Metabolic fingerprinting strategy: investigation of markers for the detection of extra virgin olive oil adulteration with soft-deodorized olive oils

Metabolic fingerprinting strategy: Investigation of markers for the detection of extra virgin olive oil adulteration with soft-deodorized olive oils

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Abstract

As extra virgin olive oil (EVOO) is a high value commodity, it might be subject of various fraudulent practices. This study is focused on a challenging authentication issue, addition of lower grade, soft-deodorized olive oil to EVOO. In the first step, sample sets of authentic EVOOs, soft-deodorized oils and their admixtures were extracted by aqueous methanol; obtained polar fractions were then analyzed by ultra-high performance liquid chromatography coupled to hybrid quadrupole time-of-flight high-resolution tandem mass spectrometry (UHPLC-QTOF-HRMS/MS). Subsequent chemometric evaluation of metabolic fingerprints enabled suggestion of several ions that might be characteristic for deodorized oils; most of tentatively identified compounds were oxidized fatty acid derivatives. In the second phase, the ´marker´ ions were employed for target analysis by ultra-high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UHPLC-QqQ-MS/MS) what enabled achieving lower the detection limits. Two compounds were selected as the best markers for detection of soft-deodorized olive oil addition, tentatively identified as methyl ester of hydroxy octadecenoic acid and ester derivative of oleic acid.

Keywords: extra virgin olive oil; soft-deodorized olive oil; authenticity; metabolic fingerprinting; UHPLC-HRMS; adulteration

1. Introduction

Olive oil popularity has increasing trend not only in Mediterranean countries, where its production has a long historical tradition, but also among customer in other countries across the world (Bajoub, Bendini, Fernández-Gutiérrez, & Carrasco-Pancorbo, 2018). Among olive oil categories, extra virgin olive oil (EVOO) represents the top-quality sub-category of virgin

oils. It is obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alterations and to be classified as EVOO in EU, its acidity level must not exceed 0.8% and the compliance with other strict criteria specified in Regulation (EU) No. 2019/1604 (European Commission, 2019) has to be met.

High price together with quantitative expansion of high-quality olive oil consumption makes olive oil vulnerable for economically motivated adulteration practices (Bajoub, et al., 2018; Casadei, et al., 2021). Fraudulent practices involve misdescription of botanical origin (Carranco, Farrés-Cebrián, Saurina, & Núñez, 2018; Filoda, et al., 2019) and/or geographical origin (Bontempo, et al., 2019; Gertz, Gertz, Matthäus, & Willenberg, 2019); addition of cheaper olive oil (e.g. refined olive oil, ROO) into higher grade olive oil (Squeo, Grassi, Paradiso, Alamprese, & Caponio, 2019; Yan, Oey, Van Leeuwen, & Van Ruth, 2018) is another common way of adulteration. In 2016, the FoodIntegrity project (www.foodintegrity.eu) launched a survey addressing all olive oil actors (producers, wholesalers, retailers, researchers, analysts, etc.) to find out their priorities in olive oil authentication issues. As the most important issue a spiking EVOO with soft-deodorized olive oil (SDOO) was rated, which is more difficult to detect than addition of ROO. This need was also evidenced in a more recent on-line survey addressed to EU and non-EU stakeholders in the olive oil sector (Casadei, et al., 2021). During soft-deodorization process, which is carried out at low temperatures under nitrogen stripping and vacuum, unpleasant sensory notes are removed (Aparicio-Ruiz, Romero, García-González, Oliver-Pozo, & Aparicio, 2017). Due to mild conditions, native oil does not significantly change its chemical structure, except for removal of volatiles (García González, Aparicio, & Aparicio-Ruiz, 2018), therefore, its identification in admixtures with EVOO is fairly complicated. Considering legal definition, after undergoing soft-deodorization, the resulting oil cannot be any longer considered as "virgin" (International Olive Council (IOC), 2019). Although some chemical markers of this

process or indirect markers (e.g. pyropheophytins (Gómez-Coca, Alassi, Moreda, & Pérez-Camino, 2020) and total amount of fatty acid methyl and ethyl esters (Gómez-Coca, Fernandes, Pérez-Camino, & Moreda, 2016; Pérez-Camino, Cert, Romero-Segura, Cert-Trujillo, & Moreda, 2008)) have been proposed, they have not been shown very effective in this type of fraud detection for the high number of possible false positives/negatives (Aparicio-Ruiz, et al., 2017). Other authentication approaches for the detection of SDOOs combine multiple analytical parameters for authentication purposes (Gertz, Matthäus, & Willenberg, 2020; Gómez-Coca, Pérez-Camino, Bendini, Gallina Toschi, & Moreda, 2020) or focused on volatile fraction of olive oils (Damiani, Cavanna, Serani, Dall'Asta, & Suman, 2020). The potential of application of ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS) was also studied for addressing the discussed challenging authentication issue - the addition of soft refined oils (i.e. soft-deodorized and soft-deacidified) into EVOOs. In this study, seven markers were selected as discriminative for this type of adulteration, but they were not products of soft-deodorization process but markers of low-quality olive oils (Cavanna, et al., 2020).

The main aim of this study was to investigate the possibility to recognize dilution of EVOO by SDOO. To identify ´marker compounds´ of this lower grade product presence, UHPLC-HRMS was employed for untargeted screening (metabolic fingerprinting) of polar fraction of olive oils samples. Authentic EVOOs, SDOOs and mixtures thereof were involved in the study. In the final phase of the study we aimed to document the applicability of identified markers for recognition of EVOO - SDOO admixtures by simple target analysis.

2. Materials and methods

2.1. Reagents and chemicals

Deionized water was obtained from a Milli-Q system supplied by Merck (Darmstadt, Germany). Methanol (purity ≥99.9%) was purchased from Honeywell (Charlotte, NC, USA). 2-Propanol (purity ≥99.9%) and ammonium formate (purity ≥99.0%) were purchased from Merck (Darmstadt, Germany) and formic acid (purity ≥99.9%) was obtained from VWR Chemicals (Radnor, PA, USA).

2.2. Samples

In total, the sample set consisted of 102 samples of various categories of olive oils.

Main part of samples was originally prepared for OLEUM EU project (http://www.oleumproject.eu/) by Institut des Corps Gras (ITERG, Canéjan, France) and consisted of: (i) EVOOs (n=2) differing in high and low ´fruitiness´; (ii) defective virgin or lampante olive oils (n=10) with specific sensory defects (rancid, fusty/muddy sediment, frostbitten, musty, winey-vinegary); (iii) SDOOs (n=10) prepared from the abovementioned defective olive oils by mild temperature processing; (iv) blends (n=60) of the two EVOOs and the ten SDOOs mixed in three different ratios (30/70, 50/50 and 70/30 % *v/v*) (Gómez-Coca, Pérez-Camino, et al., 2020).

To confirm that selected markers are not present in other EVOOs, authentic samples of EVOOs (n=20), obtained within cooperation with Barilla and University of Parma, were analysed (Cavanna, et al., 2020).

2.3. Sample preparation

The extraction procedure was previously optimized by Vaclavik et al. (2009). Briefly, oil samples were weighed (1 g) into 15 mL polypropylene cuvettes and extracted with 3 mL of methanol/water (80:20, *v/v*). Samples were automatically shaken for 20 min (240 rpm) and

afterwards centrifuged (5 min, 10,000 rpm). The upper layer was transferred into a 2 mL vial for analysis. Some samples were prepared in duplicate to control method robustness (extraction procedure reproducibility), quality control samples (QC) were prepared by pooling the aliquot of all extracts to monitor the overall instrument performance.

2.4. Untargeted analysis (UHPLC-HR-MS)

Untargeted method has been based on our previous research work by Hurkova et al. (2017). The ultra-high performance liquid chromatography (UHPLC) system Dionex UltiMate 3000 (Thermo Fisher Scientific, Waltham, MA, USA) was equipped with a Waters Acquity UPLC® BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm), maintained at 60 °C. The mobile phase consisted of 5 mM ammonium formate and 0.1% formic acid in (A) water/methanol (95/5, *v/v*) and (B) isopropanol/methanol/water (65/30/5, *v/v/v*). The flow rate was 0.3 mL/min during whole run in ESI+ (0.4 mL/min in ESI-) and the gradient was for both ionization modes as follows: 0.0 min (95% A), 1.0 min (95% A), 8.0 min (0% A), 13.0 min (0% A), 13.1 min (95% A), 15.0 min (95% A). The sample injection volume was 2 µL. Chromatographic conditions were the same for both positive and negative modes of ionization.

The quadrupole-time-of-flight TripleTOF® 6600 mass spectrometer (SCIEX, Concord, ON, Canada) was coupled to the chromatographic system. The ion source Duo Spray™ was employed, using electrospray ionization (ESI) for the measurements of metabolic fingerprints and atmospheric-pressure chemical ionization (APCI) for the exact mass calibration of the instrument. The source parameters were as follows: nebulizing gas pressure: 55 psi; drying gas pressure: 55 psi; curtain gas 35 psi, capillary voltage: +5,000 V (ESI+) and −4,500 V (ESI−), temperature: 480 °C; declustering potential: 80 V.

For the acquisition of MS and MS/MS data, Full scan and Information Dependent Acquisition (IDA) methods were used. While full MS data were recorded from *m/z* 100 to 1,200, product ion MS/MS data were collected from *m/z* 50 to 1,200. The collision energy was 35 V with

energy spread of ±15 V, resulting in both low and high energy fragments in a single spectrum. Every 7 samples, automatic *m/z* calibration was performed using positive or negative APCI calibration solution (SCIEX, Concord, ON, Canada). The resolving power was >40,000 full width at half maximum (FWHM), *m/z* 829.5393 for positive ionization mode and m/z 933.637 for negative ionization mode.

To avoid false sample clustering, the samples were injected in a randomized order. During the sequence, the QC matrix samples and blank samples were analysed to monitor the potential drift of retention data and changes of detector response together with potential carry-over effects.

Analyst 1.7.1 TF software (SCIEX, Concord, ON, Canada) was used for instrument control and data acquisition and PeakView 2.2 software equipped with MasterView (SCIEX, Concord, ON, Canada) was used for data evaluation.

2.5. Data analysis

Data processing was performed with MarkerView software 1.3 (SCIEX, Concord, ON, Canada). For peak detection, the parameters were set as follows: minimum peak width 0.02 Da, noise threshold 10, substraction multiple factor 1.5. Peak alignment parameters were set to: mass tolerance 0.01 Da, retention time tolerance 0.1 min. After peak detection and alignment, total area sum normalization and Pareto scaling were performed.

Further chemometrics evaluation was performed in SIMCA 13.0 software (Umetrics, Umea, Sweden; https://umetrics.com/), including principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). The model performance was expressed by the $R^2(X)$ parameter, so-called goodness of fit, and by the Q^2, the predictive ability parameter. Both parameters were calculated by the SIMCA software using a 7-fold internal cross-validation. PLS-DA models were used to select possible marker ions by loading plot and VIP

(Variable Importance in Projection) plot. To evaluate predictive ability of markers, receiver operating characteristic (ROC) curves were constructed and area under curve (AUC) was calculated for each marker using MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/). AUC-ROC assess the utility of a marker, values 0.9-1.0 meaning excellent marker.

Tentative identification of compounds behind the marker ions was based on the estimation of summary elemental formula (considering accurate mass, mass error, isotopic profile for respective *m/z* values in MS) and on the comparison of MS/MS spectra with data in online databases (e.g. Metlin, www.metlin.scripps.edu/index.php; mzCloud, www.mzcloud.org) and in scientific literature.

2.6. Targeted analysis (UHPLC-MS/MS)

For the targeted analysis of potential markers, the UHPLC system Agilent 1290 Infinity II (Agilent Technologies, USA) coupled to the tandem mass spectrometer QTRAP 6500 (SCIEX, Canada) was employed. The type of separation column and mobile phase conditions were set exactly the same as those for untargeted screening. The mass spectrometer was operated in both ionization modes and the parameters were as follows: needle voltage 5,500 V (ESI+)/-4,500 V (ESI-), turbo gas temperature 400 °C (ESI+)/400 °C (ESI-), curtain gas 40 psi, nebulizer and turbo gases 55 psi. Scheduled multiple reaction monitoring (MRM) methods were used in both ESI(+) and ESI(−) with the cycle time 0.5 s (ESI+) and 0.1 s (ESI-). MRM transitions were selected according to HRMS/MS spectra measured by the TripleTOF 6600 mass spectrometer. Declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) were optimized for all ions. The selected parameters are summarized in **Supplementary Table A1**.

Data evaluation was performed using Analyst software 1.7.1.

3. Results and discussion

The soft-deodorization process aims mainly at removing volatile compounds responsible for flavour defects of virgin olive oil, while most of other non-volatile components remain apparently unchanged in the treated oil. On this account, the detection of SDOO addition to high grade EVOO is a challenging analytical task. In the recent years, the popularity of untargeted approaches for food authentication has been increasing, however, the implementation of these authentication approaches based on fingerprinting concept into routine analyses practice is questionable and rather complicated, mainly due to the low reproducibility of metabolomics studies (Creydt & Fischer, 2020), demanding data processing and building of large datasets. Therefore, many approaches incline to use chemometric tools for the selection of suitable characteristic marker(s), which could be afterwards used as a single parameter to assess authenticity. For that reason, after discovery of unique biomarkers by untargeted approach, targeted strategy was applied to demonstrate the possibility of routine analysis based on targeting selected markers.

3.1. Metabolic fingerprinting

For the untargeted analysis, UHPLC-HRMS was employed to generate data enabling classification of olive oil samples according to the manufacturing process. The sample preparation strategy prior to instrumental analysis was similar to that described in an earlier study focused on olive oil authentication by Vaclavik et al. (2009), in which the evidence of EVOO adulteration with hazelnut oil could be documented by analysis of ´polar´ extract obtained by aqueous methanol.

All samples were measured in both electrospray ionization modes, total ion chromatograms of QC sample are shown in **Figure 1**. As shown here, apart from the polar compounds which were of main concern in this study, residual amounts of triacylglycerols and diacylglycerols were partitioned into aqueous methanolic extracts.

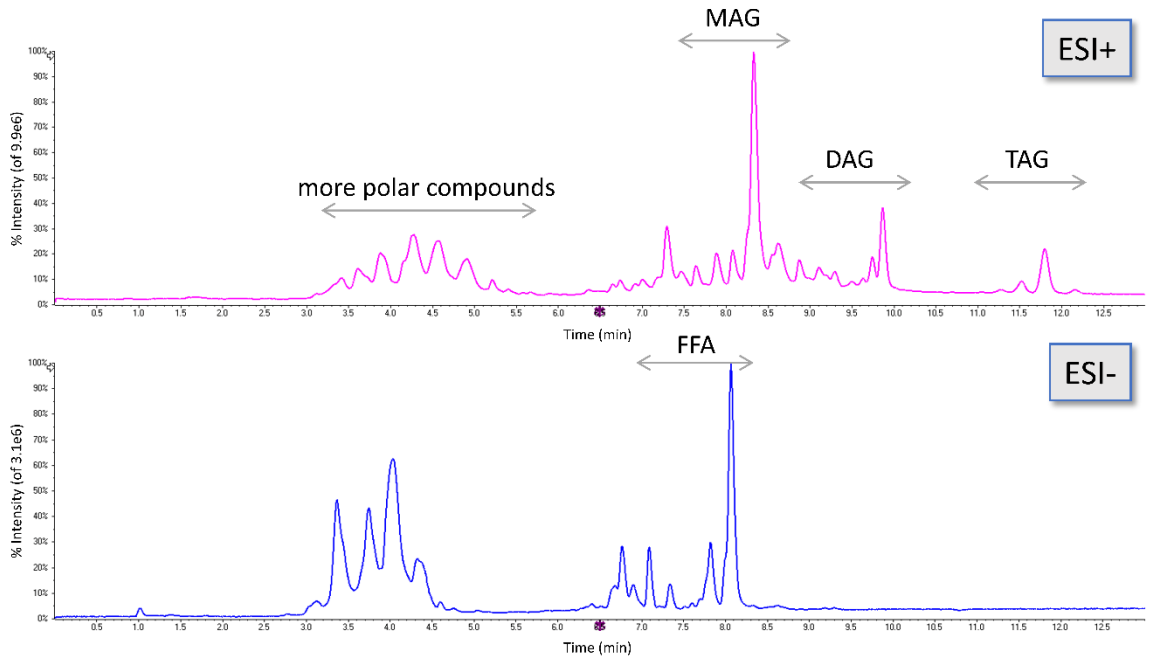

Fig. 1. Total ion chromatograms of QC sample in positive (ESI+) and negative (ESI-) ionization mode. MAG – monoacylglycerols, DAG – diacylglycerols, TAG – triacylglycerols, FFA – free fatty acids.

As mentioned in Introduction, as temperatures for removing undesirable volatiles do not exceed 100 °C, only minimum of ´modified´ compounds originated in oil treated this way can be expected. These facts were in depth discussed by Aparicio-Ruiz et al. (2017).

3.2. Chemometric analysis

Raw data generated by UHPLC-HRMS were processed by MarkerView software. After peak picking and peak alignment (see parameters in 2.4), 7,464 features in positive ionization mode and 1,762 in negative ionization mode were detected. Using MarkerView software, isotopic peaks and background peaks occurring in the blanks were excluded from the dataset to reduce data dimensionality, leaving 4,927 features (i.e. detected and aligned ions across samples with unique *m/z* value and retention time) in ESI+ and 1,324 features in ESI-. These numbers of

features are comparable to the features obtained by Cavanna et al. (2020). Further chemometric processing, including further features reduction, was performed in SIMCA software.

Prior to the statistical model construction, Pareto scaling was applied to the dataset to reduce the importance of large values. This type of scaling is recommended when searching for features with metabolic significance (Di Guida, et al., 2016). Principal component analysis (PCA) was applied in the first step of metabolic fingerprinting as it reduces the number of components while preserving the data structure. The PCA scores plots (**Figure 2**) shows the initial clustering within the sample set, where the blends are located between EVOOs and SDOOs samples. Although, some slight trend could be observed - blends with 30% SDOO addition were closer to EVOO, nevertheless, the SDOO samples were very variable in composition, thus clustering of blends according to the percentage of SDOO did not occur. Considering the score plots, it can be assumed that the two EVOO, which were used to make blends, differed considerably in composition, which, on the other hand, resulted in a greater variation within the samples in our statistical model. Worth to notice that tight clustering of QC samples documented consistent measurement of the instrument without major drifts during the sequence.

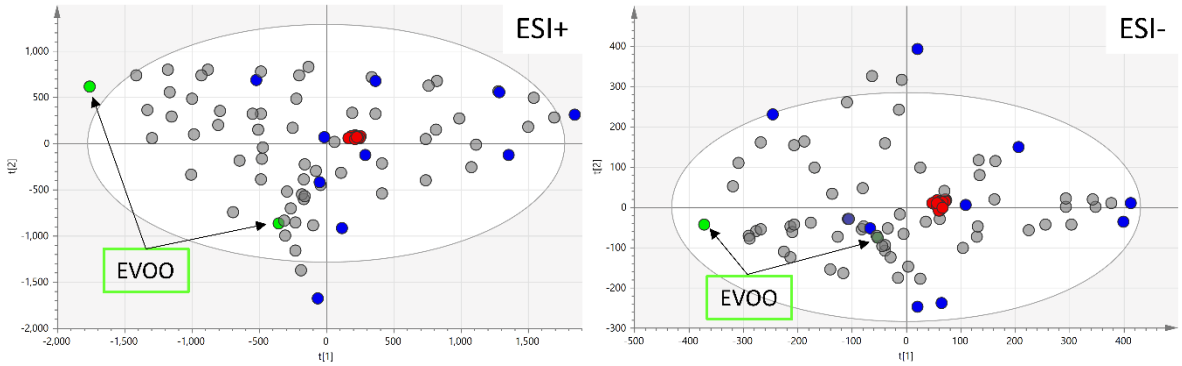

Fig. 2. Scores plots of PCA analysis. Red: QC samples; green: EVOO; blue: SDOO; grey: blends.

To search for suitable markers of soft-deodorization process, chemical fingerprints of EVOOs and SDOOs were statistically evaluated (note: to cover existing variability of EVOOs, another 20 authentic samples were involved in measurement and data assessment) and PCA and PLS-DA (partial least square discriminant analysis) score plots were created (**Figure 3**). The PCA score plots did not show sufficient clustering of the two sample groups (EVOOs vs SDOOs), when first two principal components explained only 0.384 (ESI+) and 0.486 (ESI-) variance. This means that different variables other than soft-deodorization process contribute more to the samples clustering. In the group of EVOOs (in ESI+ data), cluster of seven samples can be seen. Based on the analysis of metabolic fingerprints, this might be due to higher amounts of monoacylglycerols in those samples. For the PLS-DA model formation, only features with the highest Variable Importance for Projection (VIP higher than 1) were used (492 features in ESI+, 120 features in ESI-) and the quality parameters of the respective PLS-DA models were calculated by the SIMCA software using a 7-fold internal cross-validation. The model in ESI+ was characterized by R^2X=0.379, R^2Y=0.828, Q^2=0.608 and the quality of the model in ESI- was described by R^2X=0.476, R^2Y=0.543 and Q^2=0.182. In comparison with study by Cavanna et al. (2020), PCA and PLS-DA score plots have had lower quality parameters, what can be caused by inclusion of defective samples after more processing steps (soft deodorized, soft deacidified and/or both). Because these parameters were not sufficiently high to enable the use of such models for unknown samples classification, features with the highest VIP scores were further investigated in order to discover a suitable marker, which would be characteristic for soft-deodorization process. Also features found by Cavanna et al. (2020) in a similar study focused on SDOO based fraud were investigated to confirm applicability of those markers (see below).

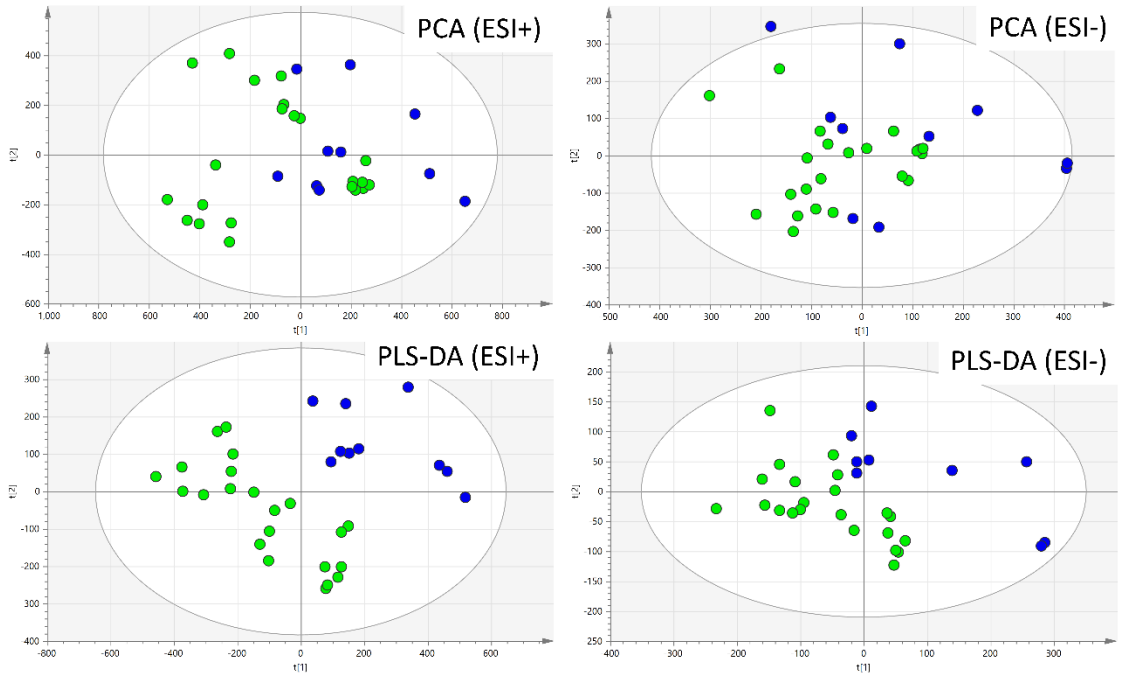

Fig. 3. PCA and PLS-DA score plots of EVOOs (green) and SDOOs (blue).

Additionally, models for SDOOs and defective olive oils before soft-deodorization process were created. The goal was to observe compounds emerging in olive oils during this ´mild´ refining step. The clustering of samples in PCA score plots (**Figure 4**) illustrates that soft-deodorization process did not significantly affect chemical composition to enable separation of particular oil pairs (prior and after deodorization). However, there is the evidence of pair separation in PC1 dimension promising for sample separation in PLS-DA (features important in PC1 dimension were those with the highest VIP score in PLS-DA). When using PLS-DA with only features with VIP score higher than 1 (536 features in ESI+, 176 features in ESI-) (**Figure 4**) models with following parameters were obtained: R^2X=0.493, R^2Y=0.895, Q^2=0.306 for ESI+ and R^2X=0.835, R^2Y=0.896, Q^2=0.384 for ESI-. The most characteristic ions for SDOO according to VIP score and the position in loading plots were further investigated for the possibility to be used as marker compounds.

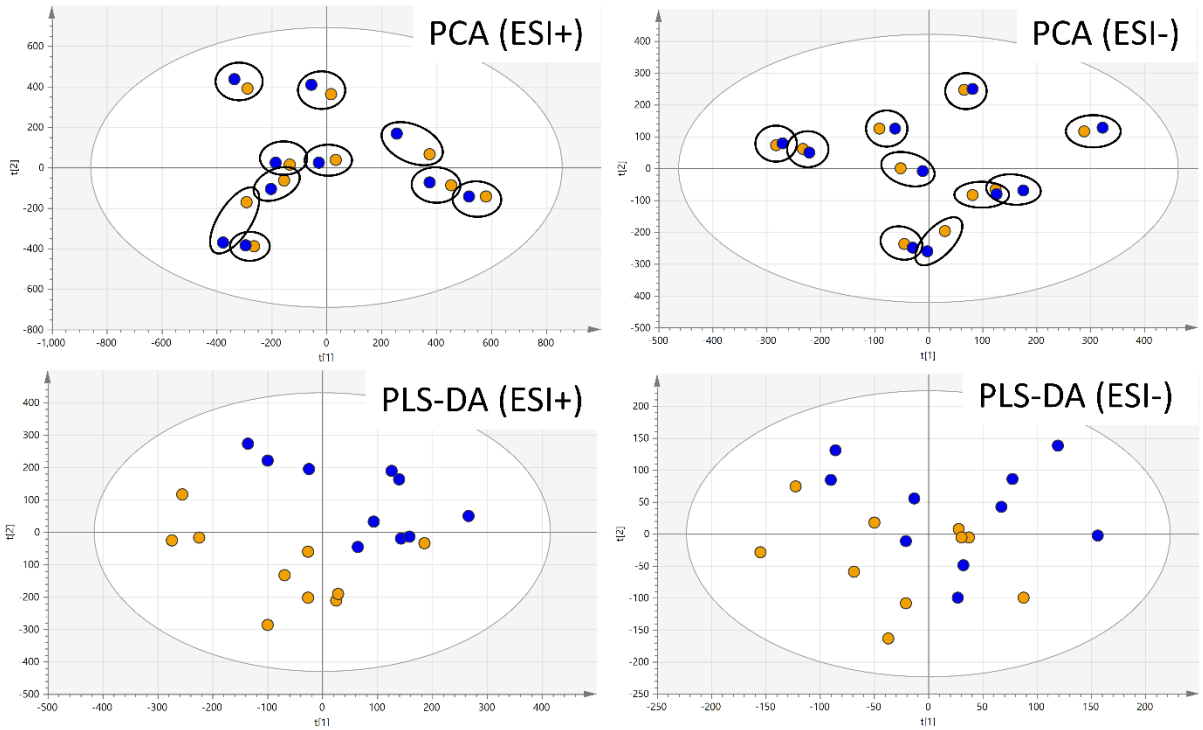

Fig. 4. PCA and PLS-DA score plots of defective oils (orange) and SDOOs (blue). Samples in black circles in PCA plots represent the particular defective oil prior and after deodorization.

3.3. Markers selection, confirmation and identification

For markers confirmation, trend plots of pre-selected markers were studied to visualize the presence of features in EVOO and SDOO samples. The important criterion was that these ions are not detectable in any EVOO. Additionally, it was checked whether respective marker compounds are formed during soft-deodorization process by comparing the presence of the marker in the herein analysed oils before and after soft-deodorization, meaning that it is the marker of soft-deodorization process which is formed due to elevated temperature. On the other hand, as mentioned in the comprehensive study by Aparicio-Ruiz et al. (2017), as far as the marker occurs in a comparable amount in both defective and SDOOs and, at the same time, it is not present in EVOOs, then it can be a marker of undergoing fermentative process in bad quality olives.

From the loading plots of PLS-DA models of EVOOs vs SDOOs, ions with following *m/z* values were selected: 283.2621 (ESI+), 299.2572 (ESI+) and 365.1237 (ESI-). These features were also one of the markers found by our above-mentioned joint research study with Cavanna et al. (2020), which confirms their applicability also for another sample set. However, these ions were found to be present also in defective oils, i.e. before soft-deodorization, what may mean that these compounds are the second type of markers, i.e. markers of lower-quality olives. For the selection of soft-deodorization process markers, loading plots of PLS-DA model of defective vs SDOOs were examined in detail. Based on VIP score, ions formed during soft-deodorization were selected, i.e. which were neither present in defective oils before soft-deodorization, nor detected in any EVOO. In total, 7 ions fulfilled these criteria.

All selected markers (No. 1-3 markers of low-quality olive oils, No. 4-10 markers of soft-deodorization process) were evaluated applying receiver operating characteristic (ROC) curves. These curves display true positive rate against false negative rate at various threshold settings and therefore evaluates the discriminatory capability of markers (Riedl, Esslinger, & Fauhl-Hassek, 2015). Resulting AUC-ROC values measure how a marker can distinguish between two groups in terms of reliability: the closer AUC-ROC value is to 1, the better is the model (Creydt & Fischer, 2020; Inoue & Toyo'oka, 2015). The complete list of markers with the AUC-ROC values is in **Table 1**.

Table 1: List of markers selected by metabolic fingerprinting strategy.

Compound No.	*m/z*	Retention time (min)	Ionization mode	AUC-ROC	p-value	Suggested elemental formula (M)	Ion species	Mass error (Δppm)	Tentative identification
1	283.2621	7.63	ESI+	0.79	1.86e-5	$C_{18}H_{34}O_2$	[M+H]+	3.4	propylene glycol-1 stearate (Cavanna, et al., 2020)
2	299.2572	7.67	ESI+	0.73	0.021	$C_{18}H_{34}O_3$	[M+H]+	3.9	propyl-12-hydroxy-9-octadecanoate (Cavanna, et al., 2020)
3	365.1237	3.82	ESI-	0.77	0.001	$C_{18}H_{22}O_8$	[M-H]-	0.5	NI
4	225.1943	5.89	ESI+	0.68	0.009	$C_{13}H_{24}N_2O$	[M+H]+	3.7	NI
5	295.2632	7.75	ESI+	1.00	2.75e-13	$C_{19}H_{34}O_2$	[M+H]+	2.9	methyl ester of hydroxylated octadecenoic acid
6	335.2558	7.74	ESI+	0.89	3.36e-4	$C_{19}H_{36}O_3$	[M+Na]+	0.1	NI
7	360.3254	8.34	ESI+	0.77	0.039	$C_{22}H_{43}NO$	[M+Na]+	1.1	NI
8	364.3570	8.72	ESI+	0.88	4.41e-4	$C_{24}H_{42}O$	$[M+NH_4]+$	0.3	NI
9	369.3011	7.89	ESI-	0.84	0.002	$C_{22}H_{42}O_4$	[M-H]-	3.3	ester derivative of oleic acid
10	393.2982	8.44	ESI+	1.00	1.43e-13	$C_{22}H_{42}O_4$	[M+Na]+	0.8	

*NI – not identified

Identification of respective ions was performed using PeakView software, which predicted the molecular formula based on the accurate *m/z* value of the molecular ion (5 ppm mass error tolerance), isotopic pattern and fragment ions information. The tentative results were searched against the METLIN (https://metlin.scripps.edu/), HMDB (https://hmdb.ca/) and ChemSpider (http://www.chemspider.com/) online databases. According to the Metabolomics Standards Initiative, the certainty of metabolite identification was level two, tentatively annotated compounds (without reference standard but based upon MSMS spectral similarity with spectral library), or level three, tentatively characterized compound classes (based upon MSMS spectral similarity with a class of compounds) (Sumner, et al., 2007).

Compounds 1 and 2 were tentatively identified within our joint paper with Barilla partners by Cavanna et al. (2020), as in-source fragment ions of compounds propylene glycol-1 stearate

(**Compound 1**) and propyl-12-hydroxy-9-octadecanoate (**Compound 2**). Because measurements reported in that paper were performed using the same ionization source and mass spectrometer conditions as in this study, it was assumed that the ion markers are in-source fragments as suggested.

Compound 5 was tentatively identified as $C_{19}H_{34}O_2$, methyl ester of hydroxylated octadecenoic fatty acid. It is assumed that methyl ester is formed by transesterification of linoleic acid which is oxidized to form hydroxylated monoenoic acid (Biedermann, Bongartz, Mariani, & Grob, 2008).

Compound 9 was observed in both ionization modes (in ESI+ as **Compound 10**), different retention times in ESI+ and ESI- were caused by different mobile phase flow. Suggested elemental formula was $C_{22}H_{42}O_4$, forming sodium adduct in ESI+ and deprotonated ion in ESI-. From the fragmentation spectra, it was deduced that it is an ester derivative of oleic acid, probably diethylene glycol monooleate. Since it is assumed only based on in-silico fragmentation, further confirmation is needed.

Other compounds were not identified.

3.4. Targeted analysis

Although high resolution mass spectrometry (HRMS) has become an important tool in food commodities authentication, for routine control the use of targeted analysis based on triple quadrupole instruments is often preferred due to a better reproducibility of measurements, lower detection limits and simpler data handling. Therefore, the possibility to measure selected markers on an QTRAP (SCIEX, Canada) mass spectrometer with QqQ mass analyser has been investigated, which is commonly used in control laboratories for trace analysis. At first, we optimized the declustering potential and the collision energy to achieve maximum signal responses. Secondly, the whole set of samples was measured targeting only the selected ions

and their MRM transitions, which were selected based on high-resolution MS/MS spectra (**Supplementary Table A1**).

The results of targeted measurement are summarized in **Table 2**. While this instrument allowed lower detection limits, it scans in low resolution, which could cause errors due to mass interferences (some MRM transitions suggested from HRMS/MS spectra could not be used because of mass interferences). The best recognition ability showed Compound 5 and Compound 9, which were detected in every blend of EVOO and SDOO, even in the 30% SDOO addition. Method to reveal addition of 30% SDOO was successfully demonstrated only in study of Gómez-Coca et al. (2020), while the method developed by Cavanna et al. (2020) detected mixtures containing at least 40% of soft refined oils. Study by Gertz et al. (2020) claims that described method is able to recognize as low as 10% addition of soft-deodorized oils, however, the deodorization experiments were performed only on three virgin olive oils and the method is based on the estimation of 12 analytical parameters, which can be rather difficult and time consuming.

Table 2: The presence of selected markers within groups of samples measured by UHPLC-QqQ-MS/MS (positive findings/total number of samples in group).

Compound No.	*m/z*	Defective oils	SDOOs	EVOOs	Blends	Marker quality *
1	283.3	10/10	10/10	22/22	60/60	-
2	299.3	10/10	10/10	22/22	52/60	-
3	365.1	10/10	10/10	22/22	60/60	-
4	225.2	10/10	10/10	22/22	60/60	-
5	295.3	0/10	10/10	0/22	60/60	++
6	335.3	0/10	9/10	0/22	44/60	+
7	360.3	1/10	10/10	0/22	60/60	+
8	364.4	0/10	10/10	0/22	50/60	+
9	369.3	0/10	10/10	0/22	60/60	++
10	393.3	0/10	10/10	0/22	48/60	+

* (++) excellent, (+) promising, (-) weak

The trend plots of two ′best′ marker ions are shown in **Figure 5**. On the other hand, by employing targeted analysis and lowering detection limits, compounds 1-4 could be detected in trace levels in each EVOO sample. Therefore, these markers suggested by Cavanna et al. (2020) would need to be quantified and assessed by concentration range in defective oils to be used for authentication purposes. Unfortunately, certified standards are currently not available. The intensities of all ions and their presence within groups of samples are illustrated in the form of box plots in **Supplementary Figure A1**. Only three types of blends, with 70, 50 and 30% of soft-deodorized oils, were available for experiments, so we could not determine directly the lowest detectable amount of its addition. Nevertheless, with regards to relatively high signal intensities of candidate marker ions in some samples with 30% soft-deodorized oils content, even fairly lower addition can be further tested for detectability.

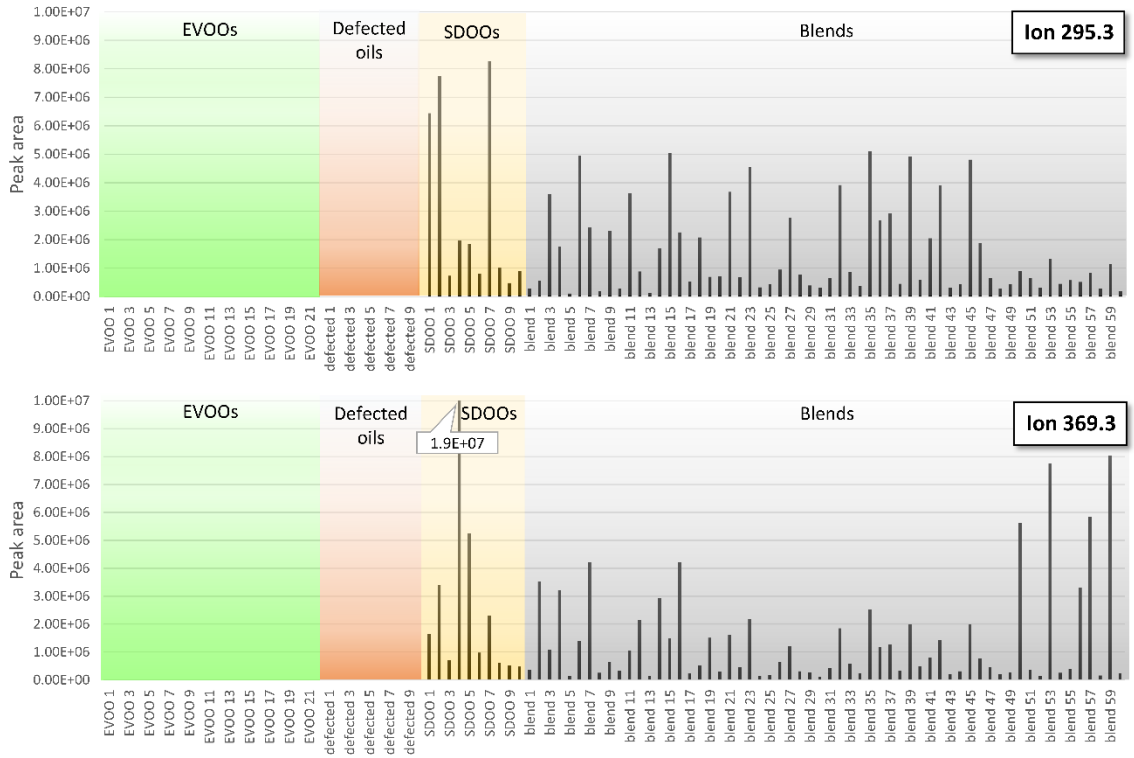

Fig. 5. Peak areas of two marker ions in all measured samples. Upper graph: areas of Compound 5 (*m/z* 295.3); Bottom graph: areas of Compound 9 (*m/z* 369.3).

4. Conclusion

This study demonstrates the complementarity of two alternative mass spectrometry based approaches aimed at detection of soft-deodorized olive oil addition to extra virgin olive oil. Employing untargeted analysis, metabolic fingerprinting, performed by UHPLC-QTOF-HRMS technique, allowed selection of ten candidate authentication markers (ions) of soft-deodorization oils addition, of which seven originated as the result of soft-deodorization process.

In the next phase, target analysis conducted by UHPLC-QqQ-MS/MS enabled a study of preselected marker ions selectivity. Thanks to the lower detection limits achieved by target approach, some compounds (no. 1-4) were found also in trace amounts in extra virgin olive oils.

In any case, all candidate ions could be detected in most blends and were able to reveal even 30% addition of soft-deodorized oils. Based on the relatively high signal intensities of marker ions in the prepared blends, it is assumed that this approach could reveal even 10 % blend. The best results in terms of selectivity were documented for ions 295.3 (ESI+) and 369.3 (ESI-). These ions, tentatively identified as methyl ester of octadecenoic acid and ester derivative of oleic acid, in combination with other ions presented in this study, could be used as markers applicable for the control of extra virgin olive oil adulteration with soft-deodorized olive oil in routine practice.

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