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Title: Effect of O2 control and monitoring on the nutraceutical properties of Extra Virgin Olive Oils

Article Type: Review Article

Keywords: Extra-Virgin Olive Oil; Malaxation process; Volatile compounds; Phenols; Nutraceutical properties.

Corresponding Author: Prof. Pietro Catania, Ph. D.

Corresponding Author's Institution: University of Palermo

First Author: Pietro Catania

Order of Authors: Pietro Catania; Mariangela Vallone; Aboud Farid; Claudio De Pasquale

Abstract: The presence of oxygen during malaxation has key role in improving Extra Virgin Olive Oil (EVOO) quality in terms of volatile and phenolic compounds. The objective of this study was to evaluate the influence of the head space malaxer oxygen concentration on the physicochemical characteristics and nutraceutical properties of EVOO from the Nocellara del Belice olives. The acidic compounds were related to the concentration of the oxygen presented in the machine headspace considering a new software application for the online oxygen management opportunely designed and applicable to all the existing plants. The right timepoint and oxygen concentration of the malaxation process was established. The best results were obtained when 30 L of oxygen were blown after 25 minutes from the beginning of the malaxation process. The application of the novel software in the Oxygen Control Monitoring (OCM) system showed very interesting results in terms of nutraceutical properties.

Cover Letter

Dear Editor,

in response to your invitation, I send you our manuscript entitled “A new electronic tool to improve nutraceutical properties of Extra Virgin Olive Oils” after a careful revision following the reviewers’ comments (reported in the “Detailed responses to reviewers” file), using red letters for the deleted words and green for the new ones. The new title is “Effect of O_2 control and monitoring on the nutraceutical properties of Extra Virgin Olive Oils”.

Best regards,

Pietro Catania

***Detailed Response to Reviewers**

Reviewer 1

Reviewer 1: *"I would consider though that the authors remove figures 2 and 3 from the text and change figure 4 from screen view mode to a O2 concentration versus time plot."*

We do not agree to eliminate Figures 2 and 3 because they show the OCM system managed by the software application developed by the authors. We agree to delete Fig. 4, where the trend of O_2 concentration in the headspace of the malaxer is represented, since this information is in the subsequent figure. Lines 159-160 were consequently deleted.

Reviewer 2

1. *The title reads "A new electronic tool to improve nutraceutical properties ..." I think the main contribution is in the characterization of the oxygen effects, rather than in the development of a electronic tool to monitor and induce step-like oxygen concentration. The electronic tool is more or less standard in nowadays process laboratories. I suggest changing the title to reflect more clearly the nature of the contribution. It could be read like "Effect of oxygen concentration in the nutraceutical properties...."*

   The title was changed in: "Effect of O_2 control and monitoring on the nutraceutical properties of Extra Virgin Olive Oils".

2. *The electronic tool performs only prescribed or open-loop control of oxygen concentration. Oxygen is injected at some specific times and subsequently allowed to deplete by the effect of malaxation. It would be interesting to test the effects of prescribed oxygen concentration profiles (e.g., ramps) in the properties of the olive oil. Maybe, closed-loop (i.e., feedback-based) control strategies can be used to obtain maximal effects. Please, comment this point.*

   We are in agreement with the suggestions provided by the reviewer. In lines 105-107 the objective of this research is clearly described, in agreement with the reviewer remarks. The control strategies (closed-loop) will be implemented in the next years using the data obtained by the authors in this and in previous studies.

3. *The conclusions should be self-contained, so references should be removed.*

   The references in the Conclusions section were deleted.

Reviewer 3

1. *In manuscript title authors claim for a "new electronic tool"; in highlights authors claim that they propose a "novel software". However, the new electronic tool and novel software is an oxygen sensor with data acquisition software which exits at least 20 years ago (LabView software).*

   The title was changed as suggested by the reviewer 2, too. The LabView software exists for several years but to date its application to the management of data measured by an oxygen sensor applied to a malaxer for Extra Virgin Olive Oil extraction has never been performed.

2. *In highlights authors claim the manuscript is the "first study in which the acidic components were related to the concentration of the oxygen present in the malaxation machine headspace". However the fact that oxygen in the process is crucial for EVOO final quality it is well known. Even oxygen monitoring and control system during malaxation is reported (Aiello et al. 2012). Therefore, the correlation between headspace oxygen concentration and product is obvious from Henry´s law.*

   In the olive oil campaign 2010/2011 the authors developed a "Real time continuous oxygen concentration monitoring system during malaxation for the production of Virgin Olive Oil" which consisted in the detection of O_2 concentration in the malaxer headspace, evaluating free fatty acids content, peroxide value, spectrophotometric indexes and total phenols (Aiello et al., 2012). Subsequently the study was extended to the volatile and phenolic component of EVOO (Catania et al., 2013). In this study a software application for the O_2 management in the malaxer headspace was implemented evaluating for the first time EVOO quality also in terms of fatty acid composition.
   Henry's law concerns the relations of the state of the gases as a function of pressure and temperature. Furthermore, Henry's law takes into account the liquid state of matter; by definition, the olive paste is a suspension in which two phases coexist: liquid and solid. Our study is based on the survey of the concentrations of saturated and unsaturated fatty acids at different times of inerting of the malaxation machine. Therefore we decided not to consider the O_2 dissolved in the two-phase system of the olive paste. Furthermore, the concentration of oxygen in the olive paste inside the malaxation machine is not directly linked with that present in the final product (EVOO) because O_2 active some enzymatic reactions in favor of the volatile and acidic component of EVOO improving its quality.

3. *However if the present manuscript would be reorganized and presented as the effect of oxygen concentration in malaxation machine headspace on EVOO quality, with a proper statistical design (like the Table 3) and with a proper statistical validation of results (not only Fig. 7 PCA, instead with MANOVA and regression analysis of proper models for to relate oxygen concentration with EVOO quality), the manuscript could be considered for publication. The data acquisition software is only the tool for this purpose.*

   Lines 99-102. The authors defined the effect of O_2 concentration in the malaxer headspace on the EVOO nutraceutical properties, as the main objective of this study. Moreover, lines 105-107 "The main goal was to establish the right timepoint and oxygen concentration of the malaxation process to obtain the higher nutraceutical properties in EVOO" were replaced with "The main goal was to establish the right timepoint for oxygen concentration variation in the malaxation process to obtain the higher nutraceutical properties in EVOO."

   Table 3 shows the data of the different tests surrogated by standard deviations for n = 3. The choice to apply PCA comes from the option to identify composite EVOO quality indicators, or common factors, able to explain the response in terms of volatile compounds by reducing the data set of variables on PC2. Therefore the sentence in lines 266-267: "Principal component analysis (PCA) were applied to investigate the relationships between the considered experimental variable and the amount of phenol and volatile compounds", was changed with "Principal component analyses (PCA) were applied in order to reduce the variable data setting and extract composite EVOO quality indicators by using phenols and volatile compound concentration obtained in each case of study".

Reviewer 4

1. *p6L90: authors may provide a table to sum up all bibliographic studies*

   The references have been included in the text following the guidelines provided by the Journal.

2. *p6L94: stated BY*

   "by" was added in line 94.

3. *p7L106: please explain why you think of a timepoint instead of O2 profile for instance*

   Line106: the sentence "The main goal was to establish the right timepoint and oxygen concentration of the malaxation process to obtain the higher nutraceutical properties in EVOO" (lines 105-107) has been changed in: "The main goal was to establish the right timepoint for oxygen concentration variation in the malaxation process to obtain the higher nutraceutical properties in EVOO."

   We refer to the timepoint because the O_2 introduction takes place at a specific point in the process that marks the end of inerting from which we monitor the profile of O_2.

4. *p7L118: what is the amount of water remaining outside the olives after washing ? It may be of importance since it can further create some emulsion.*

   The amount of water that remains on the olives after washing is negligible and can not form emulsion.

5. *p7L118: a schematic showing time duration+temperature+[O2] at all stages would be useful*

   It is not appropriate to include a scheme with the temperature trend since it was kept constant (27 ° C) for the entire duration of malaxation (line 119).

6. *p7L124: give also info on depth of product (pasta) to help consider the O2 diffusion*

   The following sentence was added: "The depth of the olive pasta inside the malaxation machine was 0.70 m".

7. *P7L130: you don't give any info on dissolved oxygen in product nor on O2 incorporation through valves*

   The aim of this study was to evaluate the influence of O_2 concentration in the headspace of the malaxer in an industrial scale plant on the nutraceutical components of the final product. The machine was equipped with double-effect valves. They do not allow any exchange with the outside except that the internal pressure exceeds the atmospheric pressure. This condition has never occurred during the tests.

8. *P8L149: what sort of O2 sensor are you talking about? Please give all specs, geometry, location, etc. I don't think it is a dissolved O2 measurement. Please discuss the effect of the pumping loop which is changing the temperature hence the O2 value.*

   Both the O_2 sensor and the pumping system are located outside the malaxer, in a thermostatic chamber in which the pump sucks from the headspace samples of atmosphere that are sent to the sensor which measures the amount of oxygen. It was widely described by the authors in Aiello et al., 2012. This reference was added in line 149, too.

9. *P9L169: how can you be sure that you have the "same degree of ripeness" ? What kind of measures are you refeering to ?*

   The following sentence was added in line 169: “The maturity index of the olives was determined by applying the “Jaen index”, varying from 0 to 7, according to skin colour (Tombesi, 1996). Harvest time was determined by examining a sample of 100 olives and dividing it into eight classes on the basis of epicarp pigmentation. The processed olives had a maturity index value of 1.90.”

10. *P13L174: You should show first 3 replicate experiments (different dates) to discuss the *true* uncertainties on measures hence on conclusions*

    The reviewer’s comment is not clear.

11. *P15: I don't see anywhere a calculation and a discussion on how many grams of O2 are necessary to oxidize all phenolic and volatile compounds... with a comparison with was is given in headspace. I don't see also any discussion on reaction rates (and the temperature effect on it) or O2 diffusion rate.*

    The O_2 needed for oxidizing all the phenolic and volatile compounds was not quantitatively discussed as the O_2 diffusion in the olive paste was not taken into account to determine nutraceutical parameters such as those highlighted in the manuscript. See also the response to reviewer 3, point 2.

*Highlights (for review)

- We propose a ~~novel~~ new software application, made by the authors, aimed at monitoring and controlling oxygen inside the malaxation machine in extra virgin olive oil production, applicable to all the existing plants.

- This paper represents the first study in which the acidic components were related to the concentration of the oxygen present in the malaxation machine headspace.

- The application of the ~~novel~~ new software application in the OCM (Oxygen Control Monitoring) system during malaxation allows to obtain very interesting results in terms of nutraceutical properties.

- The amount of 30 L of oxygen ~~blowing~~ introduced after 25 minutes from the beginning of the malaxation process showed the best results.

~~A new electronic tool to improve nutraceutical properties of Extra Virgin Olive Oils~~

Effect of O_2 control and monitoring on the nutraceutical properties of Extra Virgin Olive Oils

Catania Pietro*, Vallone Mariangela, Farid Aboud, De Pasquale Claudio

Department of Agricultural and Forest Sciences, University of Palermo,V.le delle Scienze Edificio 4, 90128 Palermo, Italy

Corresponding author: pietro.catania@unipa.it

Abstract

The presence of oxygen during malaxation has key role in improving Extra Virgin Olive Oil (EVOO) quality in terms of volatile and phenolic compounds. The objective of this study was to evaluate the influence of the head space malaxer oxygen concentration on the physicochemical characteristics and nutraceutical properties of EVOO from the Nocellara del Belice olives. The acidic compounds were related to the concentration of the oxygen presented in the machine headspace considering a ~~novel~~ new software application for the online oxygen management opportunely designed and applicable to all the existing plants. The right timepoint and oxygen concentration of the malaxation process was established. The best results were obtained when 30 L of oxygen were blown after 25 minutes from the beginning of the malaxation process. The application of the novel software in the Oxygen Control Monitoring (OCM) system showed very interesting results in terms of nutraceutical properties.

Key words: Extra-Virgin Olive Oil; Malaxation process; Volatile compounds; Phenols; Nutraceutical properties.

1. Introduction

Extra Virgin Olive Oil (EVOO) is a food of fundamental importance in the Mediterranean diet, which provides about 85% of the fat content of this diet (Pérez-Jimenez et al., 2007). Recent studies have confirmed that EVOO interacts on the prevention of the metabolic syndrome as hypercolesterolemia, hypertriglyceridemia, high blood pressure, obesity, fatty liver and insulin resistance, all closely linked to diabetes and coronary heart disease (Esposito et al., 2010, Pérez-Martinenz et al., 2011). It is generally considered to be a major contributor to human health in the Mediterranea area (Carluccio et al., 2007; Covas 2008; Covas et al., 2009).

Fatty acid composition, where oleic acid is the main component, and other components with great biological potential, including vitamin E, carotenes, squalene, chlorophyll and, in particular, a number of phenolic compounds are well-balanced and these have been related to the beneficial properties (Owen et al., 2000). It has been confirmed that some chemical components of EVOO, polyphenols and oleic acid, are inhibitors of the vascular endothelial growth factor signaling pathway (Lamy et al., 2014). These results underscore the chemopreventive properties of EVOO and highlight the importance of nutrition in cancer prevention.

All these compounds may be present in very variable quantity in EVOO. This is due to many factors such as variety, cultivation area, agricultural techniques adopted, degree of maturation and the type of olive oil extraction system (Angerosa et al., 2004; Baccouri et al., 2008; Chiaccherini et al., 2007; Gòmez-Rico et al., 2008; Inglese et al., 2011).

Considering the olive oil extraction procedures, many studies have been conducted in recent years on oil mill plant and processes for improving EVOO quality (Altieri 2010; Altieri et al., 2013; Amirante et al., 2006; Catalano et al., 2003; Clodoveo et al., 2013a; Clodoveo et al., 2013b; Leone et al., 2013; Leone et al., 2014b; Tamborrino et al., 2014a;). Malaxer is the most studied machine

among all, it is responsible for malaxation, which represents a very important and critical step in the EVOO extraction process (Selvaggini et al., 2014).

During malaxation, some important physical phenomena occur, the breaking of oil-water emulsion and coalescence of oil drops, the migration of the olives components in oil or aqueous phase, and the enzymatic transformations involving phenolic compounds and triglycerides (Migliorini et al., 2006). The processing conditions allowing a selective control of the enzymes is a crucial point of the oil mechanical extraction process strictly related to the sensory and healthy quality (Angerosa et al., 2001). The definition of restrictive parameter is a major step in the EVOO production. Recent studies on the malaxation process have targeted the optimization of temperature and oxygen concentration (Selvaggini et al., 2014), the influence of time and olive ripening stage (Jiménez B. et al., 2014), the right combination of temperature and time condition (Reboredo-Rodrìguez P. et al., 2014; Inarejos-Garcìa A.M. et al., 2009; Gòmez-Rico et al., 2009), the influence of time (Youssef et al., 2013; Chih et al., 2013; Ranalli et al., 2003), and temperature only (Parenti et al., 2008), the design of a new machine (Tamborrino et al., 2014b), the oxygen monitoring and control system (Aiello et al., 2012).

The main parameters studied concerning the malaxation process are temperature, time and oxygen in the headspace of the machine. Time and temperature have been exhaustively studied, while oxygen, defined as the third important process parameter, needs further investigation as recently stated by many authors (Leone et al., 2014a; Catania et al., 2013a; Selvaggini et al., 2014; Jimènez et al., 2014; Servili et al., 2008).

Hence, oxygen process monitoring and control are fundamental requirements in the modern EVOO processing industry.

Many studies focus on the control of the oxygen in the headspace of the malaxer, aim to determine its influence on EVOO quality considering the volatile and phenolic components. Considering these factors, the main objective of the present study was to evaluate the influence of the head space malaxer oxygen concentration on the physicochemical characteristics and nutraceutical properties of EVOO from the Nocellara del Belice olives. To the best of our knowledge this is the

first study in which the acidic components were related to the concentration of the oxygen present in the machine headspace considering a novel software for the online oxygen management opportunely designed and applicable to all the existing plants. The main goal was to establish the right timepoint for ~~and~~ oxygen concentration variation ~~of~~ in the malaxation process to obtain the higher nutraceutical properties in EVOO.

2. Materials and Methods

2.1 Olives and oil mill plant

The study was performed on typical Sicilian olive cultivar "Nocellara del Belice" in 2013 (Catania et al., 2014), manually harvested and processed within 24 hours from harvesting using an Alfa Laval oil mill plant. The oil mill plant was equipped with an olive washing machine, a disk crusher, a single-stage malaxation machine, an horizontal decanter, and a vertical centrifuge. It was operated in continuous mode.

After washing, olives were processed with a disk crusher, then the malaxation was performed in a close system for 45 minutes (Di Giovacchino et al., 2002, Servili et al., 1994) at a temperature of 27 °C (Angerosa et al., 2001 and 2004; Servili et al., 2003 and 2009).

The extraction was performed by using a triphasic centrifugal extractor without adding water. The malaxation machine used in the tests was the Alfa Laval Atmosphera 650 with a capacity of 650 L, featuring a stainless steel and airtight cylinder. Its headspace was equal to 15 % of the volume of the chamber, and the olive paste-air contact surface was 0.5 m^2. The depth of the olive pasta inside the malaxation machine was 0.70 m. The machine was equipped with a pair of inlet valves for gas, to achieve a controlled or modified malaxation atmosphere by blowing nitrogen or oxygen in the headspace, and a probe for olive paste temperature control. Also, it had a gap over the entire inner surface of the tank where hot water was circulated to control olive paste temperature. A rotary double bladed reel with spiral inside the machine realized the olive paste mixing and removed it

106 from the walls avoiding overheating. Paste loading and unloading operations were carried out by
107 means of automatic valves. The oil yield was about 20 % in all the experiments.
108 Oil samples were collected after each test, put in 100 mL dark glass bottles, stored at 12 °C and
109 transported to the laboratory where analyses were performed.
110
111 **2.2 Oxygen Control and Monitoring (OCM) system**
112
113 The software developed by the authors allows the acquisition and recording of the oxygen
114 concentration in the headspace of the malaxation machine. The application is run on the Windows
115 operating system and allows the definition of various programming input of gas (O_2 and N_2) inside
116 the machine.
117 The percentage of oxygen values inside the malaxation head space are shown in real time after they
118 have been acquired by the sensors of the OCM system. The dissolved oxygen measurements in the
119 malaxation camera are performed every 30 seconds. The data collected during monitoring are also
120 saved to file. The interface provides a screen for the electro valves manual management to allow
121 different application.
122 The oxygen concentration inside the malaxation machine is sampled by means of a gas extraction
123 system that continuously circulates. This was sampled through a closed loop pipe where the oxygen
124 sensor is located. Thus, the oxygen monitoring circuit consists of a pipeline, a gas pump, a filter
125 and an oxygen sensor (Fig. 1) (Aiello et. al., 2012).
126 The OCM software is provided with three screens; the first one (Fig. 2) allows to manage the
127 beginning of the process (Tin), to monitor the oxygen concentration in the malaxer headspace at the
128 beginning of the process (Tin-T0), the point at which nitrogen is inserted inside the machine (T0)
129 and its time of blowing (T0-T1), the malaxation time in total absence of oxygen (T1-T2), the time
130 in which oxygen is introduced in the malaxer headspace (T2-T3), the time-point when the release
131 of oxygen is interrupted (T3) and the oxygen monitoring until the end of the process.

The second screen allows to manage the electro valves in order to insert oxygen and nitrogen inside the machine (Fig. 3). The time of gas introduction depends on the diameter of the pipe and the free volume in the malaxer headspace.

~~Finally, the third software screen allows to monitor continuously the oxygen concentration in the headspace of the malaxer (Fig. 4).~~

2.3 Experimental trials

This work was designed in order to evaluate the malaxation process in industrial field with four experimental setups. The atmosphere inside the malaxation machine was modified by blowing nitrogen or oxygen (pure gases) using cylinders in the mixing chamber at specific stages of the process. Concerning the experimental conditions, except those during the T_C processes (control), the malaxation camera atmosphere was made inert by filling N_2 before the olive paste was putted in. The drupes were completely healthy and had the same degree of ripeness. The maturity index of the olives was determined by applying the "Jaen index", varying from 0 to 7, according to skin colour (Tombesi, 1996). Harvest time was determined by examining a sample of 100 olives and dividing it into eight classes on the basis of epicarp pigmentation. The processed olives had a maturity index value of 1.90. The considered variable applied in the different case of studies was the atmosphere composition in the malaxation chamber headspace, which was altered by blowing N_2 and/or O_2 at different times during the process. The experimental design is fully described with the acronyms in Table 1.

Test T_C was conducted without changing the gaseous component in the headspace of the machine. Tests $T_{5\text{-}15}$, $T_{5\text{-}25}$ and $T_{5\text{-}35}$ were carried out by blowing 5 L of O_2 at different timepoints of the process from the beginning after 15, 25 and 35 minutes respectively. Tests $T_{30\text{-}15}$, $T_{30\text{-}25}$ and $T_{30\text{-}35}$ were carried out by blowing 30 L of O_2 at the same time as described above. Nitrogen was introduced immediately after filling and before the start of mixing, thus eliminating the low amount of O_2 present in the head space of the malaxation chamber. This was done to evaluate the sole

effect of O_2 insufflations at different times of malaxation on EVOO phenolic, volatile and fatty acids compounds.

The filling of the malaxation machine lasted for 10 min. Each test configuration was replicated three times. Oil samples were collected immediately after each test and stored in 0.1 L dark glass bottles at 10 °C during transport to the laboratory.

2.4 Chemical Analytical Determinations in EVOO

2.4.1 Phenolic compounds

The olive oil phenolic composition was analyzed by HPLC-DAD. Namely, a chromatograph equipped with a Shimadzu LC-10ADVP pump, a DAD Shimadzu a SCL-10AVP system controller, a 8125 Rheodyne manual injector with a 20 µL loop and a 5-µm particle size C18 Luna column, 15 cm , 2 mm i.d., (Phenomenex, UK) was used.

Phenolic standard compounds were purchased from Sigma Aldrich (Milano, Italy). All solvents (methanol, acetonitrile, and *n*-hexane) were of HPLC grade and purchased from Fluka. Formic acid was purchased from Sigma Chemical Co. (St. Louis, MO). The ultrapure water generated by the MilliQ system (Millipore, Bedford, MA), and MilliQ water/methanol (90/10 v:v) as the most suitable solvent for the standards were used. The samples were stored in dark-brown glass bottles at 4 °C until analysis. The oil was extracted from high-quality olives and met the standards set by the European Commission (Commission Regulation (EEC) n 2568/91 of July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis, 1991) for extra-virgin quality. To clean and concentrate the sample, the polar fraction was obtained from 3 g of oil sample using an SPE diol cartridge (Vac RC 500 mg, Waters, Milford, MA). 6 mL of *n*-hexane, 6 mL of methanol: water (80:20), and 3 mL of acetonitrile was used to achieve the activation of stationary phase. The oil was washed with 10 mL of *n*-hexane under vacuum to remove the nonpolar fraction. Afterward, phenolic compounds were eluted with 8 mL of methanol:

186 water and 4 mL of acetonitrile. The vacuum was maintained at less than 30 kPa. The eluent was
187 evaporated to 2 mL under a gentle N_2 flow. A 13-mm PTFE 0.45 *u*m membrane filter, purchase
188 from Waters, were used to filter the sample. 20 µL was injected into the liquid chromatography.
189 The entire process was performed in darkness conditions with brown glass material.
190 The HPLC-DAD column was kept at ambient temperature. The mobile phase consisted of a binary
191 solvent system using water acidified with 0.1% formic acid (solvent A) and 100% acetonitrile
192 (solvent B), kept at a flow rate of 0.5 mL min^{-1}. The gradient program started with 90 % eluent A
193 and 10 % eluent B, which ramped linearly to 25 % in 12 min. This percentage was maintained for 7
194 min, and eluent B was ramped again linearly to 40 % at 30 min and to 60 % at 40 min. Each
195 phenolic compound was expressed with its standard and the linearity of the calibration method was
196 fortificated by triplicate analyses.
197
198 *2.4.2 Volatile Organic Compounds (VOCs)*
199
200 The volatile fraction was isolated and identified by using HS-SPME and GC-MS. An HP 5890 GC-
201 MS equipped with the mass selective detector HP 5973 was used in order to identify the target
202 compounds. An HP5-MS, 5% diphenyl-95% dimethylpolysiloxane, capillary column (30 m 0.2
203 mm, 0.25 mm film thickness) was used as stationary phase. Chromatographic conditions were
204 splitless injection, using He as carrier gas at 10^{-3} L min^{-1}. The injector temperature was 250 °C.
205 Oven temperature program: 8 min of 60 °C isotherm followed by a linear temperature increase of 4
206 °C min^{-1} up to 180 °C held for 2 min. MS scan conditions: source temperature 230 °C, interface
207 temperature 280 °C, E energy 70 eV, mass scan range 39–350 amu.
208 Linear retention indices were calculated with references to *n*-alkanes (C6–C22), obtained from
209 FLUKA, and run under the chromatographic condition described above. Standard mixtures of
210 selected essential oils were also injected in the GC inlet and retention indices determined. Response
211 factors of reference compounds from different classes of monoterpenes, sequiterpenes,

monoterpene alcohols and aldehydes, ester were determined and found to range from 0.85 to 1.2 versus *n* hexanol, averaging 1.0. Response factors were therefore taken as 1.0 for all compounds. Experiments of singular components standard addition have been carried out in order to evaluate matrix interference. Identification of the oil components was done using a commercial library (NIST 2005) and an FFC (flavor and fragrance components) bank provided with linear retention indices determined on the same column, to be used interactively with MS data for compound identification. All analyses were carried out in triplicate and the total content of the single compounds was calculated as percent of total chromatographic area (Mondello et al., 1995).

2.4.3. Fatty Acid Methyl Esters *(FAME)*

Fatty acids in olive oil samples (100 mL) were directly methylated with 2 mL of 0.5 M $NaOCH_3$ at 30 °C for 15 min, followed by 1 mL of 5 % HCl in methanol at 50 °C for 15 min. Fatty acid methyl esters (FAME) were recovered in hexane (1.5 mL). One microliter of each sample was injected by autosampler into an HP 6890 gas chromatography system equipped with a flame-ionization detector (Agilent Technologies Inc., Santa Clara, CA). Fatty acid methyl esters from all samples were separated using a 100 m length, 0.25 mm i.d., 0.25 μm capillary column (CP-Sil 88; Chrompack, Middelburg, the Netherlands). The injector and the detector temperature were kept at 255 °C and 250 °C respectively, with an H2 flow of 40 mL min-1, air flow of 400 mL min-1, and a constant He flow of 45 mL min-1. The initial oven temperature was held at 70 °C for 1 min, increased at 5 °C min^{-1} to 100 °C, held for 2 min, increased at 10 °C min^{-1} to 175 °C, held for 40 min, and then finally increased at 5 °C min^{-1} to a final temperature of 225 °C and held for 45 min. Helium, with a head pressure of 158.6 kPa and a flow rate of 0.7 mL min^{-1} (linear velocity of 14 cm s^{-1}), was used as the carrier gas. Fatty acid methyl ester standard solution in hexane mix solution was used to identify each FA. To quantify total FA, C23:0 (Sigma-Aldrich) was added to each sample (4 mg g^{-1} of oil) as the internal standard. The health-promoting index (HPI) was calculated

as the ratio between saturated (SFA), unsaturated (UFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid and related to the O_2 application and concentration.

2.5 Statistical analysis

Chemical analyses of EVOO were performed on three EVOO samples for each case of studies within one week from extraction. The data were subjected to the Student's *t* test for mean comparison at the 95% confidence level (Statgraphics Centurion, Statpoint Inc., USA, 2005). ~~Principal component analysis (PCA) were applied to investigate the relationships between the considered experimental variable and the amount of phenol and volatile compounds~~. Principal component analyses (PCA) were applied in order to reduce the variable data setting and extract composite EVOO quality indicators by using phenols and volatile compound concentration obtained in each case of study. The amount of each compound was considered as the dependent variable of the considered experimental parameters. The obtained principal components were considered as significant if their Eigen values were >1. All the statistical analyses were carried out using Statistica 6.0 for Windows (Stat Soft Italia).

3. Results and Discussion

Following Tc (control), where malaxation was performed without any gas addition, the initial oxygen concentration was 20 %; then, at the end of the process (45 minutes) this concentration decreased to about 14%. Worthy of note is that in the last 10 minutes (approximately 22 % of the total operating time), malaxation was performed with a constant oxygen concentration around 14 %, without further decreases (Fig. ~~5~~ 4).

When 5 L of oxygen were blown ($T_{5\text{-}15}$, $T_{5\text{-}25}$ and $T_{5\text{-}35}$) the oxygen concentration never exceeds 10 %. After that, oxygen concentration never decreases below 5 %.

When 30 L of oxygen were blown ($T_{30\text{-}15}$, $T_{30\text{-}25}$ and $T_{30\text{-}35}$) there was a sudden increase of oxygen up to values of approximately 20 %. After a few minutes, the amount of oxygen begins to decrease, always remaining above 15 %.

Phenolic compounds are the secoiridoid derivatives such as Hydroxytyrosol (3,4 DHPEA), p-HPEA, 3,4-DHPEA-EDA, p-HPEA-EDA and 3,4-DHPEA-EA and the lignan derivatives (+)-1-acetoxypinoresinol and (+)-pinoresinol, as well as the sum of all the phenolic fractions should be considered in order to investigate the influence of procedure parameter. Literature evidence as these compounds are natural antioxidants which provide to olive oil the oxidation resistance and, consequently, its duration over time (Owen et al., 2000; Servili et al., 2003 and 2009). Moreover, the importance of the healthy properties of these compounds in the prevention of cardiovascular disease (Covas, 2009) and some cancers (Garcia-Villalba et al., 2012) have found evidence. The phenolic compounds define the quality of VOOs as they represent the impact compounds that define the “pungent” and "bitter" sensory notes (Servili et al., 2009). The average concentration of secoiridoids and lignans in VOO is highly variable and depends both on the agronomic characteristics and on the extraction processes (Servili et al., 2008).

The oxygen blowing inside the malaxation machine headspace produced a low values in the sum of the phenolic fractions. The reduction was lower when 5 L of oxygen were introduced. These results are in agreement with those obtained by other authors (Migliorini et al., 2006; Parenti et al., 2007; Servili et al., 2008; Catania et al., 2013a).

These data apparently confirmed the considerable oxidative degradation, both chemical and enzymatic, of the compounds with an ortho-diphenolic structure during malaxation in the presence of oxygen due to LOX activity and PPO activity (Ranalli et al., 2003; Toscano et al., 2003). Lignans (pinoresinol and acetoxypinoresinol) did not decrease, and appeared to have a marginal role in the oxidation resistance of EVOO (Servili et al., 2003).

Therefore the oxygen blowing and the time-point showed a significant values over the phenolic composition of EVOO. The PCA (Fig. ~~6~~5) reduced the number of total variables to only few retaining the major part of the information on the systems variability. The amount of variables was

290 reduced to only two (PC1, PC2 and PC3) which retained 75.5 % of the total variance (Fig. ~~6~~5). The
291 olive oil was produced with lower amount of blow oxygen (T5-15, T5-25 and T5- 35) retained
292 positive score on PC1 and was positively related to the presence of p-HPEA, 3,4-DHPEA-EDA, p-
293 HPEA-EDA. The other ($T_{30\text{-}15}$, $T_{30\text{-}25}$ and $T_{30\text{-}35}$) were placed directly in the opposite position
294 respect to the PC1 and was more represented to the variation of lignan derivatives.
295 Volatile compounds such as saturated and unsaturated aldehydes and alcohols were correlated
296 with the "cut grass" and "fruity" oil sensory notes (Servili et al., 2003; Olias et al., 1993), and they
297 were originated during the mechanical extraction process of VOO from the LPO pathway
298 (Angerosa et al., 2004).
299 The quantity of the volatile compounds was increased in the tests were oxygen was introduced
300 during malaxation compared to the control. The highest values were obtained where 30 L of
301 oxygen were used in tests $T_{30\text{-}25}$ and $T_{30\text{-}35}$ (Table 3). This could be attributed to the presence of
302 oxygen in the final part of malaxation that would promote the polyphenols oxidation processes and
303 the activation of the endogenous enzyme complex in the lipoxygenase pathway acting cascade, that
304 is, one on products of derivation of the previous. This leads to the to the formation of C5 and C6
305 saturated and unsaturated aldehydes and alcohols, and esters. The addition of a low amount of
306 oxygen (5 L) did not cause any statistically significant differences between the control and the
307 other tests. The PCA (Fig. ~~7~~6) reduced the number of total variables to only two retaining the major
308 part of the information on the systems variability. The amount of variables was reduced to only two
309 (PC1, PC2) which retained 72.99 % of the total variance (Fig. ~~7~~6). On the base of oxygen
310 concentration the investigated concentration was appeared to be separated on the of PC2.
311 Following the VOC concentration of EVOO, the oxygen inside malaxation camera was positively
312 related to the 2-Esenale (E). Conversely the lower oxygen concentration was positively associated
313 to alcohols concentration.
314 Fatty acids have a fundamental role from the nutraceutical point of view as anti-cancer and
315 cholesterol-lowering. They stimulate the immune system and prevent the onset of diabetes and
316 chronic non-communicable diseases (Perez-Jimenez et al., 2007). EVOO is mainly composed of

317 triglycerides (98-99%) (Conte et al., 2010). The most abundant fatty acids in EVOO are, palmitic
318 stearic acids (9-14 %), monounsaturated fatty acids, palmitoleic and oleic acid (66-80 %),
319 polyunsaturated fatty acids, linoleic and linolenic acid (6-10 %) (Aguilera et al., 2005). High
320 concentration of fatty acids in the human blood reduces efficiency and number of membrane
321 receptors which are responsible for recognizing the specific proteins of low-density lipoprotein
322 (LDL). LDL have the function of carrying near 50 % of blood-cholesterol (Viola and Viola, 2014).
323 Furthermore an exceed of polyunsaturated fatty acids in the human organism start peroxidative
324 processes with production of free radicals which oxidize LDL via chain reactions. Therefore,
325 saturated, monounsaturated and polyunsaturated fatty acids play important structural and functional
326 roles in the human organism (Visioli and Galli, 2002).
327 The Oxygen content in the malaxer headspace in different time-points and it concentrations during
328 the process influences the EVOO fatty acids composition. When 30 L of oxygen were insufflated
329 SFA, i.e. palmitic and stearic acids were significantly decreased compared to the control. The
330 reduction of SFA gives to the EVOO a higher fluidity and digestibility. A major reduction about
331 28 % was recorded in $T_{30\text{-}25}$, where UFA and MUFA were increased to 5 and 7 % respectively.
332 PUFA was decreased about 12 % respect to the control. Moreover, the Oleic acid reached the
333 highest values in $T_{30\text{-}25}$ and $T_{30\text{-}35}$. That was a fundamental standpoint and a high oleic acid content
334 improved EVOO oxidation resistance that making it more stable during the storage period (Youssef
335 et al., 2010). This aspect compensate the decreasing of polyphenols. Therefore, the same values
336 were found with the addition of oxygen during 25 or 35 minutes. Youssef et al. (2013) studied the
337 effect of malaxation time on fatty acid composition, announcing that the highest oleic acid values
338 are obtained in malaxation time of 15 min (67.57) and 30 min (67.89). Longer malaxation time,
339 equal to 45 and 60 min, causes a decrease of oleic acid content with values of 65.05 and 65.20
340 respectively. Comparing these values with those obtained in our study, although of different olive
341 varieties, it could be confirmed that oxygen monitoring in the malaxation machine headspace
342 allow to obtain higher oleic acid values 72.49 and 71.24 in $T_{30\text{-}25}$ and $T_{30\text{-}35}$ respectively with
343 malaxation time of 45 min. However, the absence of oxygen at the beginning of malaxation

344 improves the EVOO oleic acid content. Therefore, the OCM application allows to prolong the
345 malaxation time without compromising the EVOO oleic acid.
346 A higher health-promoting index (HPI) (Bonanno et al., 2013),was obtained in test T_{30-25} (Fig. ~~6~~7).
347 The HPI were calculated as the ratio of UFA/SFA and MUFA/SFA (Tur et al., 2005) and it is
348 recognizable as a diet quality Index adapted to the one of most important Mediterranean dietary
349 food stuff. When the amount of oxygen is 5 L no significant statistical difference occurs between
350 the inertize samples and the control.
351

352 **4. Conclusions**

353 This study confirms that oxygen plays a decisive role during malaxation. It influences both
354 phenolic and volatile compounds in EVOO ~~(Catania et al. 2013a, Selvaggini et al. 2014, Jimènez et~~
355 ~~al. 2014, Leone et al. 2014a, Servili et al. 2008)~~. However, there was a lack on its influence on fatty
356 acid composition. It has been demonstrated a significant influence of oxygen on fatty acid
357 composition through the implementation of a new electric tool which improve an electronic
358 management and control of the oxygen in the head space of the malaxer. The software allowed to
359 control the atmosphere inside the malaxer camera with particular reference both to the amount and
360 to the time-point in which oxygen is blown. The oxygen monitoring and control in the malaxer
361 headspace increase the concentration of polyphenols, volatile compound and fatty acid content and
362 the nutraceutical value of Nocellara del Belice EVOO. The best results were obtained when 30 L of
363 oxygen are blown after 25 minutes from the beginning of malaxer procedure. The application of the
364 novel software in the OCM system during malaxation allow to obtain very interesting results in
365 terms of nutraceutical properties.
366

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Tables and Figures

Table 1. Case of studies and acronyms.

Test	Description
T_C (control)	Malaxation in un-modified atmosphere
$T_{5\text{-}15}$	5 L of oxygen introduced 15 min after malaxation start
$T_{5\text{-}25}$	5 L of oxygen introduced 25 min after malaxation start
$T_{5\text{-}35}$	5 L of oxygen introduced 35 min after malaxation start
$T_{30\text{-}15}$	30 L of oxygen introduced 15 min after malaxation start
$T_{30\text{-}25}$	30 L of oxygen introduced 25 min after malaxation start
$T_{30\text{-}35}$	30 L of oxygen introduced 35 min after malaxation start

Table 2. EVOO phenolic composition (mg kg^{-1}) in the different tests. 3,4 dihydroxyphenolethanol (3,4-DHPEA); tyrosol (p-HPEA); dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA); dialdehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EDA); oleuropein aglycon (3,4-DHPEA-EA). Data are means ± st. dev.(n = 3)

	Tc	T5-15	T5-25	T5-35	T30-15	T30-25	T30-35
3,4-DHPEA	4.1 ± 0.04	4.6 ± 0.1	4.4 ± 0.1	3.9 ± 0.1	5.7 ± 0.2	5.4 ± 0.04	6.0 ± 0.04
p -HPEA	3.2 ± 0.1	6.6 ± 0.1	6.3 ± 0.1	6.0 ± 0.1	5.0 ± 0.0	3.9 ± 0.03	7.3 ± 0.02
3,4-DHPEA-EDA	266.9 ± 2.0	191.0 ± 0.7	238.5 ± 2.2	253.6 ± 3.1	164.2 ± 23.5	231.9 ± 3.3	245.3 ± 0.1
p-HPEA-EDA	40.7 ± 0.9	38.8 ± 0.4	40.5 ± 0.6	40.8 ± 0.2	40.1 ± 0.1	30.5 ± 0.4	35.4 ± 0.1
(+)-1-acetoxypinoresinol	19.8 ± 0.1	18.6 ± 0.1	19.7 ± 0.2	20.5 ± 0.9	18.6 ± 0.4	19.1 ± 0.3	20.9 ± 0.2
(+)-pinoresinol	29.8 ± 0.2	29.4 ± 0.1	28.7 ± 0.04	29.2 ± 0.1	30.5 ± 0.1	30.5 ± 0.5	29.9 ± 0.1
3,4-DHPEA-EA	77.1 ± 2.9	64.2 ± 1.1	70.9 ± 0.6	76.2 ± 4.8	56.5 ± 1.7	60.1 ± 2.3	63.9 ± 0.8
∑ phenols fractions	441.5 ± 3.7	353.1 ± 1.4	409.1 ± 2.3	430.1 ± 5.8	320.5 ± 23.6	381.4 ± 4.1	408.6 ± 0.9

Table 3. EVOO volatile composition (μg kg^{-1}) in the different cases of study. Data are means ± st. dev.(n = 3).

	Tc	T5-15	T5-25	T5-35	T30-15	T30-25	T30-35
1-Penten-3-one	466 ± 8	630 ± 4	192 ± 4	154 ± 17	526 ± 74	553 ± 35	359 ± 45
Hexanal	306 ± 11	299 ± 1	327 ± 6	479 ± 20	478 ± 19	447 ± 16	547 ± 42
Pentenal-2-(E)	191 ± 5	215 ± 9	115 ± 10	113 ± 12	194 ± 2	204 ± 11	186 ± 13
1-Penten-3-ol	393 ± 9	382 ± 36	313 ± 25	297 ± 37	408 ± 6	364 ± 16	329 ± 8
Hexanaldehyde-2-(E)	9635 ± 119	10635 ± 92	9798 ± 82	11285 ± 78	26008 ± 1230	14950 ± 113	16144 ± 919
2,4-Hexadienal, (E,E)	1393 ± 19	1410 ± 26	1137 ± 16	1331 ± 31	1668 ± 82	1686 ± 58	1603 ± 5
1-Pentanol	38 ± 0	13 ± 1	73 ± 1	60 ± 5	8 ± 3	10 ± 1	35 ± 1
2-Penten-1-ol, (E)-	48 ± 1	53 ± 1	51 ± 7	32 ± 1	49 ± 1	51 ± 4	45 ± 4
1-Hexanol	716 ± 6	172 ± 4	900 ± 6	1785 ± 50	153 ± 5	174 ± 11	1280 ± 101
3-Hexen-1-ol, (E)	17 ± 1	6 ± 1	23 ± 2	26 ± 0	3 ± 0	5 ± 1	19 ± 1
3-Hexen-1-ol, (Z)	3385 ± 27	1108 ± 17	4878 ± 168	5669 ± 60	844 ± 58	1054 ± 72	4134 ± 317
2-Hexen-1-ol, (E)	219 ± 1	76 ± 4	2302 ± 95	649 ± 19	155 ± 16	120 ± 29	725 ± 53
1-Heptanol	13 ± 4	5 ± 6	14 ± 1	19 ± 0	9 ± 1	4 ± 6	19 ± 1
1-Octanol	49 ± 2	49 ± 4	64 ± 3	56 ± 3	56 ± 4	36 ± 3	57 ± 6
2-Heptanol (E)	118 ± 10	134 ± 11	145 ± 4	147 ± 11	172 ± 7	143 ± 1	154 ± 5
Benzyl alcohol	12 ± 0	12 ± 0	12 ± 1	16 ± 1	9 ± 1	14 ± 1	19 ± 2
Phenethyl alcohol	76 ± 1	65 ± 1	96 ± 11	106 ± 0	91 ± 2	101 ± 7	102 ± 9
Phenol	16 ± 2	15 ± 1	16 ± 1	17 ± 3	23 ± 2	17 ± 1	17 ± 2
Ethyl acetate	49 ± 1	55 ± 2	55 ± 1	62 ± 1	59 ± 5	59 ± 1	52 ± 5
3-hexenyl acetate (Z)	267 ± 5	332 ± 7	349 ± 5	384 ± 7	348 ± 23	375 ± 4	292 ± 24

Table 4. Fatty acids composition SFA (saturated fatty acid), UFA (unsaturated fatty acid), MUFA (monounsaturated fatty acid), PUFA (polyunsaturated fatty acid), OA (oleic acid). Data are the mean values of three independent experiments ± standard deviation. Values in each row having different letters are significantly different from one another at $p < 0.05$.

	Tc	T30-15	T30-25	T30-35
SFA	18,64 ± 0,27 bc	17,17 ± 0,30 d	15,34 ± 0,09 f	16,53 ± 0,36 e
UFA	79,05 ± 0,36 c	80,35 ± 0,20 b	82,93 ± 0,19 a	81,09 ± 0,30 b
MUFA	70,55 ± 0,26 d	72,23 ± 0,11 c	75,47 ± 0,20 a	73,31 ± 0,12 b
PUFA	8,50 ± 0,10 b	8,12 ± 0,10 bc	7,46 ± 0,08 d	7,78 ± 0,25 cd
OA	67,91 ± 0,06 bc	69,12 ± 0,10 b	72,49 ± 0,43 a	71,24 ± 0,25 a

	Tc	T5-15	T5-25	T5-35
SFA	18,64 ± 0,27 bc	19,22 ± 0,04 ab	19,37 ± 0,11 a	18,49 ± 0,24 c
UFA	79,05 ± 0,36 c	77,52 ± 0,18 d	78,19 ± 0,59 cd	78,70 ± 0,22 c
MUFA	70,55 ± 0,26 d	68,28 ± 0,32 e	69,99 ± 0,52 d	70,30 ± 0,32 d
PUFA	8,50 ± 0,10 b	9,23 ± 0,15 a	8,20 ± 0,10 b	8,40 ± 0,10 b
OA	67,91 ± 0,06 bc	65,98 ± 0,53 d	66,89 ± 0,80 cd	67,06 ± 0,56 cd

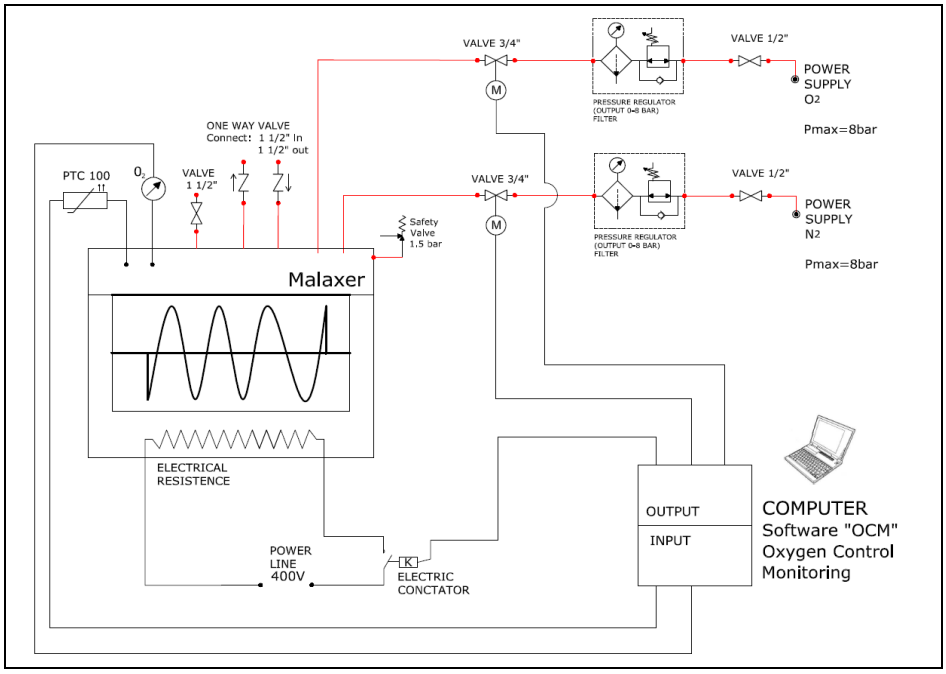


Fig. 1. Software included in the Oxygen Control and Monitoring (OCM) system.

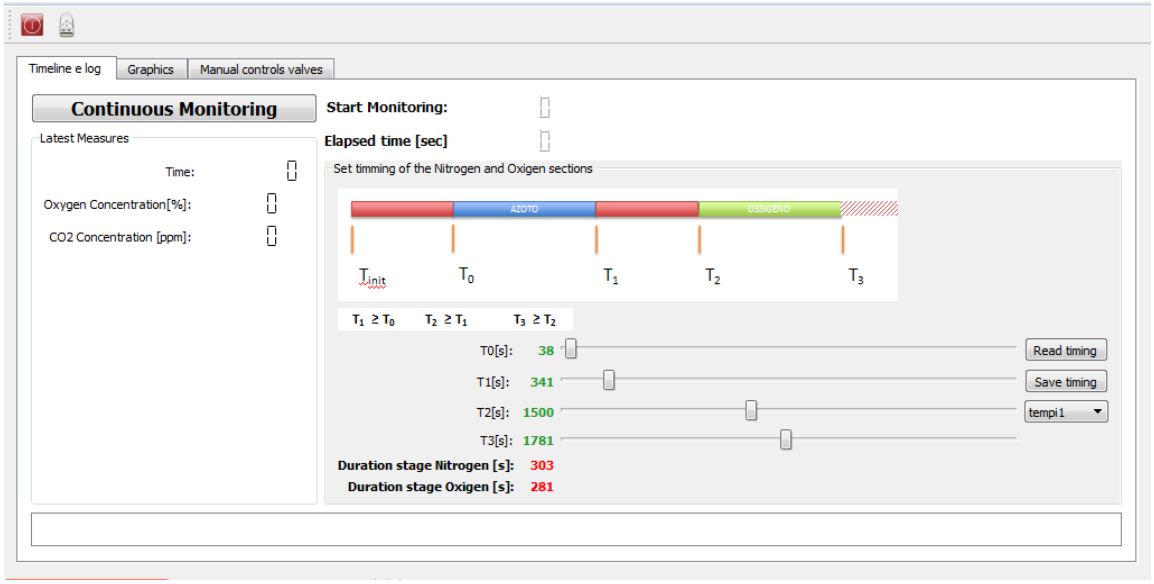


Fig. 2. Software screen to continuously control oxygen and nitrogen in the malaxer headspace.

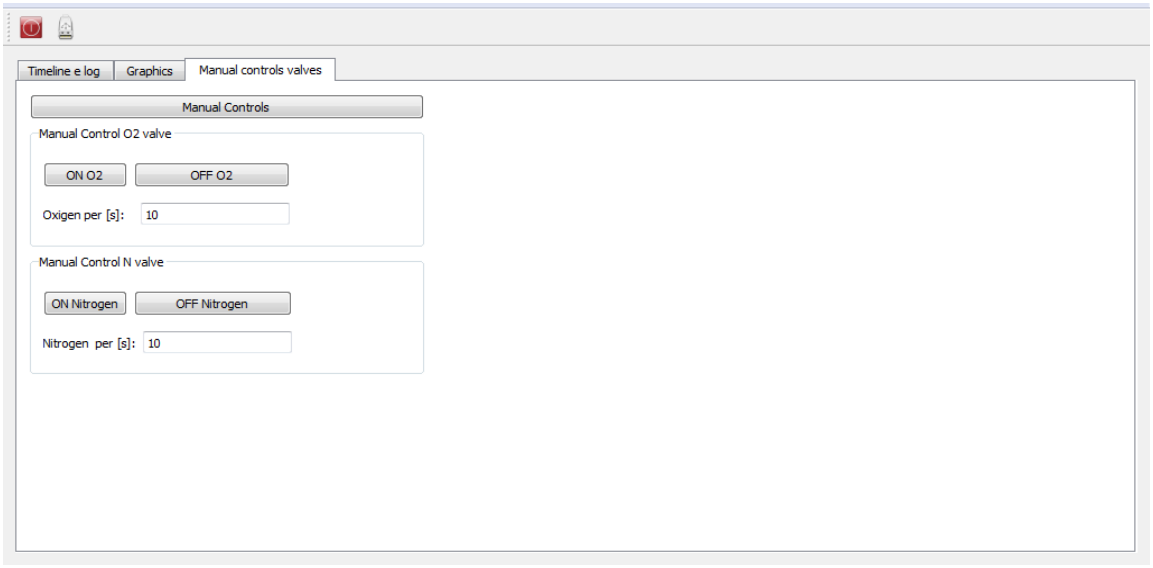
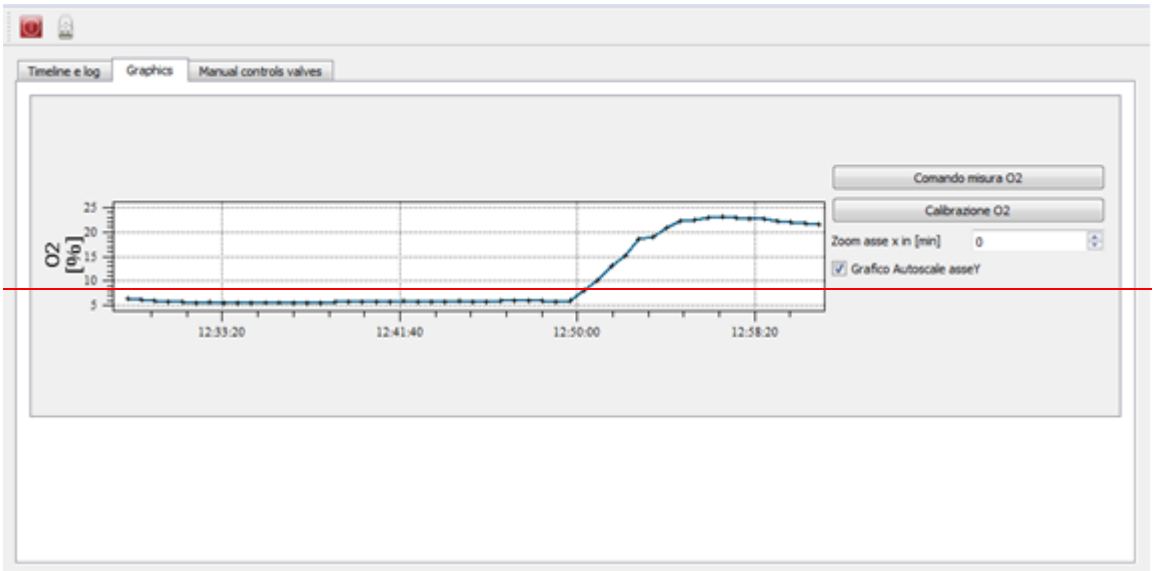
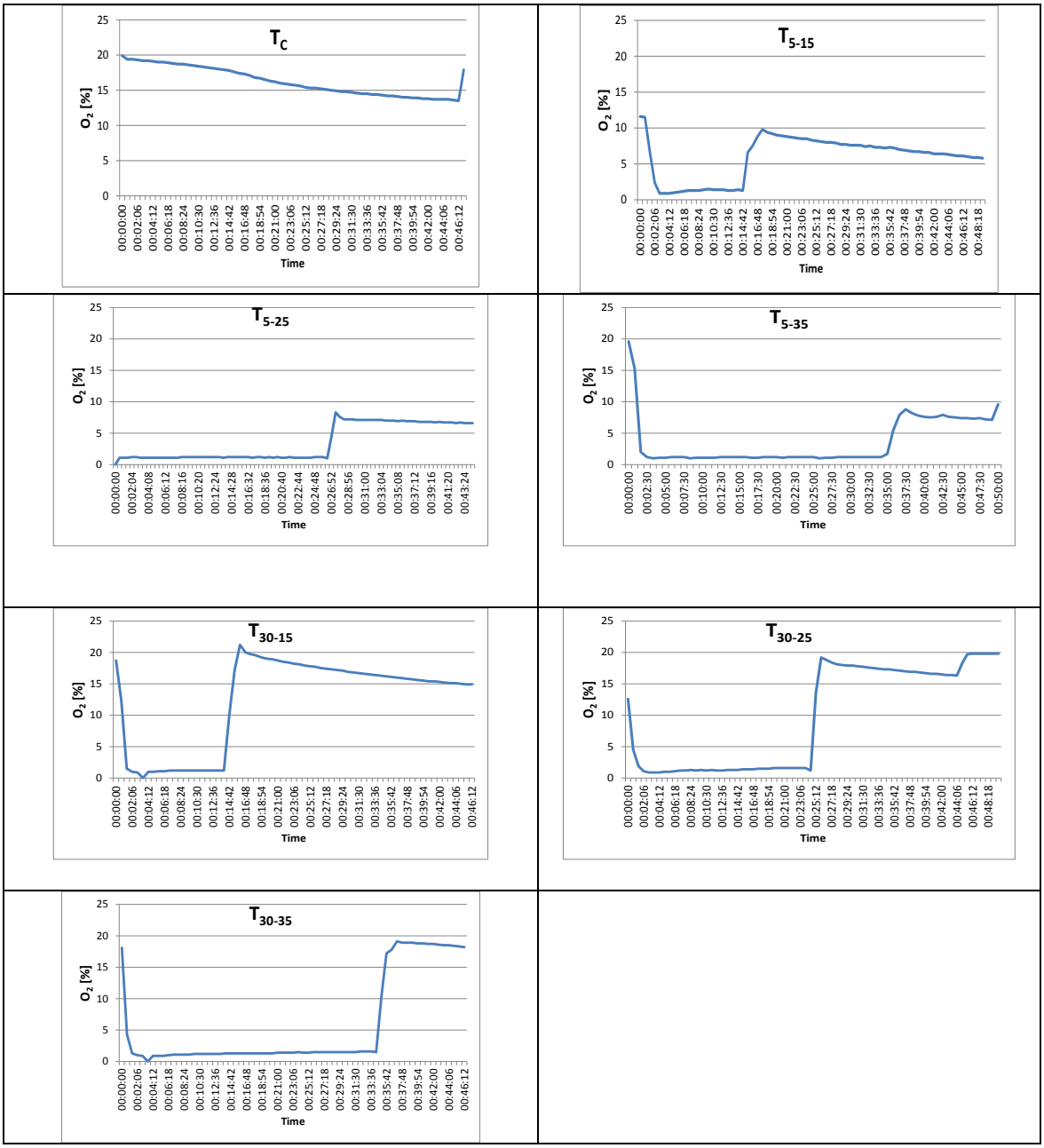


Fig. 3. Software screen for electro valves management to automatically insert oxygen and nitrogen in the malaxation machine headspace.



~~**Fig. 4.** Software screen allowing the on line monitoring of the oxygen concentration in the headspace of the malaxer.~~



574 **Fig. ~~5~~ 4.** Evolution of the oxygen concentration (T_C = Malaxation in un-modified atmosphere; T_{5-15}= 5 L of oxygen introduced 15 minutes after malaxation start; T_{5-25}= 5 L of oxygen introduced 25 minutes after malaxation start; T_{5-35}= 5 L of oxygen introduced 35 minutes after malaxation start; T_{30-15}= 30 L of oxygen introduced 15 minutes after malaxation start; T_{30-25}= 30 L of oxygen introduced 25 minutes after malaxation start; T_{30-35}= 30 L of oxygen introduced 35 minutes after malaxation start inside the malaxation machine during olive paste processing in the experimental tests. Each point of the lines is the mean value of three replicates.

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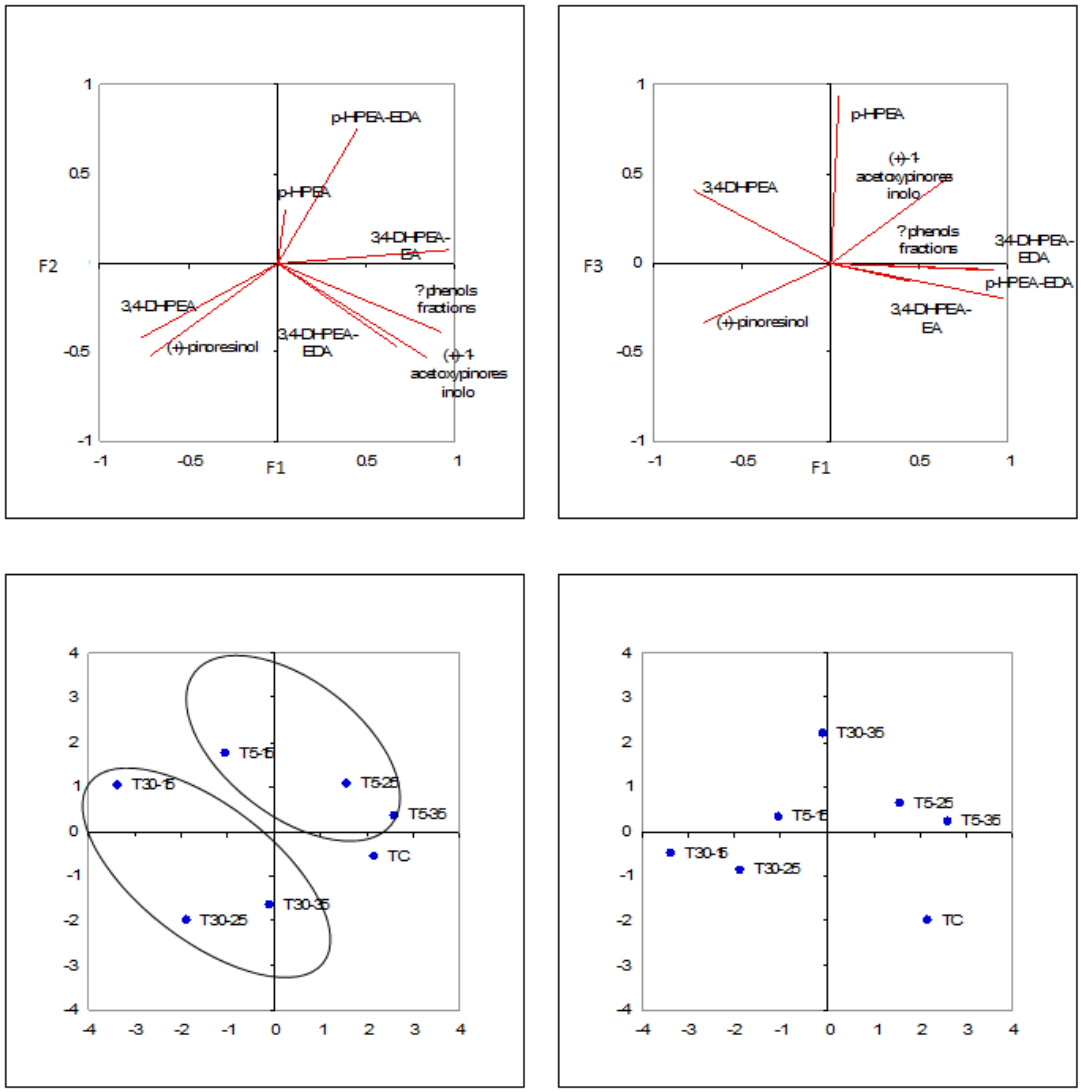
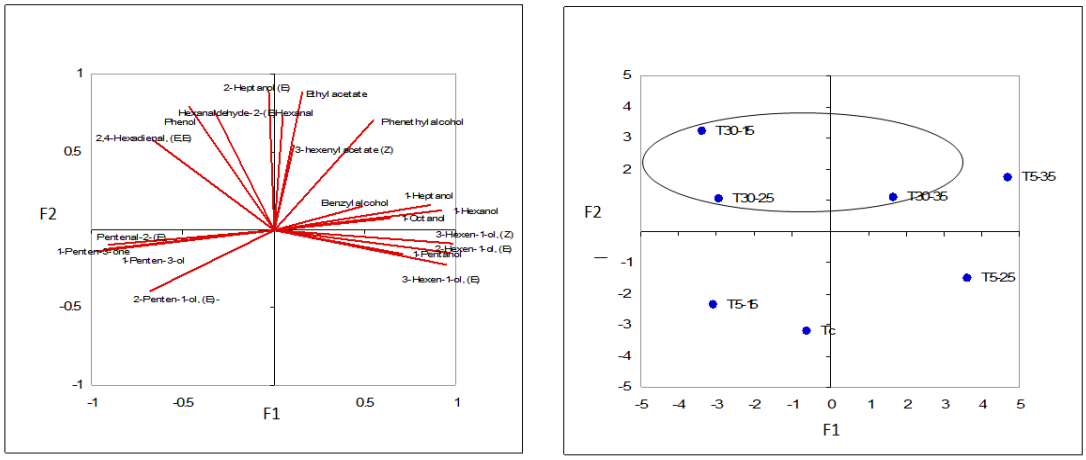


582

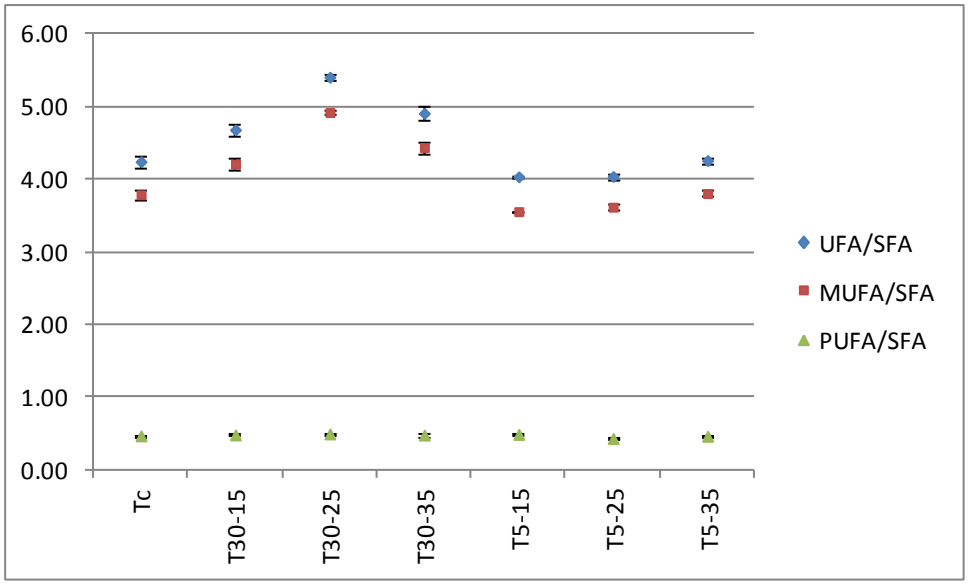
583 **Fig. ~~8~~.5.** Principal component analyses of phenolic compounds



584

585 **Fig. ~~7~~.6.** Principal component analysis Volatile organic compounds (VOCs)

586



587
588 **Fig. ~~6~~.7.**.HPI index in EVOOs from the different tests.