

Research Article

MALDI-SpiralTOF technology for assessment of triacylglycerols in Croatian olive oils**Željka Persurić¹, Junichi Osuga², Tihana Galinac Grbac³, Jasna Peter-Katalinić¹ and Sandra Kraljević Pavelić¹**¹ Department of Biotechnology, Centre for High-Throughput Technologies, University of Rijeka, Rijeka, Croatia² JEOL SAS, Espace Claude Monet—1, Croissy-sur-Seine, France³ Faculty of Engineering, University of Rijeka, Rijeka, Croatia

Traceability and authenticity of olive oils in Croatia were probed by a versatile analytical approach using spiral MALDI-TOF MS system for the triacylglycerol (TAG) analysis. Molecular ion patterns were shown to represent specific data for statistical analysis, in which differences of cultivar regions of Croatian coast were recognized. This pilot project allows indications to be followed in high throughput procedures with high number of samples to get statistically relevant data for discrimination of olive oils from different geographic sites. Additionally, the fragmentation in the TOF/TOF MS provided distinct highly valuable data for assignment of acyl residues. In this way molecular species with different fatty acid substituents can be clearly assigned and possible isomeric mixtures identified.

Practical applications: Olive oil TAG analysis is important to determine authenticity of olive oil and detect adulteration. This work shows that spiral MALDI-TOF MS analysis of TAG composition is a promising method for tracing geographical differences in olive oils.

Keywords: Fragmentation by TOF / Geographical origin / MALDI-SpiralTOF MS / Olive oil analysis / TOF MS / Triacylglycerol

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1 Introduction

In Croatia an important economic value is generated from olive oil from Istria, due to its quality and a long olive growing and oil producing tradition [1]. Olive oil from Istria is already known for its high nutritional quality, in particular due to high content of oleic acid [2]. Nevertheless, for routine olive oil geographical characterization, standard analytical

methods are still laborious and time-consuming [3, 4] while this issue is a raising concern both among consumers and authorities [5]. Indeed, the origin of food has important consequences on the final food product quality. In particular, olive oil properties are significantly influenced by the geographical origin [5, 6], that is, at higher altitude the oils show higher oleic acid content [7, 8] whereas at lower altitude the oils have higher tocopherol and linoleic acid contents [7] or palmitic acid content [8]. Traceability and authenticity of olive oil have been thoroughly studied in recent years, and the general idea was to identify physicochemical parameters and/or develop analytical methods that might provide clues on the geographical origin of this valuable food product [4, 5]. Major and minor components of olive oil including fatty acids, triacylglycerols, sterols, tocopherols, and phenolics, usually in combination with chemometrics, have been extensively analyzed in the context of geographical origin [6–16]. Olive oil is composed mainly of triacylglycerols (TAG), important for physical, chemical, and nutritional properties of oils [17] that might be suitable for assessment of

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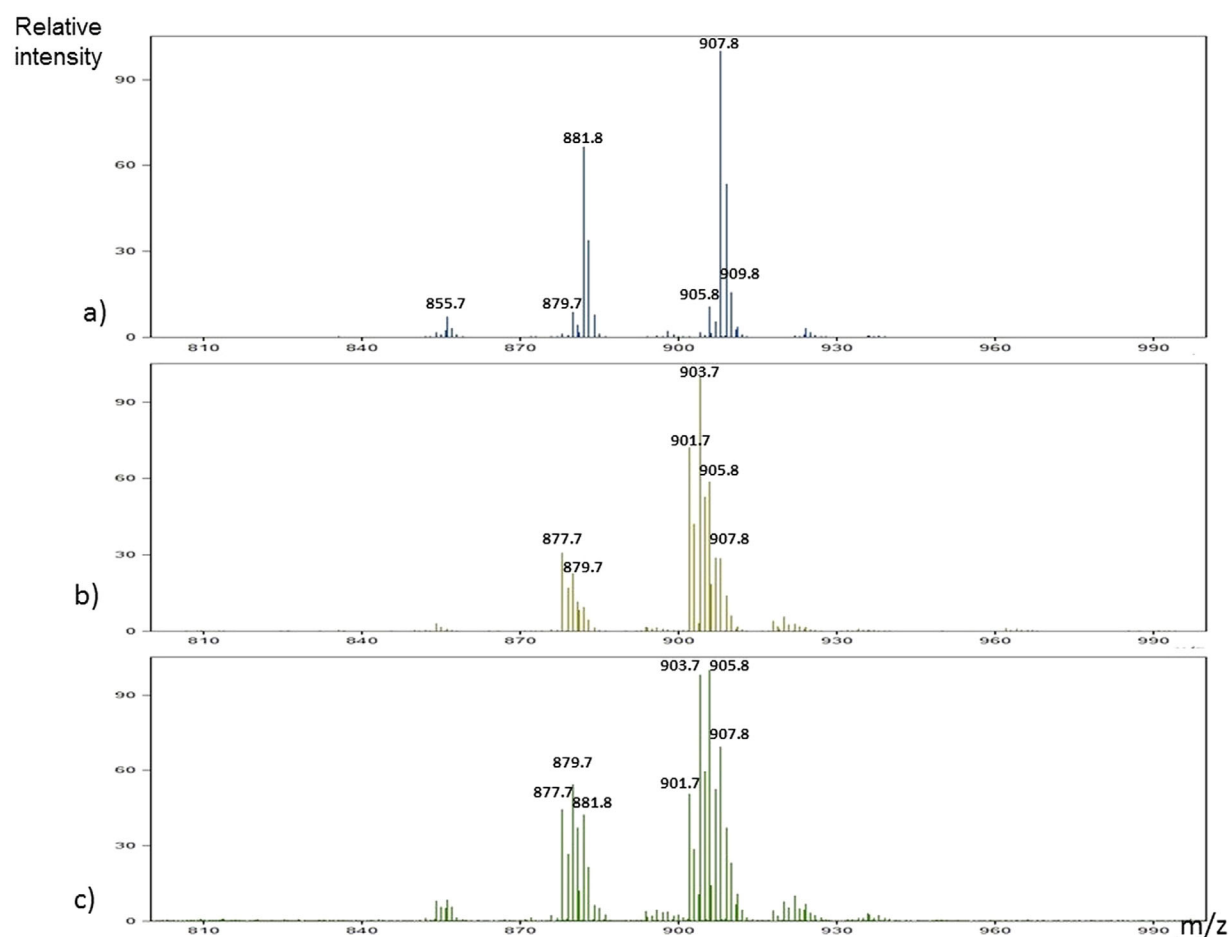
Abbreviations: CID, Collision-induced dissociation; L, linoleic acid, 18:2 n-6; Ln, linolenic acid, 18:3 n-3; MALDI-TOF, Matrix-assisted laser desorption ionization time-of-flight; MALDI-TOF/TOF, Matrix-assisted laser desorption ionization tandem time-of-flight; m/z, Mass/charge number; O, Oleic acid, 18:1 n-9; P, Palmitic acid, 16:0; PCA, Principal component analysis; S, Stearic acid, 18:0; TAG, Triacylglycerol(s); TOF/TOF, Linear or spiral time-of-flight/reflectron time-of-flight

Table 1. Geographical origin of Croatian olive oil samples collected during four harvest years

Region	Harvest year	Number of samples	Code of sample
Istria	2012	4	I2
Istria	2013	10	I3
Istria	2014	9	I4
Istria	2015	10	I5
Kvarner	2012	4	K2
Kvarner	2014	3	K4
Dalmatia	2012	1	D2
Dalmatia	2014	5	D4
Dalmatia	2015	4	D5

olive oil type and origin. Indeed, triacylglycerol composition can be regarded as a fingerprint of olive oils, being a useful tool for the detection of adulteration and for the determination of authenticity [4, 5]. The determination of difference between actual and theoretical content of triacylglycerols

with the Equivalent Carbon Number 42 (ECN42) is effective in detecting even low levels of olive oil adulteration. The ΔECN_{42} can be used to detect the presence of 1% sunflower, soybean, cotton, corn, walnut, and safflower oil. Detection of olive oil adulteration with sesame oil is possible at levels of 1.5%, with canola oil at level of 3% or with the rapeseed at level of 4%. However, the presence of hazelnut, mustard, almond, and peanut oil up to level of 5% cannot be detected [18]. Olive oil TAG analysis can be performed by use of different analytical methods, including soft ionization mass spectrometry (MS) techniques [3, 19]. In particular, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) may offer some advantages over other analytical techniques due to fast and easy sample preparation with no necessity of analyte purification, chemical modification, or derivatization [3]. Moreover, the resolution and mass accuracy in triacylglycerol mass range achieved by MALDI-TOF analysis yields detailed information about oil TAG composition without previous separation [19, 20]. MALDI-TOF mass

**Figure 1.** A comparison among TAG molecular ion region in the MALDI-TOF spectra from (a) olive oil from Istria, mix of varieties, (b) sunflower oil, (c) sesame oil. Presented spectra have been smoothened.

spectrometry has been applied for analysis of the TAG profiles and relative composition within the vegetable oils, and successfully used to distinguish different vegetable oils [21, 22], but with some limitation in detecting vegetable oil adulteration [23]. The molecular composition of TAG mixture is typically very complex as it comprises a variety of fatty acids, differing in chain length, degree of unsaturation, and distribution between the *sn*-1, *sn*-2, and *sn*-3 position of the glycerol backbone [16]. For complete structural analysis of TAG from oils, a matrix-assisted laser desorption ionization tandem time-of flight (MALDI-TOF/TOF) system with high-energy collision-induced dissociation (CID) was applied [24, 25]. In this study, the high *m/z* region of the CID spectra dominated by charge remote fragmentation peaks of fatty acid substituents in favorable cases enabled determination of the position of double bonds, while product ions in low *m/z* region allowed identification of positional distribution of the fatty acid substituents on the glycerol backbone. One limitation of the high-energy CID MALDI TOF/TOF application for the TAG analysis was shown to be the limited precursor ion gating of species separated only by one double bond which is due to insufficient flight path of MS1 [24]. Recently, MALDI-SpiralTOF/TOF with multi-turn type ion optical system as the first TOF mass spectrometer (MS1) and an offset parabolic reflectron as the second TOF mass spectrometer (MS2) has been developed. The advantages of this system are high precursor ion selectivity due to a 15-m spiral ion flight path length and possibility of eliminating post-source decay ions [26]. As shown by authors Kubo et al. [25] mono-isotopic precursor selection at good sensitivity enables the generation of mass spectra without interference from species that differ by only a single double bond.

Therefore, our goal was to test MALDI-SpiralTOF/TOF technology with monoisotopic precursor selection for analysis of TAG from samples of olive oils produced in different Croatian regions with special emphasis on Istrian olive oils. We also tested this method on different non-olive vegetable oils in order to pinpoint the differences between olive oil and other vegetable oils with regard to the overall triacylglycerol composition. As Croatia still lacks a comprehensive olive oil MS database, results of this study might contribute to understanding molecular differences of olive oils related to geographical regions.

2 Materials and methods

Olive oils were purchased directly from the producers, or in local stores. Sunflower and sesame oil were purchased in a supermarket. All samples of vegetable oils were cold pressed, extracted by mechanical processes, without heat or solvents.

Fifty olive oil samples (Table 1) were collected from different Croatian Adriatic regions including Istria, Kvarner (islands of Pag and Cres) and Dalmatia. Samples are

monocultivar olive oils or blends of varieties with emphasis on distinctive olive varieties for Adriatic regions (Buža, Bjelica, and Leccino for Istria; Simjaca, Plominka, and Oblica for Kvarner; Oblica for Dalmatia). Samples from Istria were collected from diverse geographical localities, ensuring that all main Istrian olive growing areas are represented. The olive oils samples had a label extra virgin olive oil. The categorization of olive oils was based only on the product label. Sampling was carried out during four successive harvest years; from 2012 to 2015 (harvest period was between months October and December). Bottled oils were kept in dark at 15°C until analysis.

Olive, sunflower and sesame oils were dissolved in THF (tetrahydrofuran) at concentration of 10 µL/mL (v/v). Matrix DHB (2,5-dihydroxybenzoic acid) and sodium trifluoroacetate (cationization agent) were dissolved in THF at concentration of 10 mg/mL and 1 mg/mL, and added to the samples at a volume ratio 2:1:1. A total of 0.5 µL of solution was deposited onto the MALDI sample plate and air-dried. All reagents and 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol standard used in this study were purchased from Sigma (Sigma-Aldrich, St. Louis, Missouri, USA).

All measurements were performed on a JMS-S3000 SpiralTOF mass spectrometer (JEOL Ltd, Akishima, Japan) equipped with tandem time-of flight (TOF/TOF) options and with an Nd-YLF pulsed laser operated at a wavelength of 349 nm. The MS1 measurements were performed in positive ion spiral mode at an accelerating potential of 20 kV. The extraction delay was optimized to 260 ns to provide a mass spectrometric resolution of 50–60 k FWHM (full width at half maximum) for the TAG peaks in MS1. For product ion

Table 2. General attribution by calculation of TAG to the corresponding *m/z* value observed in the MALDI-MS spectra of olive oils

Experimental <i>m/z</i> ^a	Calculated <i>m/z</i> ^a	Carbon number: number of double bonds	TAG ^b
853.7	853.7	C53: 2	PLP
855.7	855.7	C53:1	POP
875.7	875.7	C55:5	PLLn
877.7	877.7	C55:4	PLL
879.7	879.7	C55:3	POL
881.8	881.8	C55:2	POO
883.8	883.8	C55:1	POS
901.7	901.7	C57:6	LLL
903.7	903.7	C57:5	LLO/LOL
905.8	905.8	C57:4	LOO
907.8	907.8	C57:3	OOO
909.8	909.8	C57:2	SOO
911.8	911.8	C57:1	SOS

^a*m/z* values are for sodium adduct ion.

^bP, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; and Ln, linolenic acid.

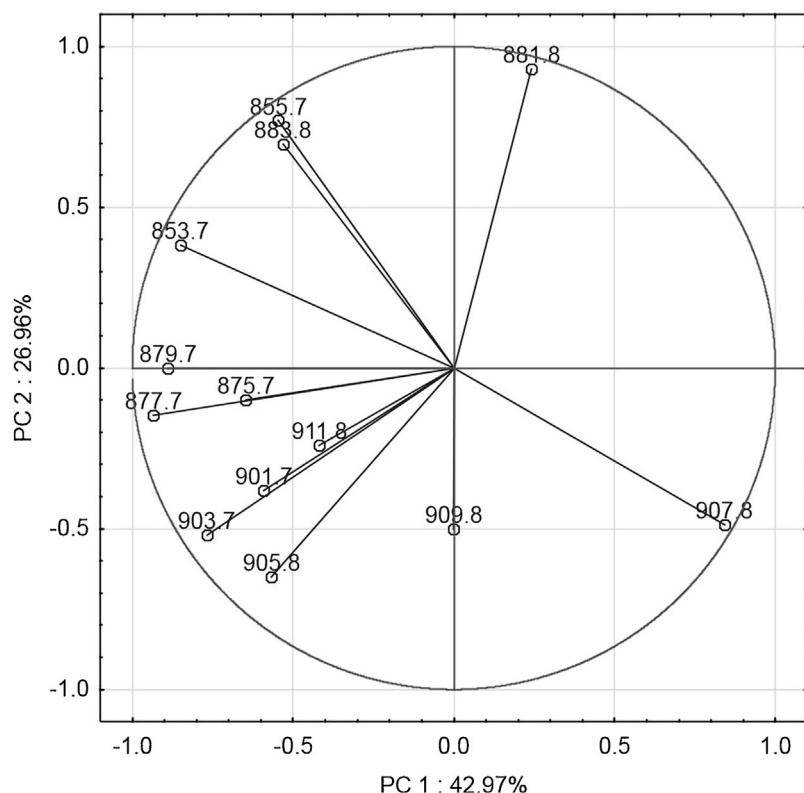


Figure 2. Projection of the variables on the factor-plane. The first two principal component factors explain ~43 and ~27% of total variance in expression data, respectively. Based on factor coordinates of variables (m/z values) for the first two factors olive oil TAG may be divided into two distinctive groups: two TAG are representative for majority of differences among tested olive oil samples.

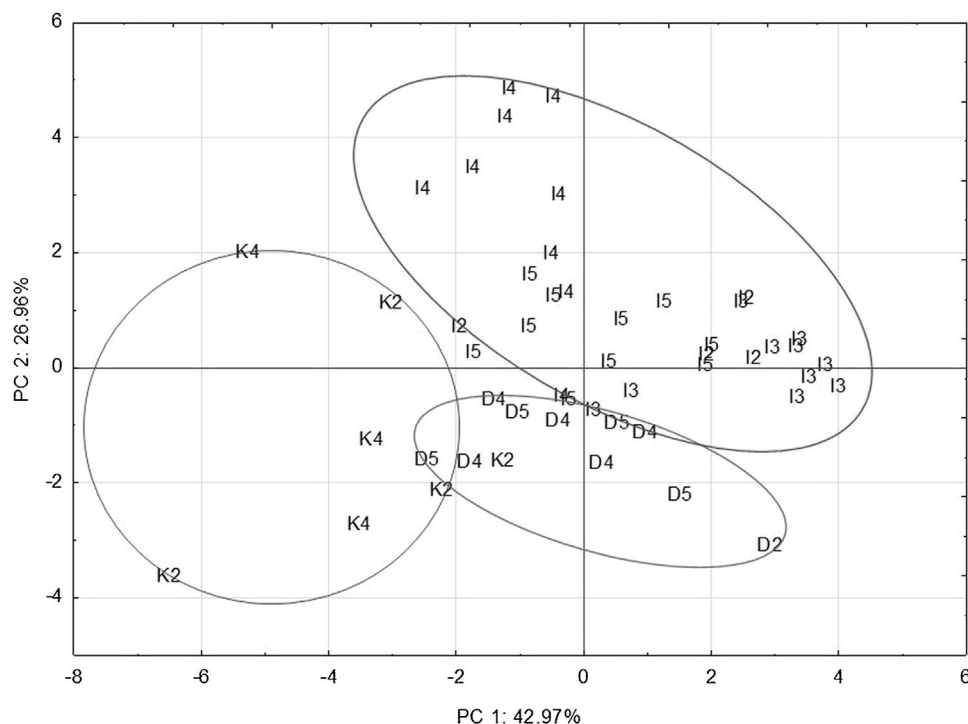


Figure 3. Measurements on projected factors with grouping label region of olive growth. Region of growth is labeled as follows: K3, olive oils from Kvarner (2013); I3, olive oils from Istria (2013); D3, olive oil from Dalmatia (2013); K4, olive oils from Kvarner (2014); I4, olive oils from Istria (2014); D4, olive oil from Dalmatia (2014); I5, olive oils from Istria (2015); and D5, olive oil from Dalmatia (2015).

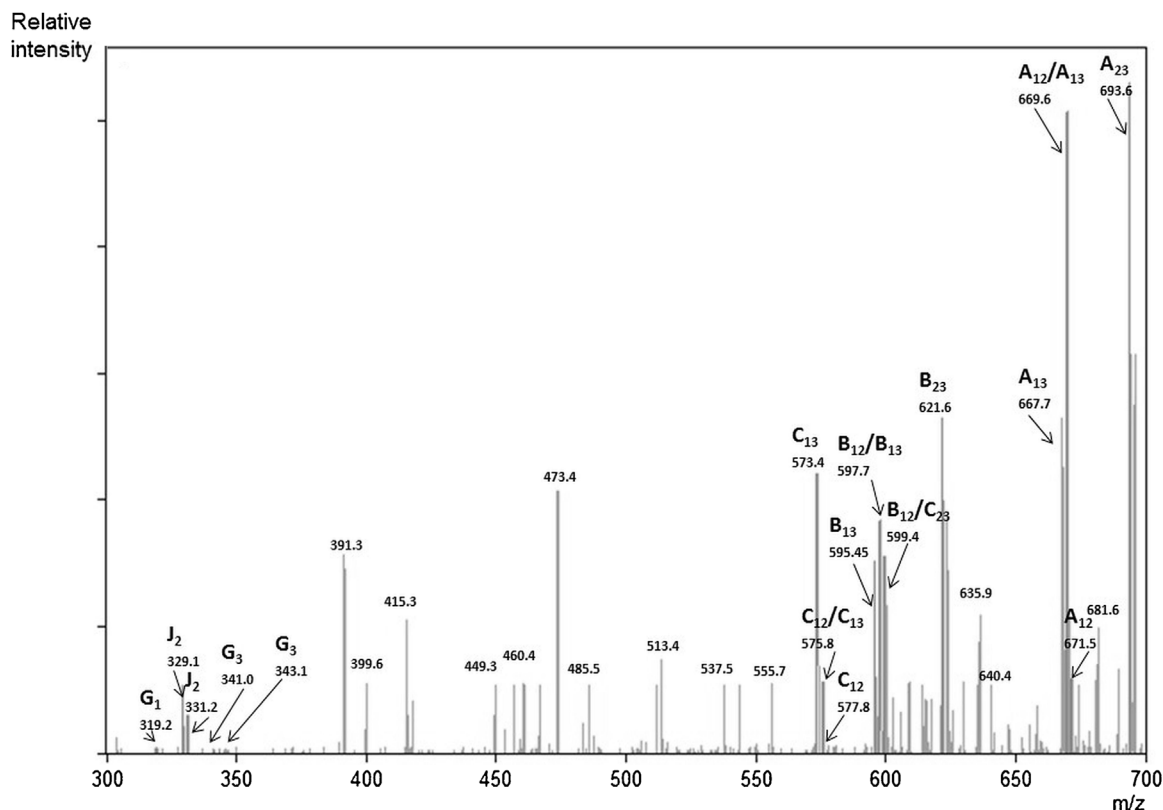


Figure 4. Product-ion mass spectra for the precursor at m/z 877.7 from olive oil (Simjaca) from the Kvarner region.

mass spectrum acquisition, helium as collision gas was introduced to attenuate 50% of original precursor ion signal value. A total of 100 spectra with 250 Hz repetition rate gave 12500 shots for MS spectra. For MS/MS spectrum, 1000 spectra were accumulated. The resulting spectra were smoothened by use of Savitzky–Golay algorithm. Average resolution values obtained in MS2 were approximately 1500 FWHM for product ions.

3 Data analysis

MS data acquisition was performed by msTornado Control. Final treatment of MALDI-TOF spectra was performed using mMass [27]. The theoretical molecular mass of the optional TAG was calculated [28] and was used on the basis of comparison to identify TAG of oil samples present in MALDI-TOF spectra. The five major TAG constituting fatty acids in olive oil, according to literature [17, 29], were considered for molecular mass calculated. The peak intensities were additionally corrected for the isotope contribution even though the resolution of the instrument proved sufficient for mono-isotope TAG analysis.

Statistical data analysis was performed on the data from MS1 using software package Statistica version 10—academic

license. We use Principal Components Analysis (PCA) [30] to better understand the impact of olive growth region on olive oil triacylglycerol composition. We are mainly interested in finding relations that might be better suited for assessment of olive oil origin.

4 Results and discussion

Obtained spectra clearly showed TAG composition for tested vegetable oils, including olive, sunflower, and sesame oils. TAG as sodium adduct ions were clearly identifiable in the m/z region between 800 and 1000, which is in line with previously published data [25, 31]. Expectedly, obtained TAG patterns differed among tested vegetable oils (Fig. 1). All olive oil samples yielded highly similar TAG profiles and a minor difference was observed only in the relative abundance of m/z ions. A total of 13 peaks were assigned to theoretical TAG species in all olive oil samples on the basis of the calculation of molecular mass (Table 2) where the most intense peak was located at m/z 907.8. This peak represents triolein (OOO) and was followed by peak at m/z 881.8 identified as 1-palmitoyl-2,3-dioleoyl-glycerol (POO).

The Principal Component Analysis was used to evaluate, compare, and separate Istrian olive oil samples from other

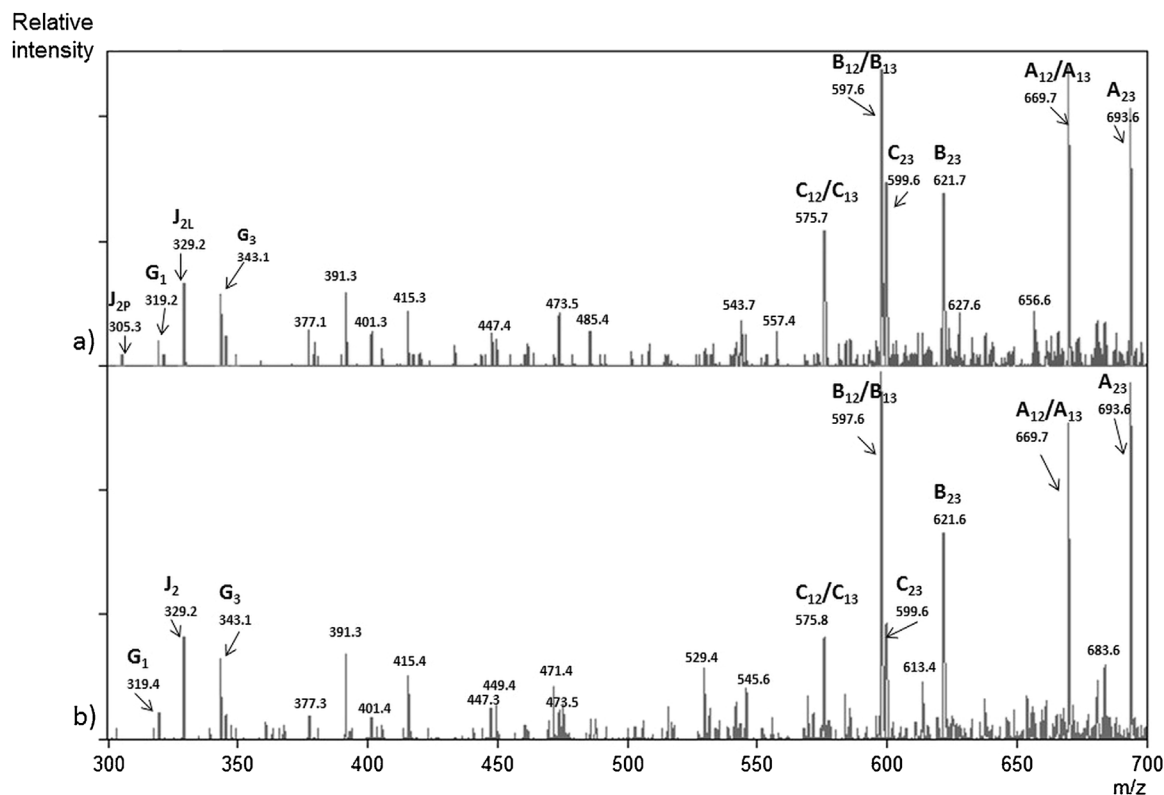


Figure 5. Product-ion mass spectra for the precursor at m/z 877.7 from (a) sunflower oil, (b) sesame oil.

olive oils according to geographical origin. For the PCA, triacylglycerol composition of olive oils was used and each test sample was formed from 13 identified TAG. Peaks' relative intensities from spectra were considered, calculated as percentages of each TAG relative intensity in the sum of total TAG intensities.

The first two principal component factors explain ~ 43 and $\sim 27\%$ of total variance in expression data, respectively. The first principal component accounts for $\sim 43\%$ of the variance in the triacylglycerol composition changes. The PC1 is most positively correlated with TAG at m/z 881.8 and 907.8 (Fig. 2). As both triacylglycerols have higher oleoyl residue content; 907.8 with three oleoyl residues (OOO), and 881.8 with two oleoyl residues (POO), we may consider this factor as oleoyl residue content related factor in triacylglycerol composition of olive oils.

The second principal component accounts for $\sim 27\%$ of the variance in the triacylglycerol composition changes. The PC2 is most positively correlated with TAG at m/z 855.7, 881.8, and 883.8, attributed to the TAG compositions POP, POO, and POS, respectively (Fig. 2). All these TAG, contained palmitoyl and oleoyl residues in their structure.

On the projection of olive oil samples onto the plane defined by the first and second principal components (presented in Fig. 3), we may observe that olive oils samples were separated according to their geographic origin. The

samples from Istria region have mostly a positive shift along the second component PC2, which is exactly opposite to olive oils from Dalmatian region. Istrian olive oils also separated from the Kvarner region olive oils. From the projection of olive oil samples in PC1 versus PC2, it may be also observed modest visual clustering of Istrian olive oils according to the harvest year.

This distribution of olive oils in PC1 versus PC2 plot allowed clustering related to the geographical origin on the basis of TAG composition and relative intensities in the mass spectra, even when TAG composition was influenced by the harvest year. A more extensive evaluation with olive oil samples not just from Croatian coastal regions but also from other Adriatic regions will be necessary to validate obtained findings. The accurate structure of identified TAG was analyzed by MALDI-SpiralTOF/TOF. The evaluation of sensitivity in the CID mode was done using 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol standard. It was found that concentration below $100 \mu\text{L/mL}$ does not give all the product ion peaks for detail assignment. In other words, detection limit for CID mode is approximately $100 \mu\text{L/mL}$. For complete structural characterization of TAG, product ions were predicted as A-, B-, C-, E, G-, and J-type ions, according to the described nomenclature [24, 32, 33]. High mass charge remote fragmentation peaks of fatty acid substituents in the obtained spectra indicated the position

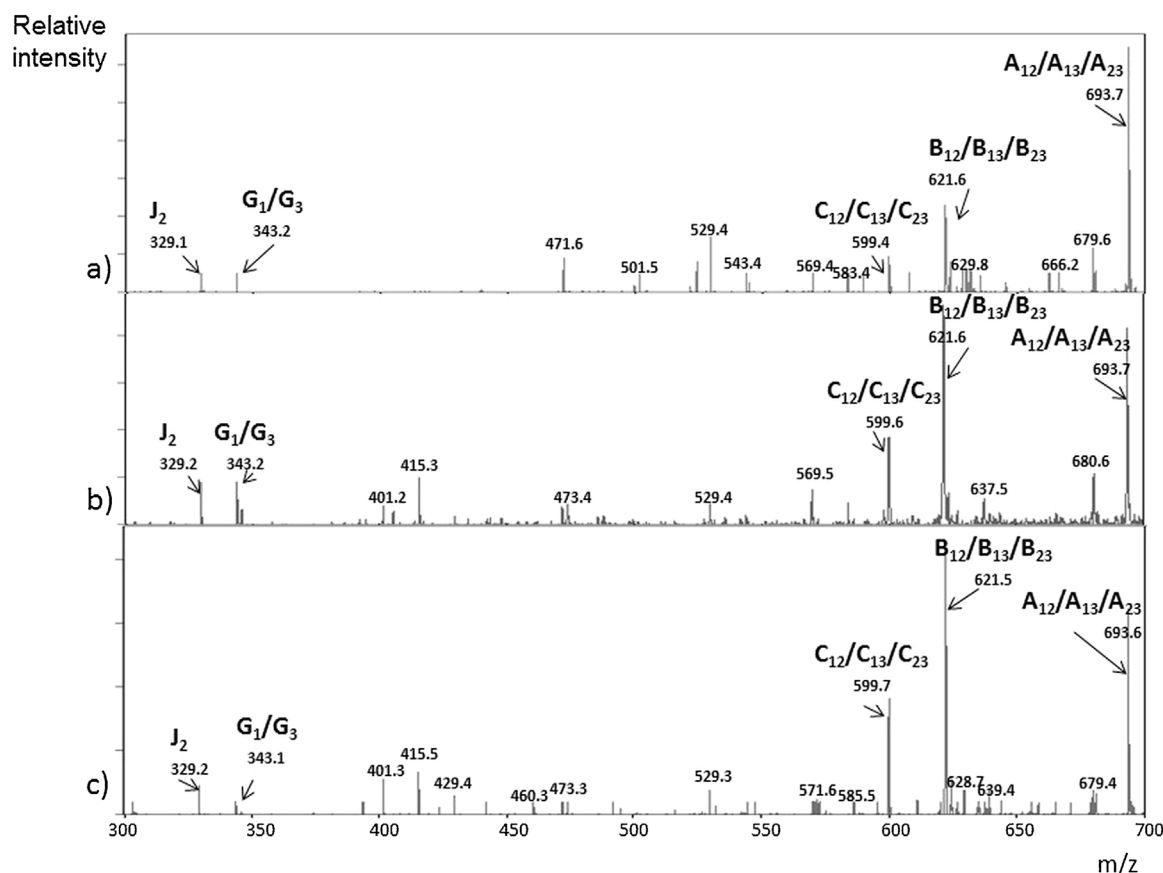


Figure 6. Product-ion mass spectra for the precursor at m/z 901.7 from (a) olive oil from the Kvarner region, variety Oblica, (b) sunflower oil, (c) sesame oil. The ions associated with the peak at m/z 901.7 in all samples represent a TAG with three linoleoyl residues.

of the double bond (Fig. S1). However, in some spectra no unequivocal confirmation of the double bond location for TAG was possible due to the absence of complete and suggestively abundant series of ions. In all vegetable oils sodiated TAG ions were located at m/z 877.7 and 901.7. It is assumed that at position m/z 877.7 TAG is composed by one palmitoyl residue and two linoleoyl residues, and at m/z 901.7 TAG is composed by three linoleoyl residues on the glycerol backbone. To confirm the structure of these TAG, MALDI-TOF/TOF was used to acquire product-ion mass spectra. The product-ion mass spectra for the precursor at m/z 877.7 from olive oil sample from Kvarner showed certain specificity in general peak abundance presented in Fig. 4.

Positional distribution of the fatty acid substituents may be deduced from peaks predicted as G1/G3- and J2-ions, characteristic for fatty acid fragments. These ions may help to determine the total number of carbon chains and unsaturated bonds in each fatty acid residue [25]. The substituents at sn -1 and sn -3 are indistinguishable by mass spectrometry as they are analogue in the steric structure of TAG [31].

The product-ion mass spectrum for precursor ion at m/z 877.7 from olive oil (Kvarner) shows low abundant fragment ions assigned as G1/G3-ions at m/z 319.2, 341.0, and 343.1 suggesting that fatty acid residues linked to sn -1/ sn -3 are palmitoyl, linolenoyl, and linoleoyl, respectively. The product-ion mass spectrum shows J2-ions containing linoleoyl and oleoyl residues at 329.1 and 331.2 m/z . Even though the abundance of some diagnostic ions was low, spiralTOF technology seem to provide a rather high selectivity [25] and poor precursor ion selectivity should not be an issue.

Product ions assigned as A-, B-, C-type, made by elimination of one acyl residue, indicate that m/z 877.7 is a mixture of the 1-palmitoyl-2,3-dilinoleoyl-glycerol (PLL) and 1-palmitoyl-2-oleoyl-3-linolenoyl-glycerol (POLn). In previously reported MALDI-TOF analyses of olive oils, only one triacylglycerol species with one palmitoyl and two linoleoyl residues on the position m/z 877.7 was present [16, 17, 19].

On the other hand, product-ion mass spectrum for precursor ion at m/z 877.7 from sesame oil showed only G1/G3-ions at m/z 319.4 and 343.1 indicating that at the positions sn -1/ sn -3 palmitoyl and linoleoyl residues are

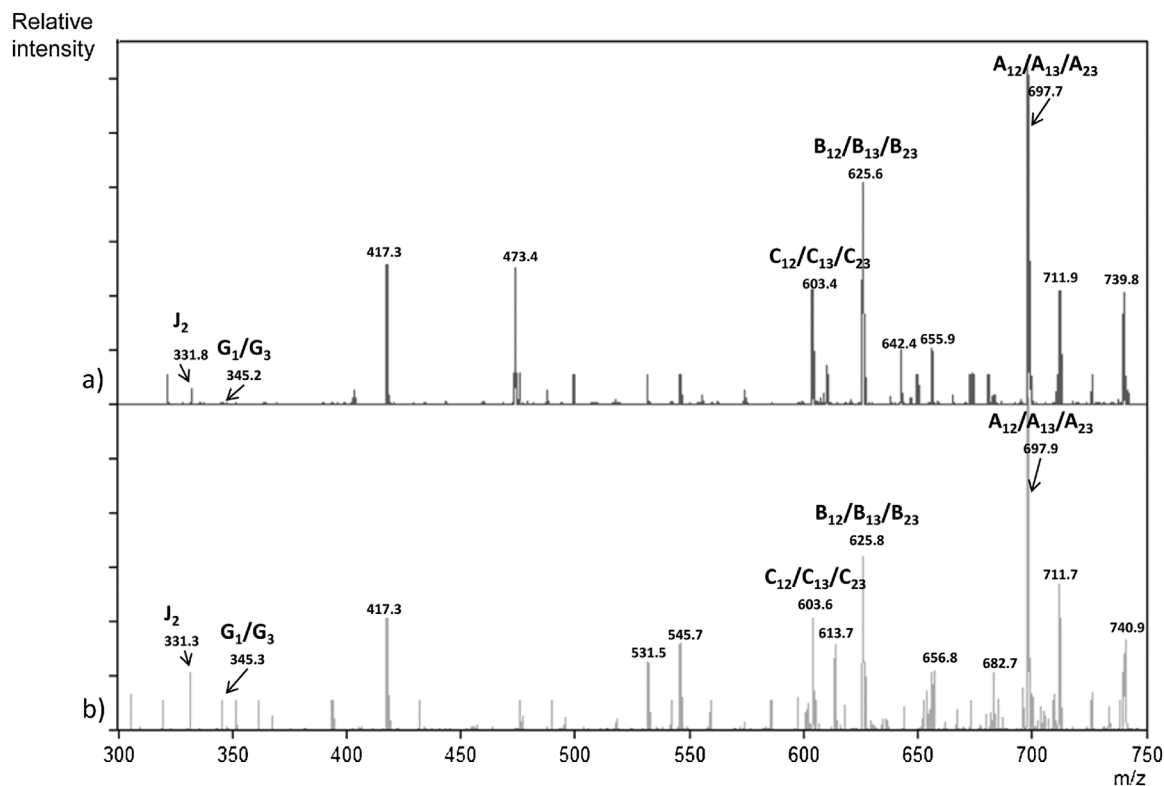


Figure 7. Product-ion mass spectra for the precursor at m/z 907.8 from two samples of olive oil from Istria (a) variety Buža and (b) variety Bjelica.

attached and a J2-ions at m/z 329.2 indicating that a linoleoyl residue is attached at the *sn*-2 position. The product-ion mass spectrum shows A-, B-, C-type ions that correspond only to triacylglycerol 1-palmitoyl-2,3-dilinoleoyl-glycerol (PLL) (Fig. 5b).

The product-ion mass spectrum for precursor ion at m/z 877.7 from sunflower oil shows the same fragment ions as in sesame oil, except that an additional peak at the m/z 305.3 was present and assigned as a J2-ion containing palmitoyl residue. The spectrum shows A-, B-, C-, G-, and J-type ions that correspond to TAG with two linoleoyl and one palmitoyl residue as a mixture of two positional isomers, 1-palmitoyl-2,3-dilinoleoyl-glycerol (PLL), and 1,3-dilinoleoyl-2-palmitoyl-glycerol (LPL), respectively (Fig. 5a). According to these results, the same precursor ion at position 877.7 m/z is shown to represent different TAG structural isomers, strictly depending on the vegetable oil origin. In this context, it can be proposed, that for analysis of TAG in olive oils, the use of versatile methods like TOF/TOF fragmentation and the precursor ion selection by MS1, namely the spiral TOF device for confident assessment of chemical structure is required.

Figure 6 shows the product-ion mass spectra for the precursor at m/z 901.7 from the olive (Oblica, Kvarner

region), sunflower and sesame oil. The ions associated with the peak at m/z 901.7 in all samples of vegetable oils are TAG with three linoleoyl residues, which confirm our previous results. The same procedure was applied to the analysis of other olive oil samples. The most abundant peak at m/z 907.8 in all olive oils was selected as a precursor ion, and the product-ion mass spectrum was acquired (Fig. 7). The product-ion mass spectrum shows A-, B-, C-, G-, and J-type ions that correspond to a TAG containing three oleoyl residues, in accordance with previously published data [24, 25]. Although the TOF/TOF analysis confirmed a proposed substitution pattern, these data are not supportive for the geographical classification of the investigated olive oils investigated.

5 Conclusions

MALDI-SpiralTOF is a fast and effective method to determine TAG composition of diverse vegetable oils, as well as of olive oils from different geographic areas. MALDI-TOF spectra show specific TAG fingerprints for sesame, sunflower, and olive oils, therefore, confirming this analytical high-throughput technique as a reliable tool for screening the

oil types. As shown by results of Principal Components Analysis, TAG profiles obtained by MALDI-TOF MS allowed a differentiation of Istrian olive oils in comparison with olive oils from other Croatian coastal regions. Moreover, MALDI-SpiralTOF/TOF proved efficient in determination of accurate TAG structure in vegetable oils, including differentiation between TAG structural isomers. High-energy CID MALDI-TOF/TOF TAG analysis is a promising method for vegetable oil origin certification and we propose this approach for comprehensive evaluation of all olive oil TAG in relation to geographical origin studies. Larger and long-term studies are however needed as many variables may occur during olive oil analyses, including chemical composition variation due to the harvest year, environmental conditions, and extraction methods. In particular, Croatia still lacks a comprehensive olive oil database that includes MS molecular data. Therefore, molecular differences of Croatian olive oils relative to geographical regions within Croatia and other Adriatic regions are still not studied in details. The added value of this work is the first application of MALDI-TOF/TOF method for the analysis of Croatian olive oils aiming to discriminate them on the basis of their TAG composition.

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The authors have declared no conflict of interest.

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