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# Differentiation of Italian extra virgin olive oils by rapid evaporative ionization mass spectrometry

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ABSTRACT

The present work aims to differentiate Italian extra virgin olive oils through the analysis of about 100 samples for the building of a spectral database useful for the comparison beetwen top-quality oils. High-quality oils belonging to two different harvest years and coming from different Italian regions, labelled as PDO (Protected Denomination of Origin) or obtained from a single variety of olives (monocultivar), were available.

Rapid Evaporative Ionization Mass Spectrometry, in combination with a monopolar handpiece as sampling device was explored for the first time on liquid and poorly conductive samples. Multivariate analyses were applied to build different chemometric models. The monocultivar oils gave positive feedback, leading to the reliable identification of each cultivar. The recognition of PDO oils resulted more challenging (failure percentage $> 5\%$), probably due to the major intra-class variability, since oils labelled with the same PDO trademark often are produced from different cultivar in different percentages.

## 1. Introduction

Extra virgin olive oil (EVOO) is one of the most important and abundant commercial products of Mediterranean countries, mainly due to pedoclimatic factors, but also related to its nutraceutical, antioxidant and other well-known healthy effects (Fanali, Dugo, & Mondello, 2016; Foscolou, Critselis, & Panagiotakos, 2018). Because of this high commercial value, EVOO is susceptible to the risk of mislabeling and adulteration, for example by the addition of other vegetable oils of lower economic value (Jabeur, Zribi, & Bouaziz, 2016; Yang, Ferro, Cavaco, & Liang, 2013). As a consequence, authenticity and traceability are important for both consumer health preservation and commercial purposes. In order to trace back to the geographic origin of olive oils and guarantee the quality of this important product, the European Regulation, established rules on the Protection of Designations of Origin (PDO) and Protected Geographical Indications (PGI) (EC, 2006; EU, 2012). Both the quality trademarks indicate "the name of a region, a specific place or, in exceptional cases, a country, used to describe an agricultural product or a foodstuff". They differ in the quality definition, as for PDO "the quality or characteristics are essentially or exclusively due to a particular geographical environment with its inherent natural and human factors" while for PGI "specific quality, reputation or other characteristics attributable to that geographical origin". Italy is one of the largest European producers of extra virgin olive oils labelled with these quality trademarks. In particular, 46 Italian brands are recognized by the European Union, distinguished in 42 PDO and 4 PGI. Moreover, some of these quality products can be obtained from 100% olives of only one variety, referred as "monocultivar oils" (Rotondi, Magli, Morrone, Alfei, & Pannelli, 2013), or from the skillful mixing of oils extracted from several olives cultivar, indicated as "blend" (European Commission, 2012). Monocultivar extra virgin olive oils are endowed with chemical-physical and organoleptic characteristics that give them a specific territorial peculiarity and identity; furthermore, growing interest is shown by the olive oil producers in the marketing of monocultivar oils as a way to improve the competitiveness. Instead, oils produced by the complex art of "blending" can be more harmonious and able to respond to different consumer needs, so that many PDOs are blends, as the related production regulations allow percentages of olives

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of different varieties. Despite the increasing attention paid to ensuring the quality and traceability of these products, the European regulation lacks an official methodology to evaluate the origin and the genuineness of EVOOs. Therefore, a rapid and reproducible method to obtain an olive oil fingerprinting, could be useful to monitor the authenticity and the adulteration of EVOO as a quality control approach, being attractive for industry, as well as ensuring transparency for consumer choices. Several methodologies have been proposed to achieve a rapid fingerprinting of EVOO and build a database useful for the detection of adulterations, such as IR spectroscopy (Azizian, Mossoba, Fardin-Kia, Karunathilaka, & Kramer, 2016; Georgouli, Martinez Del Rincon, & Koidis, 2017), nuclear magnetic resonance (NMR) (Klikarová et al., 2019) and mass spectrometry (MS)-based techniques (Jergovic, Peršurić, Saftić, & Kraljević Pavelić, 2017). Between them, ambient MS showed unparalleled qualities due to the possibility to obtain intact ions directly from their native environment without sample preparation or chromatographic separations. In the present work, an innovative instrumental setup, called iKnife, was tested on EVOO samples coming from seven different selected areas of the north, central and south Italian Regions (Apulia, Calabria, Sicily, Lazio, Tuscany, Garda). This approach is obtained by the coupling between rapid evaporative ionization mass spectrometry (REIMS) with a surgical monopolar diathermy hand-held device corresponding to an electroknife (Arena et al., 2020). The latter, responsible for sampling, leads to the thermal ablation of the sample and then to the formation of an aerosol containing gaseous molecular ions. Sample conductivity is essential at this stage. The resulting vapors are directly transferred into the mass spectrometer which returns in a few seconds a chemical fingerprint of the sample. REIMS applications normally require a spectral database of reference mass spectra in order to build multivariate classification models necessary for subsequent pattern-based identification (Balog et al., 2016). Starting from the first applications in the clinical field to discriminate between healthy and tumor cells (Phelps et al., 2018; St John et al., 2017), in the last five years such a technique was conveniently applied in the food area for fast and reliable detection of food fraud, e.g. fish and meat mislabeling (Black et al., 2017; Nollet et al., 2020; Rigano, Mangraviti, Stead, Martin, Petit & Dugo, 2019), honey adulteration (Wang et al., 2019), or preservation of a PDO trademark (Rigano, Stead, Mangraviti, Jandova, Petit & Marino, 2019). One of the main advantages consists in the very short analysis time, also related to the lack of tedious and complex sample preparation procedure. For the first time, this novel technique has been applied to liquid and poorly conductive samples. Moreover, although only negative spectra are usually reported in the literature (Rigano, Mangraviti, et al., 2019), both negative and positive polarity ionization were investigated to fully explore system capability on a so fatty matrix (more than 98–99% of triacylglycerols).

The main challenge consisted in the intrinsic large variability of the investigated samples, related to the fact that the same cultivar can be present in different PDO oils and the same PDO can be produced from different percentages of the same cultivars, according to the specific production regulation.

Furthermore, all the samples come from very close regions, thus making more challenging the application here presented with respect to a previous work in which pistachio nuts from different countries were compared (Arena et al., 2019; Rigano, Stead et al., 2019).

## 2. Material and methods

### 2.1. Reagents and materials

LC-MS grade 2-propanol, acetonitrile and trifluoroacetic acid (TFA), sodium chloride (NaCl) EMSURE® ACS, ISO, Reag. Ph Eur were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany). LC grade distilled water was obtained from a Milli-Q advantage A10 system (Millipore, Bedford, MA, USA).

Polymeric MALDI targets, FlexiMass – DS plates were provided by Shimadzu (Duisburg, Germany). Three different Ionic Liquids (ILs) matrices, namelyN-isopropyl-N-methyl-N-tert-butylammonium-cyano-4-hydroxycinnamate [IMTBA-CHCA], *N,N*diisopropylethylammonium-α-cyano-4-hydroxycinnamate [DIEA-CHCA], diisopropylethylammonium ferulate [DIEA-FA] were kindly provided by Prof. Daniel Armstrong (University of Texas Arlington, Arlington, Texas, USA).

### 2.2. Samples

In this study, we used a training set of EVOOs obtained during the olives harvesting years 2017–2018, from different Italian regions in the framework of the Violin project (Valorization of Italian Olive Products Through Innovative Analytical Tools), promoted by Ager Foundation (Milan, Italy). The samples were supplied from different providers and labelled with PDO trademark or Certified Origins for monocultivar oils; both types were used to build two multivariate statistical models. The detailed list of the training set oils is reported in Tables S1 and S2 (Supplementary Material) for PDO (36 samples) and monocultivar oils (26 samples), respectively. Moreover, a validation EVOO set (additional 28 samples) was used to investigate the predictive capability of the models. It contains EVOO labelled with the same PDO trademark or obtained with the same olive variety than the training sets, but coming from a different producer or a different production lot, and also lower quality commercial oils in order to evaluate the occurrence of false positive.

### 2.3. Sample preparation

One mL of each EVOO was added into glass tubes containing 5 mL of a 50 g/L NaCl aqueous solution. The mixture was wrapped in aluminum foils and put inside special molds in order to obtain ice cubes “easy to cut” (Supplementary Material, Fig. S1).

Different salt concentrations (5, 10, 50 and 100 g/L) and oil-saline solution ratios (1:1, 2:1, 1:2, 1:4, 1:5, 1:10, *mL:mL*) were also tested during the optimization of sample preparation method. The final conditions were selected on the basis of the highest signal intensity.

During the sampling optimization, IL solutions prepared at a concentration of 10000 mg $L^{-1}$ in acetonitrile:water (2:1 mL:*mL*) with 1 mL/L TFA, were also evaluated in different ratios with EVOO.

### 2.4. Iknife conditions

A hand-held monopolar probe (Waters Corporation, Wilmslow, UK) was employed for EVOO sampling. It consists of an electrosurgical device equipped with a 6 mm long knife blade and connected to a high-frequency diathermy generator (Erbe VIO 50 C - Erbe, Tuebingen, Germany). The alternative electric current produced, at a power of 40 W and in drycut mode, was applied to heat the blade by joule effect causing instantaneous vaporization by contact with the surface of the sample.

The produced vapor was transferred by an evacuation line (3 m long, 1 cm diameter, PTFE tube) to the REIMS source (Waters, Milford, USA) of a Xevo G2 XS QTof mass spectrometer (Waters Corporation). The consequent collision of the aerosol particles with a heated kanthal coil surface of the source, set to 4.5 A and 4.2 V, led to ion formation.

A matrix of 2-propanol was infused in continuous into the REIMS source at a constant flow rate of 150 μL/min through a syringe pump (fixed luer lock LC pump priming syringe by Trajan Scientific, Crownhill, UK), to promote the ionization of lipid species and maintain source cleanliness. All analyses were carried out in REIMS TOF MS sensitivity mode with continuum data acquisition. The mass resolution was approximately 20,000 FWHM (full width at half maximum) over the mass range of interest. The cone voltage and heater bias were set at 40 V and 80 V, respectively. Mass spectrometric analysis was performed in negative and positive ionization mode over a mass range of 100–1300 *m/z* with and integration (scan) acquisition time of 0.5 s/scan. Prior to use, the instrument was calibrated using sodium formate in 2-propanol directly infused into the REIMS source.

The EVOO analyses were replicated over a period of three months, after different instrumental calibrations in order to minimize the variability related to instrumental sources. Then, around 140 spectra were acquired for each sample included in the training set (Table S1, Supplementary Material).

### 2.5. Data processing

The raw data acquired in MassLynx v. 4.1 (Waters Corporation), were processed and visualized using LiveID software (Waters Corporation), in order to evaluate the intrinsic variation of the data by means of multivariate statistical analysis. Prior to model buildings, endogenous matrix ions m/z 255.2330 (deprotonated palmitic acid) and m/z 907.7731 (sodium adduct of triolein) were used for internal lock-mass correction of MS analysis acquired in negative and positive ionization modes respectively. Moreover, a background subtraction and data resampling (binning to 0.10 Da) were applied to reduce the background and dimensionality of the data set.

Two different approaches were employed automatically by the software for the statistical analysis, namely Principal Component Analysis (PCA), and Linear Discriminant Analysis (LDA). The first approach allows the clustering of the samples in an unsupervised manner, so each recorded mass spectrum will occupy a specific position in the multidimensional space, independently of the belonging type (defined in statistical terms as a class). Afterward, LDA, that is a supervised analysis that maximizes inter-class variance, minimizing intra-class variance, is applied on the PCA in order to address the identification of unknown samples (recognition step).

In order to evaluate the robustness and predictive ability of PCA-LDA models, an *in-silico* validation was performed. In this case, the data set used for the building of each model is partitioned in 5 portions (5-fold stratified validation), everyone contains a representative proportion of each class. Four portions (80%) are applied to create a model with the same conditions present in the original one. The obtained model is used to predict the classifications of the portion (20%) that has been excluded. The validation output reports the details related to the correct and incorrect classifications and the number of outliers. The latter are calculated based on Mahalanobis distance (Mahalanobis, 1936) to the nearest class center.

Once predicted the model accuracy by the in-silico validation, a recognition step was performed using a series of samples not included in the training data set. This phase can occur in playback, *i.e.* in a subsequent phase to the analysis, or in real-time (simultaneously with their acquisition).

## 3. Results and discussion

### 3.1. Sampling method

Commonly iknife method is applied for the analysis of solid or semi-solid samples (Arena et al., 2020; Rigano, Mangraviti, et al., 2019). They are usually reduced to small pieces or crushed to increase the sampling surface and allowing the most internal portions to be burned by the heated knife. The obtained powders can be wetted with distilled water to improve electrical conductivity.

For the first time in this study, iknife was explored to achieve mass spectra from liquid and poorly conductive samples, such as olive oil. In order to find an appropriate sampling technique, different approaches have been considered. It was proposed at first, the analysis of oil mixed with IL at different concentrations on specific plates (MALDI plates). The use of IL matrices has already been investigated through MALDI technology for proteins determination (Armstrong, Zhang, He, & Gross, 2001; Crank & Armstrong, 2009); it has been demonstrated that the IL application allowed to obtain homogeneous solutions providing a better reproducibility between one sample and another, and promoting sample ionization. In the present study, IMTBA-CHCA, DIEA_CHCA and DIEA_FA (Supplementary Material, Figure S2 A) were tested as a mixture with EVOOs at different concentrations, and the resulting solutions were placed on MALDI plates to be analyzed.

The used MALDI plates are polymeric supports composed of 48 hydrophobic positions, each surrounded by a hydrophilic zone, and in which samples can be spotted (Supplementary Material, Figure S2 B). This particular conformation allows the single droplets anchorage in the sample position and prevents their spreading to the surrounding area. The mix of EVOO with DIEA_FA provided the best results in terms of signal intensity between 600 and 1000 m/z (Fig. 1). Particularly, adducts with the ubiquitous chloride ion were generated with both di- and triglycerides (DAGs and TAGs, respectively) and deprotonated fatty acids (FAs) were detected in the region 250–300 *m/z* (Fig. 1). The 1:2 ratio in favor of the IL matrix was the optimal concentration to increase ionization efficiency. Nevertheless, poor repeatability of the ionic current generated led to leave this approach and consider other options, even less expensive.

Among the other considered options, the most common sampling method was tested on the grounded counter electrode with the interposition of wet paper. It consists of a silicone plate for the electric current discharge to the ground that runs through the sample. Usually the latter presents a consistence that not requires further measures, but in the case of liquids such as EVO oil, a solid or semi-solid support was necessary to prevent its spreading. Alternatively, the sampling of EVOOs directly placed on ice was tested, but also in this case, the signal repeatability was quite low, probably due to the instantaneous burn which did not ensure a sufficient number of scans.

Therefore, the physical state of the sample was modified to obtain a product of proper consistency easy for sampling. Oil-water mixtures solidified in the form of ice cubes and in different ratios have been tested. Nevertheless, due to the presence in all the cases of low intensity mass spectra (Fig. 2A and E), the use of a saline solution has been chosen with the purpose to improve conductivity and increase signal intensity in both positive and negative ionization mode. Interestingly, the use of the IL DIEA-FA produced weaker signals when dissolved in water and frozen, without the use of the MALDI plate support, despite the same salt concentration was employed (around 6.6 mg $mL^{-1}$). The increase of the IL concentration was not taken into account due to the high cost in the case of large number of samples, as in the current project.

Then, four different mixtures of oil-NaCl solution were tested, at a saline concentration of 5, 10, 50, 100 g $L^{-1}$ (Fig. 2) and in different proportions. Fig. 2 shows the spectra obtained in both positive and negative ionization mode, along with a preliminary identification according to chemical class: the chloride adducts of DAGs and TAGs were predominant in the negative spectra, along with deprotonated FAs, while sodium adducts of TAGs and the dehydrated species of monoglycerides (MAGs) and DAGs were the major ions detected in positive, together with some protonated TAGs and their potassium adducts.

Among them, oil-50 g $L^{-1}$saline solution (in 1:5 ratio), corresponding to a salt concentration of about 40 mg $mL^{-1}$, provided the most informative spectra and the most intense peaks, both in positive and negative ionization mode (Fig. 2), while only a poor signal, even more than one order of magnitude lower than the one detected with the use of IL, was registered by using an amount of NaCl comparable to the IL (4 mg $mL^{-1}$). Therefore, even if in the present study the use of the more easily available NaCl appeared the best solution to fit the research purpose, the role of ILs for increasing the ionization efficiency of apolar compounds as well as sample conductivity could be further investigated, especially for the detection of minor components.

### 3.2. Mass spectral content

Mass spectra obtained from the analysis of EVOOs were acquired both in positive and negative ionization mode, in order to evaluate which spectral profile gave the best results, useful for an appropriate characterization and differentiation of the samples.

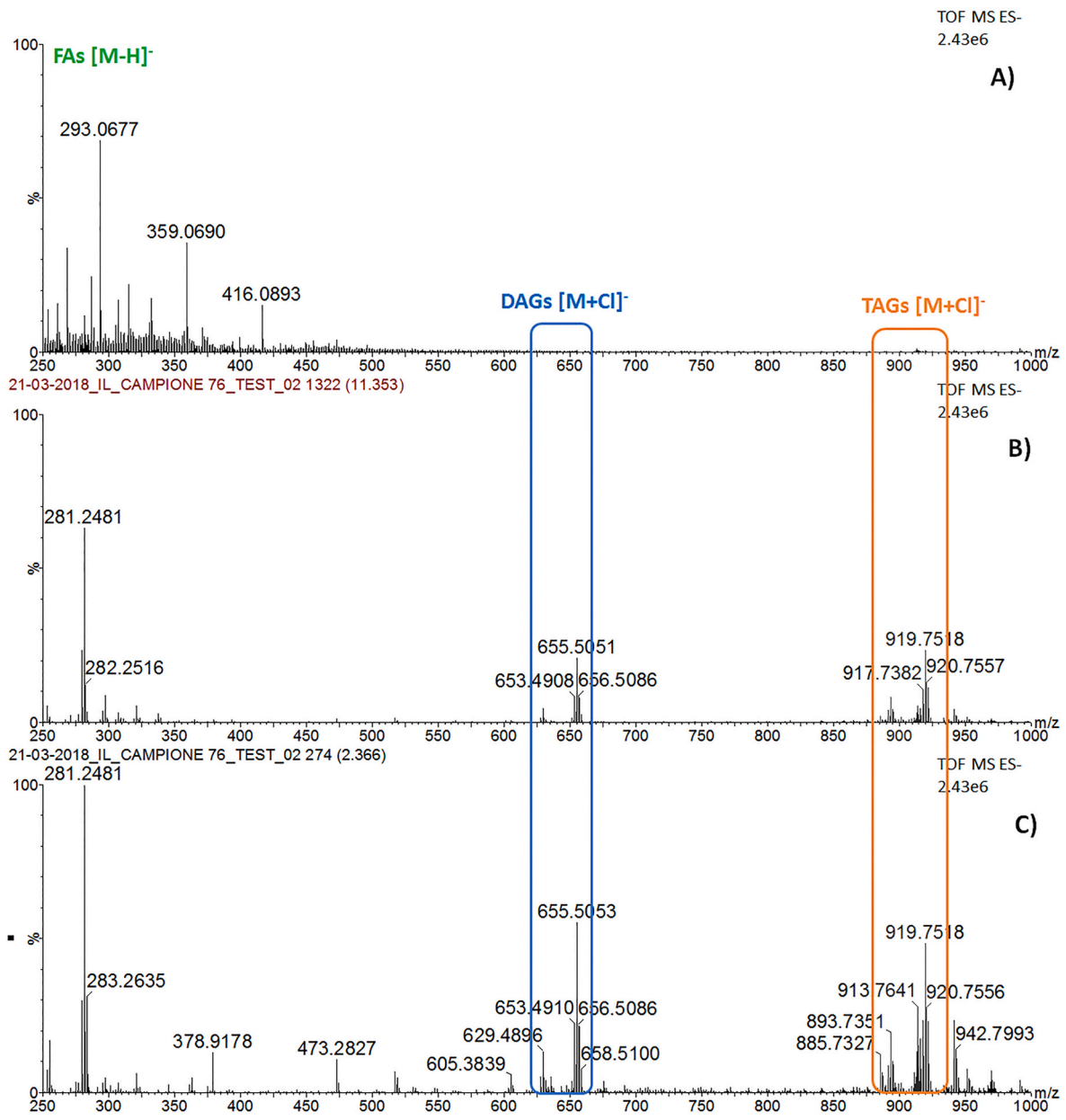


**Fig. 1.** REIMS (−) spectra (mass range 250–1000 *m/z*) obtained for an EVOO sample mixed with an Ionic Liquid (IL) solution (10000 mg $L^{-1}$) of **A**) DIEA_CHCA, **B**) IMTBA_CHCA, **C**) DIEA_FA, in the ratio 1:2 (*v:v*) EVOO:IL solution in all the cases. FA: fatty acids; DAGs: diglycerides; TAGs: triglycerides. FA: fatty acids; DAGs: diglycerides; TAGs: triglycerides.

Fig. 2 shows good quality and highly informative mass spectrum also in positive ionization mode. Therefore, both sets of data (in positive and negative ionization modes) were utilized for the chemometric analysis and each statistical model was evaluated in terms of capability in discriminating between different samples, related to the number of discriminating variables (or components) contained in the mass spectra. To this regard, Table S3 (Supplementary Material) reports the list of identified compounds in both positive and negative spectra along with their mass error, while Tables S4 and S5 (Supplementary Material) report percentage content of FAs and TAGs in PDO and monocultivar oils. As for the identification, it is noteworthy that the TOF analyzer allowed to obtain mass error lower than 5 ppm in the majority of the cases, despite the use of an "ambient" ionization source, which could negatively affect mass accuracy data due to interferences coming from the sample matrix or the environment (Arena et al., 2020; Farré & Barceló, 2015) Higher mass errors were measured for potassium adducts of some abundant TAGs detected in the positive spectra or for very low intensity ions, corresponding to compounds probably present at trace levels, such as TAG (C53:1), TAG (C53:2), TAG (C54:1) and TAG (C50:3) detected only in negative ionization mode. Table S3 (Supplementary Material) reports 59 ions, 30 of which were detected in positive mode and 29 in negative. Going into details, 15 out of 30 ions detected in positive allowed to identify 6 MAGs and 9 DAGs; the other 15 ions were identified as TAGs in the forms of protonated species, sodium and potassium adducts for a total of 12 TAGs. In some cases, the same *m/z* value was assigned to protonated molecule and sodium adducts of different TAGs with very low mass error in both cases, thus hindering a univocal identification. As for the elucidation of the negative spectrum, 6 ions were identified as deprotonated FAs and 23 ions as chloride adducts of 7 DAGs and 16 TAGs. Hence, as expected, MAGs and TAGs were better ionized in positive mode, while FAs were detected only in negative ionization. Interestingly, a major number of TAGs were identified in negative ionization, which also enabled a univocal identification for each ion. As a consequence, the MS spectra

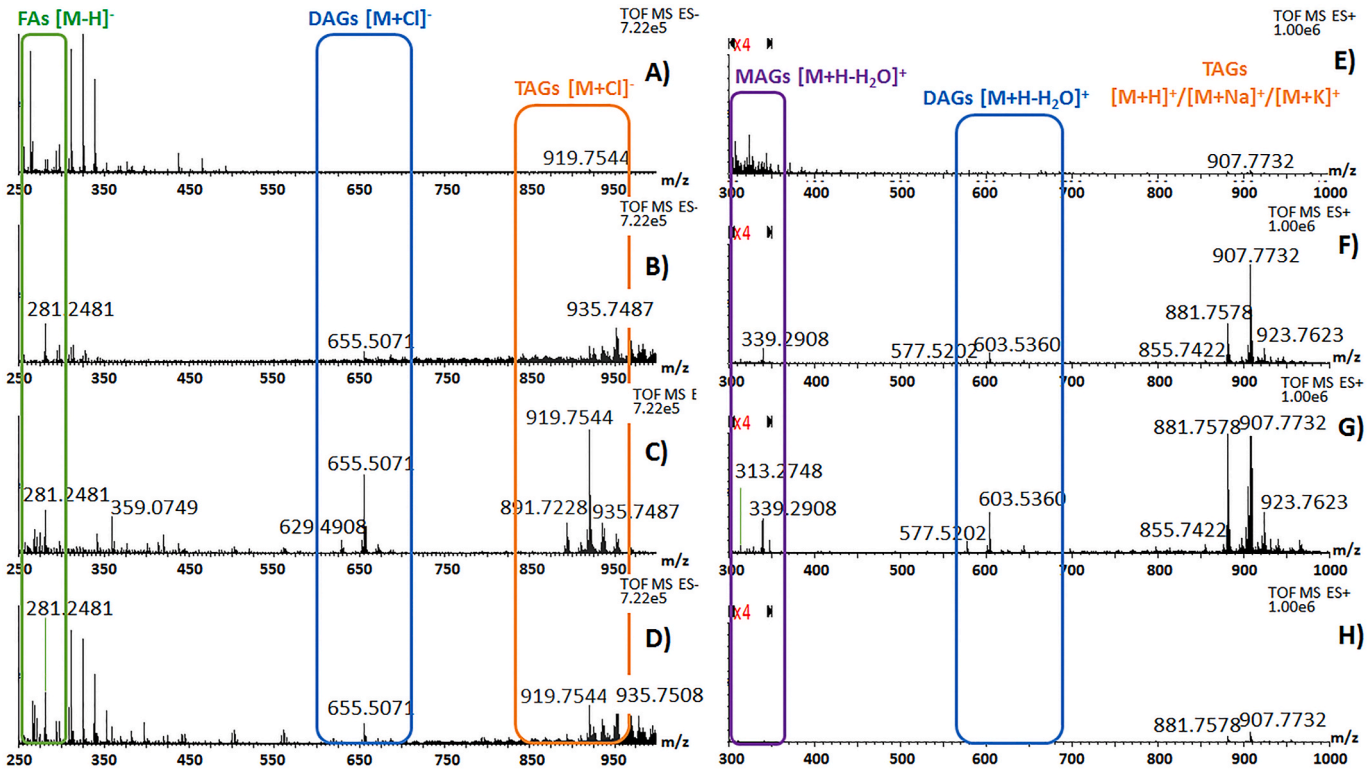


**Fig. 2. A-D)** REIMS (−) spectra (mass range 250–1000 *m/z*) obtained for an EVOO sample mixed with water in the ratio 1:5 (*v:v*, EVOO:water) with increasing concentration of NaCl (**A.** 0, **B.** 5 g $L^{-1}$, **C.** 50 g $L^{-1}$, **D.** 100 g $L^{-1}$); **E-H)** REIMS (+) spectra (mass range 300–1000 *m/z*) obtained for an EVOO sample mixed with water in the ratio 1:5 (*v:v*, EVOO:water) with increasing concentration of NaCl (**E.** 0%, **F.** 5 g $L^{-1}$, **G.** 50 g $L^{-1}$, **H.** 100 g $L^{-1}$). FA: fatty acids; MAGs: monoglycerides; DAGs: diglycerides; TAGs: triglycerides. FA: fatty acids; DAGs: diglycerides; TAGs: triglycerides.

acquired in negative were selected to perform a relative quantification of the major EVOO components, *viz.* TAGs, for the samples under investigation. Specifically Table S4 and Table S5 (Supplementary Material) report semi-quantitative results obtained for TAGs with a carbon number of 52 and 54 and double bond number ranging from 2 to 5, which most likely correspond to the combination of the most abundant FAs of EVOOs, namely oleic acid (C18:1ω9), linoleic acid (C18:2 ω6) and palmitic acid (C16:0). The obtained semi-quantitative data were in agreement with a multitude of papers dealing with the characterization of Italian EVOOs (Bucci, Magrí, Magrí, Marini, & Marini, 2002; Cerretani et al., 2006; D'Imperio, Dugo, Alfa, Mannina, & Segre, 2007; Mannina et al., 2003; Ollivier, Artaud, Pinatel, Durbec, & Guérère, 2003) and with LC-MS results on the same samples (data not shown): the species C54:3, most likely corresponding to triolein, was detected at the lowest content in Sicilian samples, specifically in the PDO Monti Iblei characterized by the cultivar Tonda Iblea, with an average of about 40% that increased up to 54% in Apulian EVOOs; on the contrary, the TAGs C52:4 and C52:3, identified as dilinoleyl-palmitoyl-glycerol and oleyl-linoleyl-palmitoyl-glycerol by LC-MS, were quantified at the lowest levels in the Apulian samples, with a relative amount equal to half the amount found in the Monti Iblei or Tonda Iblea samples (1.5 and 6% against 3% and 11%, respectively). Even the abundant C52:2 TAG, corresponding to dioleyl-palmitoyl-glycerol, spanned the range 21–30% in the samples under investigation, with minimum percentages for Apulian samples and maximum levels for Calabrian EVOOs. Within this context, it is possible to assess that, when a large number of samples is available and can be properly classified according to many variables such as the origin, the trademark and the cultivar, also the macro-constituents can be considered valid discriminants, despite only top-quality samples that strictly respect current regulations were analyzed. Of course, chemometric analysis is mandatory to evaluate the differentiation capability and the prediction rate.

### 3.3. Statistical models

The raw files generated by REIMS analysis of EVOOs from two harvest years (2017–2018) provided the starting material for the building of 4 distinct PCA-LDA statistical models. Specifically, two of them included analyses carried out on samples certified with the trademark of origin, "PDO negative" and "PDO positive", to indicate models generated in negative (in the range 250–1000 m/z) and positive ionization mode (mass range 300–1000 m/z), respectively. Both multivariate models consisted of 7 classes (Fig. 3), representative of the most productive regions of the south (Sicily, Calabria), central (Apulia, Lazio, Tuscany) and north Italy (Lombardy, Veneto, Trentino).

The comparison of the statistical models showed a better clustering for "PDO negative" compared to "PDO positive". In particular, the two-dimensional (2D) score plots shown in Fig. 3A–B pinpoint for the "PDO negative" model a clear separation along both function 2 and function 3 between EVOOs produced in the central-northern area and those of the southern part. The latter are further separated according to the specific region with the Apulian PDO Terra di Bari found at negative values of function 2 compared to Sicilian and Calabrian samples at positive values (Fig. 3A) and the Calabrian PDO Lametia found at positive values of LD1 compared to the others (Fig. 3B). The Sicilian samples (circled with dashed yellow line) are in turn well distinct each other along function 3. The central-northern EVOO, although grouped in a single area (circled with a continuous blue line), are partially separated in three small groups respect to function1 and function 2 (Fig. 3B), while two of them, namely PDO Garda and PDO Sabina are totally overlapped along function 3 (Fig. 3A). Such a closeness is probably due to the fact that the same cultivars can be included in different PDO EVOOs, as long as they comply with the percentages provided by their production regulations (Table S1, Supplementary Material).

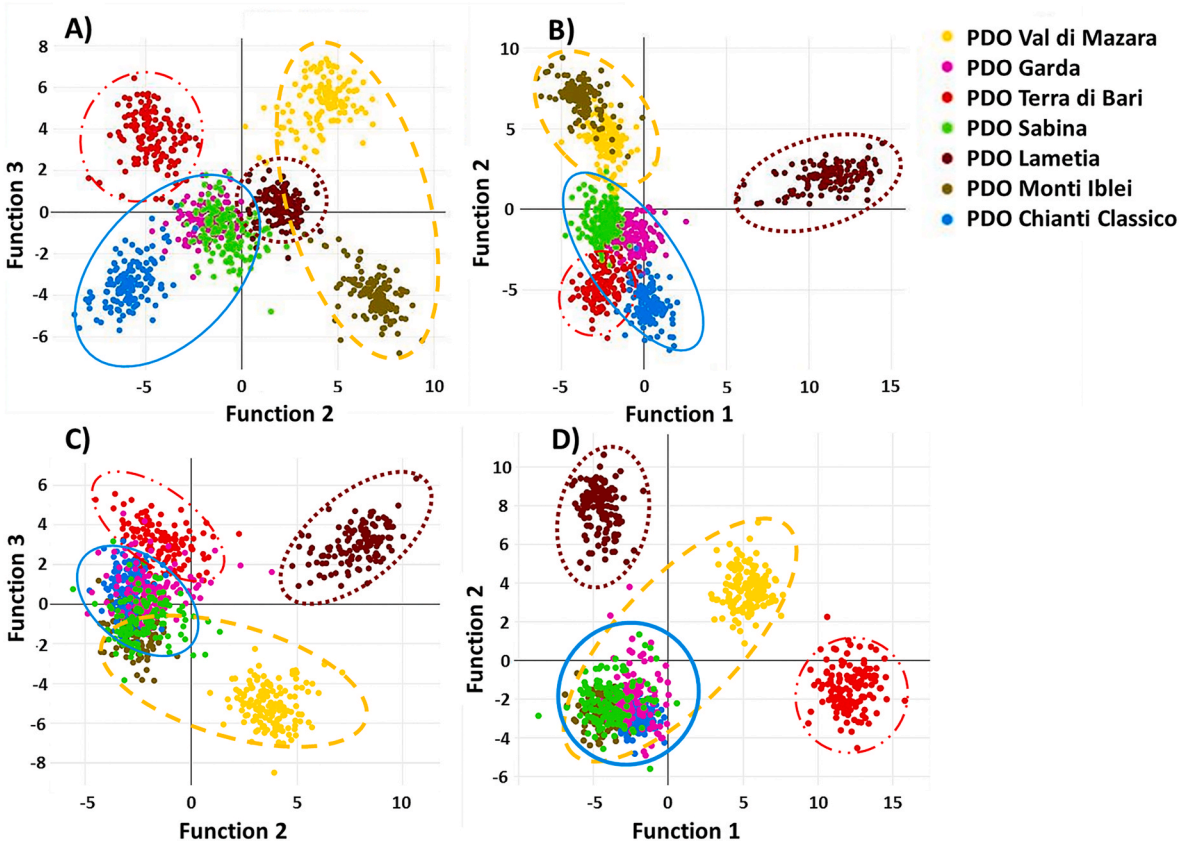


**Fig. 3.** Bidimensional visualizations of the PCA-LDA models built by the LiveID software for 7 PDO EVOOs classified according to Table S1 (Supplementary Material), in negative ionization mode (**A** and **B**) in the mass range 250–1000 *m/z* and in positive ionization mode (**C** and **D**) in the mass range 300–1000 *m/z*. Legend: –, Sicilian PDO EVOOs; …, Calabrian PDO EVOO; _., Apulian PDO EVOO; ⎽, central-northern PDO EVOOs.

In PDO positive model (Fig. 3C–D), despite the clear separation of Lametia PDO EVOO (circled with a dotted brown line) along function 2 (Fig. 3C), Val di Mazara and Terra di Bari PDO EVOOs along function 1 (Fig. 3D), an either complete or partial overlapping was observed between the central-northern Italy classes and the PDO Monti Iblei EVOO.

An in silico stratified 5-fold validation process was applied to estimate the predictive classification accuracy of both models. a high total correctness score percentage, approximately 98.0% for PDO negative and 94.5% for PDO positive, was obtained, thus confirming the observations made on the PCA-LDA score plots discussed above. In fact, the majority of failures for the "PDO positive" model regards the wrong classification of the PDO oil Monti Iblei coming from the South of Italy and classified as a central-northern EVOO in almost 9% of cases, as well as the PDO Chianti Classico which provided around 7% of failures, due to the mismatching with other central-northern PDO trademarks. Subsequently, a data set of samples not included in the validated models

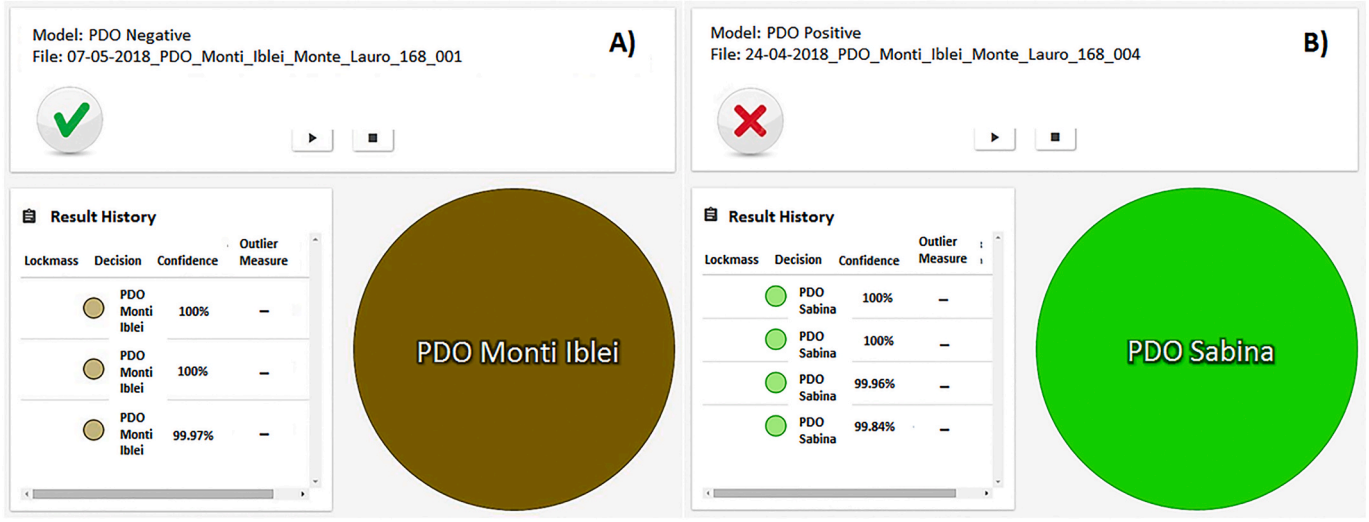


**Fig. 4.** Playback recognition by the LiveID software of a PDO Monti Iblei EVOO through the PCA-LDA models built for PDO EVOOs (as in Fig. 3) in **A**) negative ionization mode and **B**) positive ionization mode.

were processed by playback or real-time recognition. In the case of PDO negative model all the samples were correctly classified leading to an overall assignment rate of 100%, as in the case of a PDO Monti Iblei EVOO (Fig. 4A), while for PDO positive some recognitions led to false sample identifications, as in the case of the same PDO Monti Iblei sample, analyzed in positive mode (Fig. 4B).

Given the wide interest in monocultivar EVOOs as high-quality products (some of them designated with PDO or PGI quality trademarks), and starting material for blend production, two statistical models, one in positive and another in negative ionization mode, have been built with monocultivar oil only, in order to reliably identify the most common olive cultivars employed in Italy.

Both PCA-LDA statistical models include 8 classes and showed a satisfactory clustering (Fig. 5), probably due to the lower variability present in each class including oil obtained from a single olive variety. The separation between them is clearly visible in the tridimensional visualizations reported in Fig. 5. Specifically, typical cultivars of the northern Italian regions such as Caninese, Casaliva, Frantoio (circled with a continuous blue line), the Coratina variety from Apulia (circled with a dotted green line), Carolea from Calabria (circled with a dashed/dotted red line), and three Sicilian cultivars (Nocellara del Belice, Biancolilla and Tonda Iblea, grouped and circled with a dashed yellow line) were considered. As for the Sicilian cultivars, they come from different areas, namely the city of Trapani (Nocellara del Belice) in the western part of Sicily, Agrigento (Biancolilla) around the center of Sicily and Syracuse (Tonda Iblea) at the eastern Sicily. The 5-fold stratified validation confirmed a high percentage correctness score generated for both models, between 97% and 99%, and what is more meaningful is that the playback recognition of samples not included in the model gave a correct identification in both cases. To this regard, the same analyses matched against the PDO models were used for the recognition step of the monocultivar models. This was possible because the PDO Monti Iblei sample is also a monocultivar oil obtained by the cultivar Tonda Iblea. Fig. 6 highlights in both cases the right recognition for 5 subsequent cuts by the iknife, with a confidence value of 100%. In conclusion, our study seemed to demonstrate that genetic factors are responsible for a major discrimination between different high-quality EVOOs, at least when a fingerprinting analysis is performed, capable to detect mainly major compounds, such as FAs and TAGs. The obtained score plots certainly pinpoint a differentiation also based on pedoclimatic conditions, but the comparison between the PDO and the monocultivar models pointed out that classification according to the olive variety could be more effective with respect to the PDO trademark discrimination or previous attempts to identify oils according to the cultivation area, *viz.* Italian regions, as in previous works (Dugo et al., 2020), in which a maximum prediction rates of 70% was achieved by using the phenol and tocopherol content as discriminant variables. Such a conclusion is also in agreement with several papers dealing with the classification of EVOO only according to the cultivar (Azizian et al., 2016; Cerretani et al., 2006; Kosma, Badeka, Vatavali, Kontakos, & Kontominas, 2016), while only few research works focused on the discrimination according to the trademark in comparison with commercial EVOOs (Lukić et al., 2020) and/or in most cases mainly based on the different geographical origin (Antonini et al., 2015; Becerra-Herrera et al., 2018; Consonni & Cagliani, 2019; D'Imperio et al., 2007; Mannina et al., 2003).

Finally, the occurrence of false positive was carefully evaluated in all the models by matching against the monocultivar models some blend EVOOs, as well as monocultivar EVOOs typical of the same Italian regions, but not included in the model (Fig. S3, Supplementary Material), while some commercial not PDO EVOOs produced in the same regions of PDO samples were processed by the PDO models (Fig. S4, Supplementary Material). A more detailed discussion is provided in Supplementary Material.

## 4. Conclusions

Achieved results were very promising in the discrimination of different EVOOs, in spite of the great variability within the same class (different cultivars, different producers) and the geographical closeness between all the classes. In particular, the PDO model required a wider set of samples including all the possible variables (harvest year, producer and production lot, production and harvest area, employed cultivars), conversely from the monocultivar model which provided a higher correctness score after a minor number of analysis. This could mean that the olive cultivar plays a major role in discrimination. However, different sub-models could be built in order to differentiate the same PDO oil respect to the harvest and/or production geographic area. As a matter of fact, the combination of a suitable sampling device with MS and chemometrics provided a unified and unparalleled powerful tool in the field of authenticity and traceability, even within the same country, preserving not only the consumers but also competitiveness, *viz.* the global market.

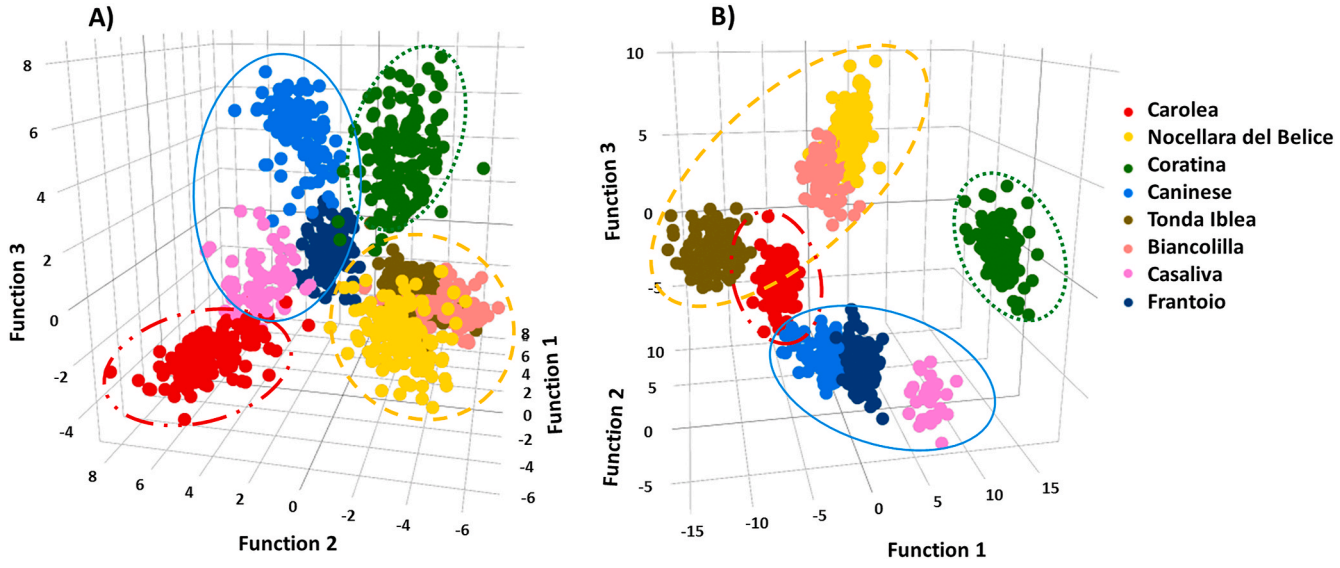


**Fig. 5.** Tridimensional visualizations of the PCA-LDA models built by the LiveID software for 8 monocultivar EVOOs classified according to Table S2 (Supplementary Material), in **A)** negative ionization mode in the mass range 250–1000 *m/z* and **B)** in positive ionization mode in the mass range 300–1000 *m/z*. Legend: –, Sicilian PDO EVOOs; …, Apulian PDO EVOO; _., Calabrian PDO EVOO; _, central-northern PDO EVOOs.

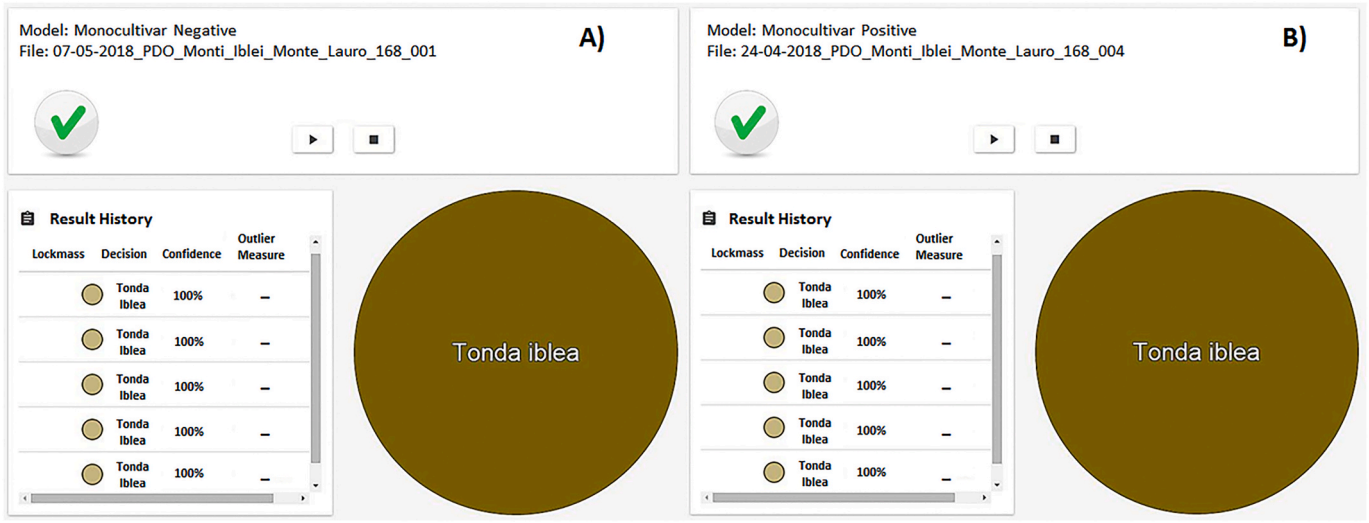


**Fig. 6.** Playback recognition by the LiveID software of a monocultivar Tonda Iblea EVOO through the PCA-LDA models built for monocultivar EVOOs (as in Fig. 5) in **A**) negative ionization mode and **B**) positive ionization mode.

## CRediT authorship contribution statement

**Domenica Mangraviti:** Methodology, Investigation, Writing - original draft. **Francesca Rigano:** Conceptualization, Validation, Supervision, Writing - review & editing. **Adriana Arigò:** Visualization. **Paola Dugo:** Supervision, Visualization. **Luigi Mondello:** Conceptualization, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.110715.

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