m*/*z* values with the expected monoisotopic masses. However, because dimeric species contain more than 100 carbon atoms, the contribution to the signals by the first ^{13}C isotope variant is higher than that of *all*-^{12}C species (^{13}C natural abundance = 1.1%). Therefore, for the dimer TAG, the monoisotopic masses are not expected to be the most abundant ions. For the same reason, in a specific cluster, the signals are the result of the superimposition of the isotopic contributions of species with different degrees of unsaturation. In this manner, in agreement with the prediction made with the "isotope abundance calculator" tool available in the Data Explorer software, the even mass sodiated dimer TAG were found to be the most abundant signals within a cluster. This apparently contrasts with the literature findings which indicate odd mass dimer TAG as the most abundant ions. In fact, this holds when the *m*/*z* values of isolated molecular entities are measured, as in the cases of model systems containing a single TAG or species previously isolated by HPLC as in the case of HPLC-ESI MS (*15*).

The oxygen atom in the dimer could either bridge two acyl chains (C−O−C) or generate oxygenated functions (hydroxy, keto, or epoxy) in the acyl chains of carbon-linked dimers, for example, a hydroperoxide of a dimer (*16*). It is unlikely that peroxy bonds (C−O−O−C) could occur in the heated oil, as they are known to readily decompose at temperatures above 100 °C. It is well-known that peroxy-linked dimers may be formed at higher temperatures. These can only occur as intermediate compounds resulting in either ether-linked or other oxygenated polar dimers (*1*).

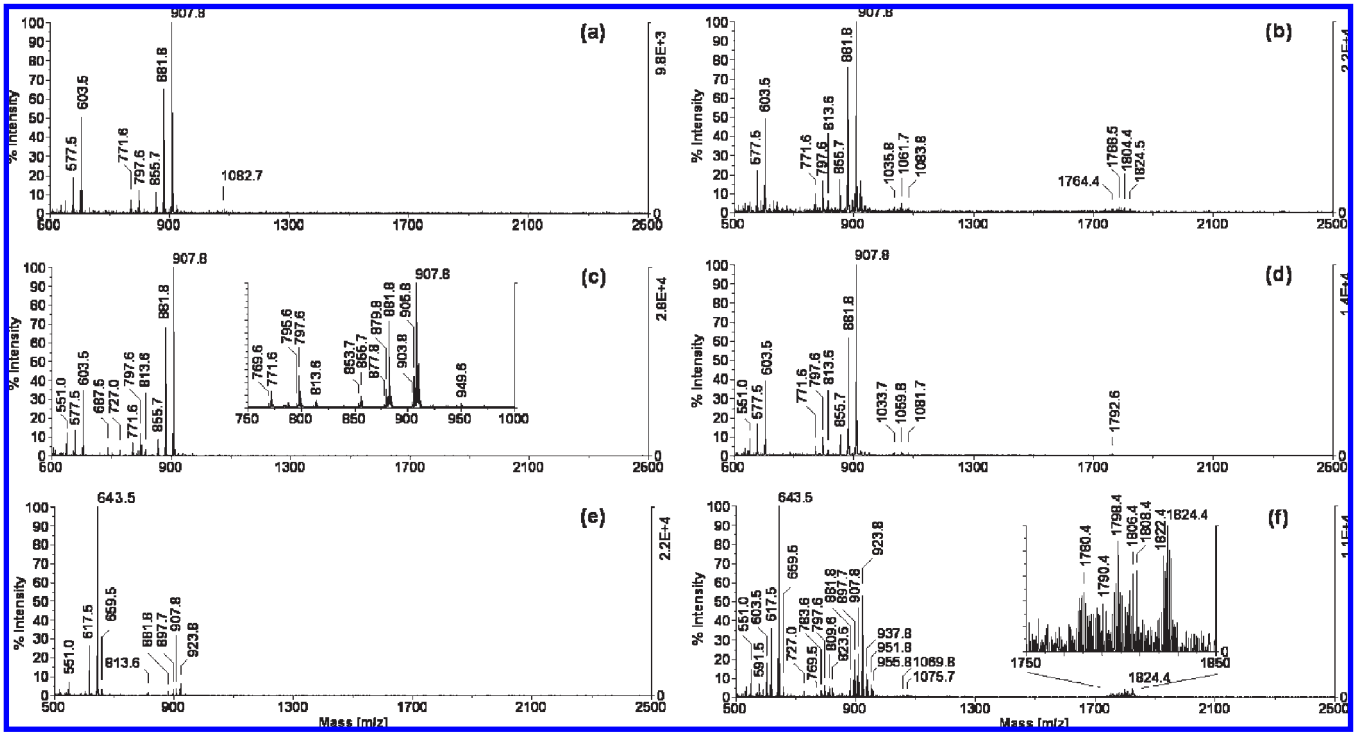

Figure 6. MALDI-TOF MS analysis of unfractionated (**a**) unheated and (**b**) thermally stressed (4 h at 180 °C) virgin olive oil (VOO), nonpolar fractions of (**c**) unheated and (**d**) thermally stressed VOO, and polar fractions of (**e**) unheated and (**f**) thermally stressed VOO.

Interestingly, oxygenated dimers and, in principle unexpectedly, nonoxygenated dimers were almost completely retained on the silica gel column and thus recovered together in the polar fraction. This is consistent with the previous findings demonstrating overall recovery by HPSEC of dimer and oligomer species in the "polar compounds" using hexane/diethyl ether 90:10 (v/v) as the eluting solvent for nonpolar compounds (*32*).

The signal clusters centered at *m*/*z* 1055.7 and 1071.7 from the nonpolar fraction or the signals at *m*/*z* 1071.7 and 1093.7 from the heated oil polar fraction (see the expanded view in **Figure 5b**) correspond to the "chain-branched addition products" already described (*16*, *35*). These compounds are most likely formed by pairing TAG and β-scission-derived aldehydes (or oxidized TAG and aliphatic short-chains) with a pathway analogous to that leading to TAG dimerization. From a different point of view, these compounds could be seen as one of the possible moieties resulting from the β-scission of TAG dimers. For instance, the most intense signal at *m*/*z* 1071.7 in the polar fraction matches the product obtained from the condensation of the $C_{54:5}$ TAG (LLO) and undecanal. When computing the expected molecular mass, the loss of two H was considered due to the C−C bond formation (*16*). Nevertheless, a contribution to the ion signals of and similarly the isobaric product generated by condensation of aliphatic undecene and oxidized $C_{54:5}$ TAG cannot *a priori* be ruled out. Undecene can arise from the cleavage of the C7−C8 bond of either oleic or linoleic acids. The signals at *m*/*z* 1055.7 and 1087.7 match the molecular mass of the species formed by condensation of the same aliphatic undecene with the nonoxidized or 2-fold oxidized TAG, respectively. However, *m*/*z* 1055.7 can arise from the condensation of oxidized $C_{54:6}$ TAG with nonenal. The signal at *m*/*z* 1087.7 most likely contributes the condensation product of oxidized $C_{54:5}$ TAG and undecanal. One should also consider the case of isobaric hydroxyl aldehyde transfer to undecanal. This reflects an alternative to 11-oxo-6-undecenal formation from the cleavage of the C8−C9 bond in either oleic or linoleic acid.

The less intense signal at *m*/*z* 1057.7 should be at least in part a result of oxidized $C_{54:6}$ TAG being condensed with nonanal, which is among the most abundant aldehyde compounds formed from thermo-oxidized TAG. Furthermore, this signal also matches the molecular mass of the mixed compound LLL-4-hydroxy-2, 3-*trans*-nonenal; this hydroxyl aldehyde is formed from 13-hydroperoxylinoleic acid and is thought to have high cellular toxicity (*36*). However, definitive identification of these compounds cannot be conclusive using mass spectrometric measurements alone. Signals at higher *m*/*z* (1085.7−1097.7) are the homologous higher mass compounds derived from longer chain β-scission fragments (**Table 1**). The expected dimeric species generated by the pairing of TAG and β-scission TAG fragments were either nonexistent or below the limit of detection.

MALDI-MS Analysis of Heated Virgin Olive Oil (VOO) and Its Polar Fraction. When VOO was heated in the conditions used for sunflower oil, no significant modification was apparent along the MALDI spectra acquired at 4 and 6 h.

To illustrate the capability of the MALDI-TOF MS to monitor the products deriving from an intrinsically less oxidizable oil, such as VOO, heated at a subreal-scale, the results for VOO heated 4 h at 180 °C are presented. MALDI analysis of the nonpolar and polar fractiond of VOO is compared for unheated and heated oil in **Figure 6c**,**d** and **e**,**f**, respectively. The results indicate that the most intense MALDI signals correspond to OOO (*m*/*z* 907.8) and OOP (*m*/*z* 881.8). Analogous to sunflower oil, the polar fraction contained a major signal at *m*/*z* 643.5 ascribable to the OO DAG. The incomplete TAG biosynthesis in VOO leaves 2−3% DAG; the signal *m*/*z* 643.5 could be 1,2-OO because the 1,3-DAG isomer occurs only in trace amounts in VOO. Heating generated TAG oxidization products in VOO more slowly than in sunflower oil. After 1 h of heating, signals of oxidized TAG were hardly detected in the spectrum of whole oil. In contrast, low levels of oxidized TAG were detected early on 1−2 h of heating in the polar fraction (not shown). At variance to what has been observed for sunflower oil, after 4 h of heating the signal of

Table 2. Assignment of the Primary Mass Signals Detected in the MALDI-TOF MS Spectra of Nonheated and Thermally Stressed Virgin Olive Oil[a]

measured MW	identification	prevalence in the apolar (a) or polar (p) fraction
	Diacylglycerols	
551.0	DHB matrix	
575.5	PL^+	(fragment) a
577.5	PO^+	(fragment) a
601.5	LO^+	(fragment) a, p
603.5	OO^+	(fragment) a, p
1075.7	$C_{54:3} + C_{11}H_{22}O$ (undecanal)	p
615.5	PL or oxidized fragment LL^+	a, p
617.5	PO or oxidized fragment LO^+	a, p
641.5	LO	p
643.5	OO	p
645.5	OS	p
	β-Scission Fragments	
769.7	$C_{52:4} - C_9H_{16} + {}^{16}O$	p
771.7	$C_{52:4} - C_9H_{16} + {}^{16}O$	a, p
783.6	$C_{54:4} - C_{10}H_{18} + {}^{16}O$	p
795.6	$C_{54:5} - C_9H_{16} + {}^{16}O$	p
797.6	$C_{54:4} - C_9H_{16} + {}^{16}O$	a, p
807.6	$C_{54:5} - C_8H_{16} + {}^{16}O$	p
809.6	$C_{54:4} - C_8H_{16} + {}^{16}O$	p
813.6	$C_{54:4} - C_9H_{16} + 2{}^{16}O$	a, p
821.7	$C_{54:5} - C_7H_{14} + {}^{16}O$	p
823.7	$C_{54:4} - C_7H_{14} + {}^{16}O$	p
	TAG/Oxidized TAG	
853.7	$C_{50:2}$ (PPL)	a
877.7	$C_{52:4}$ (PLL)	a
879.8	$C_{52:3}$ (PLO)	a
881.8	$C_{52:2}$ (POO)	a
895.7	$C_{52:3} + {}^{16}O$	p
897.7	$C_{52:2} + {}^{16}O$	p
903.7	$C_{54:5}$ (LLO)	a
905.7	$C_{54:4}$ (LOO)	a
907.7	$C_{54:3}$ (OOO)	a
921.7	$C_{54:4}$ (LOO) + ${}^{16}O$	p
923.7	$C_{54:3}$ (OOO) + ${}^{16}O$	p
935.8	$C_{54:5}$ (LLO) + $2{}^{16}O$	p
937.8	$C_{54:4}$ (LOO) + $2{}^{16}O$	p
939.8	$C_{54:3}$ (OOO) + $2{}^{16}O$	p
951.7	$C_{54:5}$ (LLO) + $3{}^{16}O$	p
953.7	$C_{54:4}$ (LOO) + $3{}^{16}O$	p
955.7	$C_{54:3}$ (OOO) + $3{}^{16}O$	p
	TAG Coupled to β-Scission Fragments	
1033.7	$C_{54:3} + C_9H_{20}$	a
1035.8	$C_{54:2} + C_9H_{20}$	a
1059.8	$C_{54:4} + C_{10}H_{20}O$ (decanal)	a, p
1061.7	$C_{54:3} + C_{10}H_{20}O$ (decanal)	a, p
1069.7	na[c]	a, p
1075.7	$C_{54:3} + C_{11}H_{22}O$ (undecanal)	p
1081.7	na	a
1083.7	na	a
	Dimers	
1780.4	$C_{54} - C_{52}$; $n = 5 + {}^{16}O$[b]	p
1782.4	$C_{54} - C_{52}$; $n = 4 + {}^{16}O$[b]	p
1784.4	$C_{54} - C_{54}$; $n = 9$[b]	p
1786.4	$C_{54} - C_{54}$; $n = 8$[b]	p
1788.4	$C_{54} - C_{54}$; $n = 7$[b]	p
1790.4	$C_{54} - C_{54}$; $n = 6$[b]	p
1792.4	$C_{54} - C_{54}$; $n = 5$[b]	p
1796.4	$C_{54} - C_{52}$; $n = 5 + 2{}^{16}O$[b]	p
1798.4	$C_{54} - C_{52}$; $n = 4 + 2{}^{16}O$[b]	p
1806.2	$C_{54} - C_{54}$; $n = 6 + {}^{16}O$[b]	p
1808.4	$C_{54} - C_{54}$; $n = 5 + {}^{16}O$[b]	p
1822.4	$C_{54} - C_{54}$; $n = 6 + 2{}^{16}O$[b]	p
1824.4	$C_{54} - C_{54}$; $n = 5 + 2{}^{16}O$[b]	p

[a] In the assignment of TAG, the possible and most represented species are reported; however, occurrence of isobaric species should also be considered. DAG, TAG, and their derivatives are detected as Na^+ adducts. Measured MW are referred to monoisotopic mass values (see text). [b] *n* denotes the total number of double bonds within the dimeric species. In the computation of molecular mass a loss of two H, due to the formation of a C−C between two different TAG molecules, has been taken into account. [c] na, not assigned.

native DAG signal (for instance *m*/*z* 643.5) was still dominating the oxidized TAG (**Figure 6f**). The ratio of signal intensities between DAG and TAG oxidation products remained practically unaltered after 6 h of heating. Taken together, these data indicate that in VOO the TAG are more protected during deep-frying. This is consistent with the nature of VOO, which is intrinsically more stable to oxidation because of its higher antioxidant content and the lower unsaturation of TAG with respect to sunflower oil. In addition to the signals at *m*/*z* 603.5 and 617.5 described above, the signal at *m*/*z* 797.7 is likely due to the TAG produced by the cleavage of the double bond of the oleic acid residue (loss of C_9H_{16}) followed by the formation of the corresponding aldehydic compound.

Schiller et al. (*37*) described the double-bond breakdown of unsaturated fatty acids in TAG in the gas phase induced by the laser soon after the ionization/desorption event. This fragmentation route is analogous to the homolytic β-scission subsequent to the autoxidation. The signal at *m*/*z* 797.7, together with that at *m*/*z* 771.7 generated from the OOP fragmentation, also occurred in the VOO sample. In the polar fraction of heated VOO, the intensity of the above fragments increased as a result of the two cooperating phenomena, that is, the oxidative β-scission and the source-induced fragmentation. A similar increase was already observed for the β-scission products occurring in the polar fraction of sunflower oil. As confirmation of the two operating pathways, the signal at *m*/*z* 813.6, corresponding to a further

oxidized compound at m/z 797.7, was the only one occurring in the polar fraction of VOO (**Figure 6e**). In comparison, signals at m/z 771.7 (and the related unsaturated species at m/z 769.7), 797.7, and 813.6 were simultaneously detected in the heated oil (**Figure 6f**). As expected for olive oil, which is high in oleic acid, the most abundant β-scission products were those derived from 9-hydroperoxide.

During heating, mono- (m/z 923.8) and dioxygenated (m/z 939.8) triolein increased in the polar fraction (**Figure 6f**). Because VOO contains LOO and LLO, the polar fractions of the heated oil contain these partly oxidized TAG (**Figure 6f**). Comparison of the peak intensity in the m/z 1750−1850 range indicated that TAG dimerization occurred in VOO to a lesser extent that in sunflower oil (**Figures 2c** and **6c**). In addition to this, the oxygenated species were detected almost exclusively in the polar fraction of heated VOO.

However, the formation of oxidized species was conditional upon both the difference in chemical composition between the two vegetable oils and the technological process of production. Whereas a small amount of TAG dimer was already identified in the commercial sunflower oil, oxidized TAG were missing in VOO: in contrast to VOO, commercial sunflower oil usually undergoes refining and deodorizing processes that can trigger an early autoxidation. The reduced degree of oxidation justifies the lower intensity signals in VOO, as compared to sunflower oil, arising from TAG coupled to short aliphatic chains generated from β-scission, observed in the range of m/z 1033.7−1083.7. As discussed for sunflower oil, the nonpolar fractions of VOO contained, in addition to native TAG, minor amounts of the newly formed β-scission products (or in source formed fragments, see above). These products, at m/z 771.7, 797.7, and 813.6, contain short-chain fatty acids (**Figure 6c**,**d**). Tentative assignments to the TAG species detected in VOO are reported in **Table 2**.

On the basis of MALDI-TOF data, the unsaturated TAG undergo modification even in the case of relatively short heating times (2−4 h). Studies on model TAG already indicated the usefulness of direct MALDI-TOF MS analysis in assessing the thermal damage associated with frying oil subjected to extended use (*23*). The described strategy, which combines on-column chromatographic fractionation with MALDI-TOF analysis, expands the amount of information available for vegetable oils subjected to the conditions of real-scale thermo-oxidation. Therefore, the above strategy should open the way for reliable MS-based determination of minor amounts of polar compounds generated from mild thermal conditions (oil refining or soft deodorization). This can also help in detecting adulteration of VOO, as this is very difficult to achieve using currently available methods.

Correlations among chemical species detected by MALDI-TOF with those identified from independent techniques (HRGC, NMR) are in progress with the aim of developing a comprehensive approach, including the quantitative determination, to assessing the thermo-oxidation of heated oil.

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