PAPER

INVESTIGATION ON MICROBIOLOGY OF OLIVE OIL EXTRACTION PROCESS

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ABSTRACT

Several batches of approx. 200 kg olives from *Frantoio* and *Moraiolo* cultivars were processed in an oil mill at two dates of harvesting. Samples were collected in several steps of extraction process for sensory, chemical and microbial analyses.

All extracted olive oil from the second olive harvesting date was affected by sensory defects and hence classified as being "non-extra virgin". A distinction between extra virgin olive oil and non-extra virgin olive oil obtained from both harvesting dates was explained by the volatile compounds content of olive oil samples and by yeast and mould counts collected at different processing steps.

- Keywords: moulds, sensory defects, virgin olive oil, volatile compounds, yeasts -

INTRODUCTION

The absence of sensory defects is necessary for olive oil to be marketed as "extra virgin" in the EU.

Extra virgin olive oil (EVOO) is characterized by pleasant sensory notes. They are mainly originated by aldehydes, esters, alcohols and ketones, which are responsible for oil sensory attributes such as "green" and "fruity" (APARICIO and MORALES, 1998; MORALES *et al.*, 2005, BENDINI *et al.*, 2012). However, several phenomena can alter the initial pleasant flavour, giving rise to unpleasant sensory notes.

The current olive oil regulations (EU Reg. 1348/2013) classify the most frequent sensory defects into four groups as follows: "fusty", "musty", "winey–vinegary", and "rancid".

Storage of olive fruits in piles before being processed is a cause of sensory alterations in EVOO. Olive transpiration during storage is known to increase pile temperature, enabling microbial cells to grow and to affect the chemical composition of olives (MORALES *et al.*, 2005). Both biogenesis of volatile compounds and transformation phenomena of phenolic compounds can be significantly influenced by microbial contamination of olives. Effects of olive microbiota on oil characteristics are considered even greater than time-temperature conditions of malaxation (VICHI *et al.*, 2011).

Oil quality may be affected by microorganisms, according to their metabolic activities. During olive crushing, microorganisms might migrate into oil through both solid particles of olive fruit and micro-drops of vegetation water (CIAFARDINI and ZULLO, 2002). Some microorganisms do not survive a long time, but others may persist and become a typical microbiota of olive oil. For example, yeasts may remain metabolically active during olive oil storage and thus modify olive oil characteristics (ZULLO *et al.*, 2010).

Enzymatic activities of yeasts and moulds isolated from either olives or EVOO have been reported to include β-glucosidase, β-glucanase, polyphenoloxidases, peroxidase and, in some cases, lipase and cellulase activities (CIAFARDINI and ZULLO, 2002; CIAFARDINI *et al.*, 2006; ZULLO and CIAFARDINI, 2008; ROMO-SANCHEZ *et al.*, 2010). Enzymes such as β-glucosidase are known to improve oil quality by increasing phenolic compound extractability, while others such as lipase, polyphenoloxidases and peroxidase are known to cause detrimental effects (PALOMARES *et al.*, 2003; ROMO-SANCHEZ *et al.*, 2010; VICHI *et al.*, 2011; MIGLIORINI *et al.*, 2012). *Penicillium* and *Fusarium* spp. isolates have been shown to produce amounts of exogenous lipoxygenase (FAKAS *et al.*, 2010) that, together with endogenous lipoxygenase, is the key enzyme of LOX pathway (ANGEROSA *et al.*, 2004).

Extraction process control should include monitoring activities on microbial contamination in olives and EVOO, as associated with sensory and chemical analyses. The study of the microbial populations occurring at different steps of EVOO extraction process, as well as their role in affecting oil characteristics, appears to be increasingly useful.

The aim of this work was to investigate both microbial ecology throughout olive oil processing and a possible relationship between EVOO volatile compound content and microbial contamination.

MATERIALS AND METHODS

Experimental design

During 2011 crop season, several batches of approx. 200 kg olives from *Frantoio* and *Moraiolo* cultivars were processed in an oil mill (Azienda Agricola Buonamici, Fiesole, Florence, Italy).

Plant for oil extraction (TEM, Florence, Italy) consisted of a cleaning and water washing system, an olive grinding cutter crusher (mod. FR350), a controlled-temperature vertical axis malaxation equipment (500 kg capacity) (mod. V500), a "decanter" (two-step mod. D1500) with 1500 kg/h maximum capacity and a cardboard filter press (15 μm cut-off). Plastic residue or "alperujo" from decanter was subjected to separation by centrifugation of stone fragments to obtain destoned pomace (Fig. 1).

Olives were processed within 12 h from harvest at two dates (HD): November 16, 2011 (HD1) and November 23, 2011 (HD2). Oil extraction trials were carried out in quadruple.

Olives were crushed at 2,500 rpm (crusher holes 6.5 mm in diameter); malaxation was carried out at half capacity under vacuum (residual pressure of 20 kPa) at 22 ± 1°C for a mean time of 15 min to work under low oxidative stress impact conditions; decanter worked with a screw conveyor rotating at a slower speed than that of the bowl.

Samples were collected in several steps of extraction process for sensory, chemical and microbial analyses, as shown in Fig. 1.

Chemical analyses

Olives

A homogeneous olive sample was crushed with a laboratory crusher, and resulting olive paste was used for chemical analyses.

The water content (g kg^{-1} of dry matter) was measured on olive paste by gravimetric method (CHERUBINI *et al.*, 2009).

The total sugar content was determined by the UNI 22608 method, modified as described in a previous study (CHERUBINI *et al.*, 2009). Results for sugar content obtained from the equipment (Compact Titrator, Crison, Modena, Italy) were expressed as g kg^{-1} of dry matter.

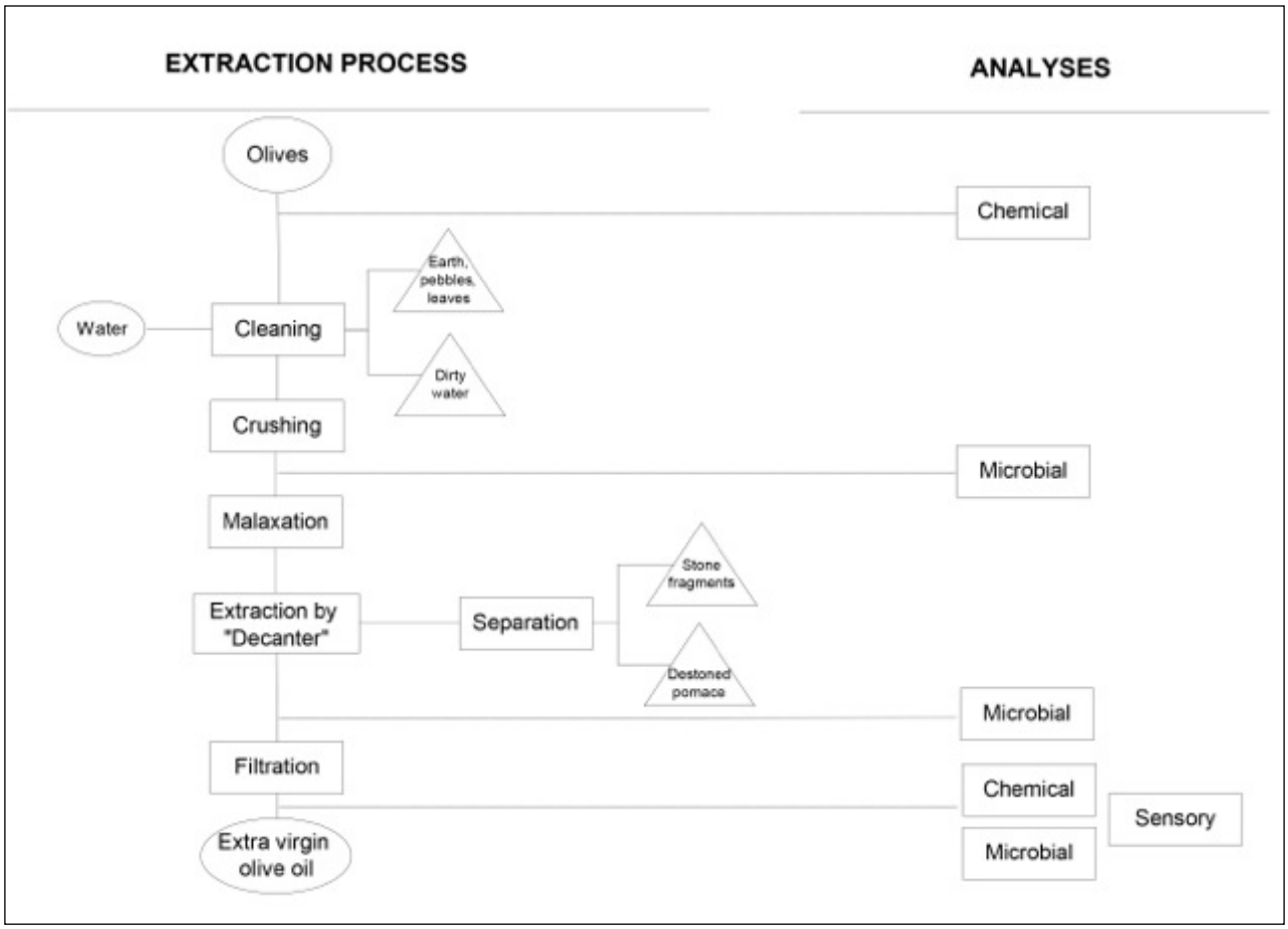

Fig. 1 - Overview of extraction process and analyses carried out.

The total oil content was determined with hexane in an automatic Randall extractor (mod. 148, Velp Scientifica, Milan, Italy), following the analytical technique described in a previous study (CHERUBINI *et al.*, 2009). Results were expressed as g kg^{-1} of dry matter.

The total phenolic compounds content was determined by weighing 4 g crushed olives and adding 80 mL Methanol:Water (60:40) solution; two series of stirring for 30 min and centrifugation at 4,000 rpm for 15 min were performed, and the supernatant was collected. The phenolic extract was adjusted to volume of 200 mL by Methanol:Water (60:40) and placed in the freezer for 2 h. After thawing, the phenolic extract was filtered. One mL filtered extract, 5 mL Folin Ciocalteu reagent, and 20 mL sodium carbonate were placed into a 100 mL flask and adjusted to volume with distilled water. Sixty minutes were waited for colour development; after one hour, UV reading (UV/VIS, Varian model Cary 1E, The Netherlands) was performed at 765 nm wavelength. The total phenolic compounds content was expressed as mg kg^{-1} of dry matter on a calibration curve.

Olive oil

Acidity (% oleic acid), peroxide value (meq O_2 kg^{-1}) and spectroscopic indices were measured according to EU official method (EC Reg. 1989/2003).

Extraction, identification and determination of phenolic compounds were performed in agreement with IOC Official Method (IOC, 2009) by an HPLC equipment consisting of a Hewlett Packard 1200 diode-array detector system and a Hewlett Packard model 1200 autosampler (Agilent Technologies, Santa Clara, California, USA). Secoiridoids, lignans, flavonoids and phenolic acids were quantified in $mg_{tyrosol}kg_{oil}^{-1}$. The total phenolic compounds content ($mg_{tyrosol}kg_{oil}^{-1}$) was determined using the sum of the peak areas of phenols recorded at 280 nm.

The tocopherol content was determined according to ISO 9936:2006 (ISO, 2006) using a Hewlett Packard mod. 1050 liquid chromatograph with quaternary pump and fluorescence detector, provided with Hewlett Packard mod.1100 autosampler (Agilent Technologies, Santa Clara, California, USA). Quantitative analysis was carried out using the external standard method. Results were expressed as mg of total tocopherols per kg of oil.

The volatile compound content was determined according to the literature (VICHI *et al.*, 2003), using HS-SPME-GC-MS technique (solid phase microextraction of the head space, cou-

pled with a gas chromatograph with a mass spectrometer as a detector). Analysis was performed using the Trace CG instrument combined with a Trace DSQ Thermo Finnigan instrument (Fisher Scientific SAS, Illkirch, France). Quantitative analysis was performed using 4-methyl-2-pentanol as an internal standard. Results were expressed as mg of volatile compound per kg of oil.

Sensory analyses

Sensory evaluation of olive oil was performed by a panel test according to the EU official method (EU Reg. 1348/2013). Samples were analyzed by a panel of professional tasters (8 tasters and a panel leader) of CCIAA (Chamber of Commerce, Industry, Handcraft and Agriculture) of Florence. The panel has been recognized by MIPAAF (Ministry of Agricultural Policies, Food and Forestry) since 2002. Intensity of both sensory defects and "fruity", "bitter" and "pungent" attributes was assessed and expressed as the median of tasters score on a scale range from 0 to 10.

Microbiological analysis

Paste and oil samples from each batch were sterilely withdrawn and then transported to the laboratory under refrigerated conditions (4°C). Ten g of olive paste or 10 mL of unfiltered oil were transferred into 90 mL of sterile saline and homogenized for 10 min with a Stomacher Lab Blender 400 (Seward Ltd, Worthing, West Sussex, UK) and a magnetic stirrer, respectively. After decimal dilutions, 100 µL suspension was plated on specific growth media for cell enumeration in triplicate using the spread plating technique. Yeasts were counted on MYPG agar (ZULLO and CIAFARDINI, 2008) integrated with ampicillin and sodium propionate in order to inhibit growth of bacteria and moulds, respectively (ROMO-SANCHEZ, 2010). The plates were incubated at 30°C for 48-72 h. Moulds were counted on MYPG agar without inhibitors (KAWAI *et al.*, 1994) after incubation at 30°C for 24-48 h. Finally, total mesophilic microorganisms were counted on Plate Count Agar (Oxoid Ltd, Basingstoke, Hampshire, UK) after incubation at 30°C for 48 h.

Filtered oil samples (100 mL) were microfiltered through nitrocellulose filters with a porosity of 0.45 µm (Minisart NML-Sartorius, Göttingen, Germany), which was able to retain yeasts and moulds. Then the nitrocellulose filters containing the microorganisms were washed with 10 mL saline and placed onto the specific media described above.

Data processing

Chemical, sensory and microbiological determinations were processed according to one-way ANOVA followed by Tukey's test (significance level: $p = 0.05$).

Principal Component Analysis (PCA) was used to classify samples by Statistica 7.0 software package (Stasoft GmbH, Hamburg, Germany). Correlation studies between microbial cell density and the volatile compounds content of oil samples were carried out by calculating both Pearson and Spearman rank correlation coefficients (significance level: $\alpha = 0.05$).

RESULTS

Characteristics of olive and olive oil samples

Chemical characteristics of processed olives are given in Table 1. They show a slight increase in olive ripening level between the two dates of harvesting. As reported in the literature (RYAN *et al.*, 2002; SERVILI *et al.*, 2004; CHERUBINI *et al.*, 2009), a significant decrease in phenolic compounds content occurred, and a decrease in sugar content, even if significant only for *Frantoio* cultivar, was also observed. No significant variations were measured in both water and olive oil contents during the harvesting interval.

Sensory and chemical characteristics of extracted olive oil are given in Table 2, while their volatile compounds content is reported in Table 3. Samples are encoded in relation to olive cultivar, harvesting date and batch.

Table 2 shows that all olive oil samples extracted from olives of the first harvesting date were extra virgin. They had much lower values

Table 1 - Olive characteristics on two harvesting dates (HD1 and HD2). SD: standard deviation; different letters in the same row indicate significant differences ($p < 0.05$) for the same cultivar; dm: dry matter.

	Frantoio Cultivar				*Moraiolo* Cultivar			
	HD1		HD2		HD1		HD2	
	Mean value	SD	Mean value	SD	Mean value	SD	Mean value	SD
Phenolic Compounds (mg/kg dm)	33000 [a]	2285	26000 [b]	1811	37000 [a]	2579	30000 [b]	2097
Sugar Content (g/kg dm)	75 [a]	5	54 [b]	4	77 [a]	5	69 [a]	5
Water Content (g/kg)	391 [b]	20	437 [a]	22	411 [a]	21	430 [a]	22
Oil Content (g/kg dm)	440 [a]	31	460 [a]	32	500 [a]	35	450 [a]	31

Table 2 - Chemical and sensory characteristics of extracted olive oil.

		Frantoio cultivar								*Moraiolo* cultivar							
		HD1				HD2				HD1				HD2			
	Batch code [1]	F1a	F1b	F1c	Mean value ± SD	F2a	F2b	F2c	F2d	Mean value ± SD	M1a	M1b	M1c	M1d	Mean value ± SD	M2a	M2b	M2c	M2d	Mean value ± SD
EU legal characteristics	Free acidity (% oleic acid)	0.20	0.20	0.22	**0.21^{a}**±0.01	0.19	0.19	0.23	0.24	**0.21^{a}**±0.03	0.20	0.22	0.22	0.22	**0.22^{b}**±0.01	0.25	0.25	0.27	0.27	**0.26^{b}**±0.01
	Peroxide value (meq O_2/kg oil)	3.6	3.6	3.5	**3.8^{b}**±0.1	6.2	4.9	5.3	4.6	**5.2^{a}**±0.7	3.9	4.0	4.3	4.6	**4.2^{b}**±0.3	5.3	4.5	5.2	4.9	**5.0^{a}**±0.4
	K_{232}	1.76	1.71	1.74	**1.74^{a}**±0.03	1.77	1.80	1.87	1.85	**1.82^{a}**±0.05	1.80	1.74	1.82	1.80	**1.79^{a}**±0.03	1.79	1.86	1.79	1.83	**1.82^{a}**±0.03
	K_{270}	0.15	0.15	0.16	**0.15^{b}**±0.01	0.19	0.19	0.23	0.24	**0.21^{a}**±0.03	0.17	0.16	0.17	0.17	**0.17^{b}**±0.01	0.15	0.17	0.17	0.17	**0.17^{b}**±0.01
	ΔK	0.00	0.00	0.00	**0.00^{a}**±0.00	0.00	0.00	0.00	0.00	**0.00^{a}**±0.00	0.00	0.00	0.00	0.00	**0.00^{a}**±0.00	0.00	0.00	0.00	0.00	**0.00^{a}**±0.00
	P.A.: Fruity* (0-10)	3.5	3.4	4.1	**3.7^{a}**±0.4	n.d.	2.9	3.3	4.0	**3.4^{a}**±0.6	3.8	3.9	4.4	3.7	**4.0^{a}**±0.3	2.9	3.2	3.4	4.1	**3.4^{a}**±0.5
	P.A.: Bitter* (0-10)	2.6	3.6	3.6	**3.3^{a}**±0.6	n.d	3.6	3.3	5.1	**4.0^{a}**±1.0	2.6	3.6	4.6	3.7	**3.6^{a}**±0.8	3.8	3.5	3.5	3.5	**3.6^{a}**±0.1
	P.A.: Pungent* (0-10)	3.7	4.5	4.9	**4.4^{a}**±0.6	4.1	4.1	4.2	5.0	**4.4^{a}**±0.4	4.0	4.2	4.7	4.6	**4.4^{a}**±0.3	4.9	5.8	5.6	5.6	**5.5^{a}**±0.4
	N.A.: Rancid* (0-10)	0.0	0.0	0.0	**0.0^{b}**±0.0	0.0	1.9	1.6	1.6	**1.3^{a}**±0.9	0.0	0.0	0.0	0.0	**0.0^{b}**±0	1.2	1.4	1.4	1.1	**1.3^{a}**±0.2
	N.A.: Fusty* (0-10)	0.0	0.0	0.0	**0.0^{b}**±0.0	2.1	1.6	1.3	1.0	**1.5^{a}**±0.5	0.0	0.0	0.0	0.0	**0.0^{b}**±0	1.1	1.2	0.8	1.0	**1.0^{a}**±0.2
	N.A.: Winey-Vinegary* (0-10)	0.0	0.0	0.0	**0.0^{b}**±0.0	1.7	1.7	0.6	1.0	**1.3^{a}**±0.5	0.0	0.0	0.0	0.0	**0.0^{b}**±0	1.2	1.4	1.4	1.1	**1.3^{a}**±0.2
Biocompounds (mg/kg)	Total phenol content	720	640	720	**690^{a}**±46	560	610	700	690	**640^{a}**±68	690	670	730	720	**700^{a}**±30	570	700	610	700	**640^{a}**±65
	Oleuropein	39	32	32	**34^{a}**±4	30	40	50	50	**40^{a}**±11	40	50	70	60	**55^{a}**±11	40	50	60	60	**50^{a}**±10
	3,4 DHPEA-EDA [2]	230	170	170	**190^{a}**±32	130	130	150	140	**140^{a}**±10	153	142	152	157	**151^{a}**±6	100	140	100	110	**110^{b}**±19
	p-HPEA-EDA [3]	92	84	84	**83^{a}**±4	110	90	60	50	**80^{a}**±27	67	59	46	47	**55^{a}**±10	40	38	34	26	**34^{a}**±6
	3,4 DHPEA-EA [4]	31	29	26	**29^{a}**±2	41	38	58	51	**47^{a}**±9	26	36	33	33	**32^{a}**±4	48	48	40	49	**46^{a}**±4
	pHPEA-EA [5]	8.4	8.1	8.0	**8.1^{a}**±0.2	8.0	8.0	19.0	18.0	**13^{a}**±6	8.6	8.2	7.7	7.4	**8.0^{a}**±0.5	17	15	11	12	**14^{b}**±2
	3,4 DHPEA [6]	0.5	0.5	0.7	**0.6^{a}**±0.1	0.4	0.6	0.8	0.7	**0.6^{a}**±0.2	0.8	0.6	0.7	0.6	**0.7^{a}**±0.1	0.6	0.7	1.2	0.9	**0.9^{a}**±0.3
	Tocopherols	290	250	240	**260^{a}**±25	292	292	287	288	**290^{a}**±3	249	256	252	257	**254^{a}**±4	280	280	250	250	**270^{a}**±17
	Fruity volatile compounds [7] (mg/kg)	13.4	14.0	13.4	**13.6^{a}**±0.4	9.8	10.2	10.9	10.5	**10.3^{a}**±0.4	13.6	13.4	13.1	14.2	**13.6^{a}**±0.5	11.7	11.5	13.0	10.9	**11.8^{b}**±0.9

HD: harvesting date; SD: standard deviation; ^{a,b} different letters in the same row indicate significant differences ($p < 0.05$) for the same cultivar; n.d.: not determined; *median of the tasters score; P.A. and N.A.: Positive and Negative Attributes.
(1) F: *Frantoio*; M: *Moraiolo*; 1: First harvesting date; 2: Second harvesting date; a, b, c, d: olive batches.
(2) Dialdehydic form of decarboxymethyl oleuropein aglycone; (3) Dialdehydic form of decarboxymethyl ligstroside aglycone; (4) Oleuropein aglycone; (5) Ligstroside aglycone; (6) Hydroxytyrosol; (7) Sum of the underlined volatile compound contents in Table 3.

than EU legal chemical limits, no sensory defects and a value of "fruity" attribute with a medium intensity of perception, as reported in EU Reg. 1348/2013.

Conversely, all olive oil samples extracted from olives of the second harvesting date were not extra virgin, as they had significant sensory defects. Despite this, they were in compliance with all legally established (EU Reg. 1348/2013) chemical characteristics and "fruity" attribute.

As a result of malaxation operating conditions at low oxidative stress impact, olive oil resulted in high phenolic compounds content and a phenolic profile characterized by slightly degraded phenolic compounds (SERVILI *et al.*, 2004; GOMEZ-RICO *et al.*, 2009). The total phenolic compound content was approx. 670 mg/kg; the dialdehydic form of decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA) was the most abundant phenolic compound, and its content was approx. 150 mg/kg; low (approx. 0.7 mg/kg) hydroxytirosol content (3,4-DHPEA) was found. No significant differences were observed between samples at the two harvesting dates; the medium intensity of "bitter" and "pungent" attribute perception can be explained by phenolic compounds values (EU Reg. 1348/2013).

Volatile compounds content of olive oil samples were subdivided into chemical classes, as reported in Table 3. Compounds that have been shown (KALUA *et al.*, 2007; DI GIACINTO *et al.*, 2010; APARICIO *et al.*, 2012) to be significantly related to oil defects are reported. Underlined volatile compounds are intermediate of LOX pathway and they are considered (DI GIACINTO *et al.*, 2010; KOTTI *et al.*, 2011; APARICIO *et al.*, 2012) to be responsible for olive oil "fruity" positive attribute.

A sum of underlined compound contents is reported in Table 2 as "Fruity volatile compounds"; "fruity" attribute, measured by panel test, can be explained by these values.

Table 3 - Volatile compounds content of extracted olive oil. HD: harvesting date; n.d. not determined.

A. Class of esters, acids and hydrocarbons

	HD	Batch code	Methyl acetate (mg/Kg)	Ethyl acetate (mg/Kg)	Butyl acetate (mg/Kg)	Cis-3-hexenyl acetate (mg/Kg)	Trans-2-hexenyl acetate (mg/Kg)	Butyric acid (mg/Kg)	Pentanoic acid (mg/Kg)	Hexanoic acid (mg/Kg)	Octanoic acid (mg/Kg)	Heptan (mg/Kg)	Octan (mg/Kg)
Frantoio													
	1	**F1a**	0.036	0.019	0.002	0.144	0.003	nd	0.010	0.274	0.070	0.007	0.048
		F1b	0.022	0.017	0.002	0.131	0.002	nd	0.013	0.333	0.089	0.006	0.043
		F1c	0.015	0.020	0.002	0.134	0.001	nd	0.011	0.255	0.047	0.004	0.038
	2	**F2a**	0.015	0.042	0.002	0.070	nd	0.011	0.007	0.210	0.121	0.005	0.053
		F2b	0.007	0.035	0.002	0.409	nd	0.010	0.008	0.243	0.135	0.002	0.039
		F2c	0.008	0.027	0.001	0.614	nd	0.012	0.013	0.237	0.170	0.003	0.036
		F2d	0.006	0.023	0.001	0.619	nd	0.010	0.010	0.235	0.166	0.003	0.036
Moraiolo													
	1	**M1a**	0.005	0.018	0.001	0.747	0.003	nd	0.004	0.236	0.095	0.003	0.032
		M1b	0.005	0.016	0.002	1.070	0.027	nd	0.007	0.259	0.083	0.005	0.031
		M1c	0.006	0.019	0.003	1.173	0.004	nd	0.008	0.277	0.065	0.003	0.033
		M1d	0.005	0.015	0.001	0.480	0.006	nd	0.004	0.214	0.065	0.005	0.028
	2	**M2a**	0.006	0.021	0.001	1.032	nd	0.013	0.012	0.277	0.185	0.003	0.035
		M2b	0.005	0.020	0.002	0.858	nd	0.013	0.011	0.261	0.160	0.004	0.040
		M2c	0.008	0.022	0.001	0.927	nd	0.012	0.008	0.201	0.119	0.002	0.036
		M2d	0.006	0.023	0.001	0.701	nd	0.014	0.015	0.262	0.171	0.003	0.037

B. Class of aldehydes

	HD	Batch code	Valeraldheyde (mg/Kg)	Hexanal (mg/Kg)	Trans-2-Pentenal (mg/Kg)	Cis-3-Hexenal (mg/Kg)	Heptanal (mg/Kg)	Trans-2-Hexenal (mg/Kg)	Octanal (mg/Kg)	Trans-2-Heptenal (mg/Kg)	2.4 Hexadienal (mg/Kg)	Trans-2-Octanal (mg/Kg)	Benzaldehyde (mg/Kg)	Trans-2-Nonenal (mg/Kg)	Trans-2-Decenal (mg/Kg)
Frantoio															
	1	**F1a**	0.128	0.586	0.031	1.421	0.025	10.090	0.131	0.038	0.292	0.015	0.031	0.190	0.229
		F1b	0.106	0.644	0.035	1.822	0.026	10.217	0.159	0.033	0.313	0.010	0.031	0.196	0.232
		F1c	0.087	0.596	0.030	1.569	0.025	9.963	0.134	0.024	0.270	0.010	0.030	0.185	0.237
	2	**F2a**	0.094	0.481	0.023	1.085	0.017	7.331	0.063	0.042	0.198	0.010	0.030	nd	0.132
		F2b	0.079	0.504	0.028	1.084	0.017	6.938	0.066	0.034	0.189	0.015	0.032	nd	0.125
		F2c	0.070	0.547	0.035	1.157	0.023	6.785	0.090	0.031	0.177	0.014	0.033	nd	0.078
		F2d	0.071	0.535	0.035	1.050	0.022	6.545	0.103	0.030	0.172	0.016	0.034	nd	0.092
Moraiolo															
	1	**M1a**	0.092	0.556	0.036	1.742	0.028	8.750	0.148	0.013	0.271	0.019	0.028	0.187	0.203
		M1b	0.079	0.443	0.036	2.016	0.022	7.684	0.137	0.009	0.262	0.016	0.029	0.197	0.18
		M1c	0.100	0.489	0.042	1.937	0.029	7.371	0.152	0.020	0.246	0.014	0.031	0.194	0.204
		M1d	0.087	0.563	0.032	2.045	0.025	9.435	0.117	0.023	0.276	0.019	0.028	0.202	0.180
	2	**M2a**	0.053	0.538	0.039	1.320	0.027	6.595	0.117	0.024	0.206	0.012	0.036	nd	0.081
		M2b	0.044	0.474	0.043	1.803	0.022	6.172	0.093	0.021	0.229	0.009	0.035	nd	0.058
		M2c	0.042	0.416	0.045	4.051	0.015	5.121	0.072	0.010	0.348	0.022	0.034	nd	0.059
		M2d	0.070	0.585	0.038	1.123	0.025	6.607	0.126	0.036	0.192	0.018	0.039	nd	0.099

C. Class of alcohols, ketones and phenols

	HD	Batch code	1-Penten-3-ol (mg/Kg)	2-Heptanol (mg/Kg)	Pentanol (mg/Kg)	1-Octen-3-ol (mg/Kg)	Trans-3-Hexenol (mg/kg)	Cis-3-Hexenol (mg/Kg)	Cis-2-Pentenol (mg/Kg)	2-Butanone (mg/Kg)	2-Octanone (mg/Kg)	1-Octen-3-one (mg/Kg)	1-Penten-3-one (mg/kg)	6-methyl-5-Hepten-2-one (mg/Kg)	Guaiacol (mg/Kg)	Phenol (mg/Kg)	Ethyl-guaiacol (mg/Kg)	4-Ethyl-phenol (mg/Kg)
Frantoio	1	F1a	0.446	nd	0.005	0.002	0.004	0.242	0.337	0.433	0.003	0.001	0.433	0.004	0.005	0.261	0.131	nd
		F1b	0.454	nd	0.005	0.002	0.004	0.260	0.358	0.477	0.004	0.001	0.477	0.005	0.005	0.275	0.124	nd
		F1c	0.475	nd	0.004	0.002	0.003	0.200	0.353	0.484	0.004	0.001	0.484	0.004	0.008	0.253	0.158	0.080
	2	F2a	0.403	0.225	0.004	0.005	0.004	0.089	0.288	0.360	0.014	0.003	0.360	nd	0.002	0.198	nd	nd
		F2b	0.491	0.294	0.004	0.007	0.005	0.259	0.310	0.481	0.012	0.002	0.481	nd	0.004	0.200	nd	nd
		F2c	0.597	0.351	0.005	0.008	0.006	0.431	0.382	0.708	0.010	0.001	0.708	nd	0.005	0.193	nd	nd
		F2d	0.582	0.346	0.005	0.010	0.007	0.454	0.376	0.712	0.035	0.004	0.712	nd	0.006	0.203	nd	nd
Moraiolo	1	M1a	0.554	nd	0.006	0.002	0.006	0.521	0.428	0.586	0.008	0.002	0.586	0.004	0.004	0.245	nd	nd
		M1b	0.569	nd	0.006	0.002	0.009	0.695	0.435	0.590	0.004	0.002	0.590	0.002	0.004	0.251	0.117	nd
		M1c	0.584	nd	0.005	0.002	0.008	0.709	0.444	0.606	0.006	0.002	0.606	0.004	0.003	0.352	0.114	0.067
		M1d	0.571	nd	0.006	0.002	0.006	0.346	0.447	0.533	0.005	0.001	0.533	0.003	0.004	0.238	nd	nd
	2	M2a	0.658	0.375	0.005	0.009	0.008	0.714	0.420	0.715	0.009	0.002	0.715	nd	0.006	0.208	nd	nd
		M2b	0.575	0.369	0.005	0.007	0.005	0.745	0.412	0.711	0.003	0.002	0.711	nd	0.004	0.210	nd	nd
		M2c	0.603	0.361	0.004	0.006	0.006	0.853	0.402	0.720	0.003	0.002	0.720	nd	0.002	0.196	nd	nd
		M2d	0.636	0.381	0.006	0.012	0.007	0.509	0.394	0.753	0.011	0.004	0.753	nd	0.006	0.205	nd	nd

A multidimensional map of all samples related to volatile compounds was obtained by PCA. The relevant sample loading and score plots are reported in Fig. 2. The model explained 60% of data variability along the first (Factor 1) and second (Factor 2) principal components.

A comparison between the score plot and the loading plot showed that olive oil samples extracted from olives of the second harvesting date were all positioned on the left side of the plot. They were characterized by high values of benzaldehyde, 2-butanone, butyric acid, 2-heptanol, octanoic acid, 1-octen-3-ol, 1-octen-3-one and 2-octanone.

All these compounds are related to olive oil defects: These compounds have been associated with "musty", "winey–vinegary" and "fusty" defects by some literature data (KALUA *et al.*, 2007; APARICIO *et al.*, 2012), whereas they have been associated with "rancid" defect by DI GIACINTO *et al.* (2010).

Microbial ecology of oil extraction process

Cell concentrations of dominant microbial populations at different steps of oil extraction process from *Frantoio* and *Moraiolo* cultivar olives are shown in Tables 4 and 5, respectively.

Yeasts and/or moulds were always the dominant populations, independently of the sampling point. Cell density of bacteria only accounted for 1% of the total microbial counts on PCA plates.

The cell concentrations in olive paste after crushing (P) and in extracted olive oil (D) ranged between values below 10 and above 10^4 CFU/g or mL. These values were higher than that obtained from filtered olive oil (O), which, in most cases, was < 10^2 CFU/100 mL.

Microbial counts of each olive batch were often affected by high standard deviation values, as it typically occurs in manufacturing processes of raw materials (such as olives) at industrial scale. A rough general pattern for microbial evolution during olive processing could nonetheless be drawn.

Mould counts in olive paste after crushing (PM) were always significantly higher than those in extracted olive oil (DM), while yeast counts showed a different behaviour.

In most olive batches (from both *Frantoio* and *Moraiolo* cultivars) of the first harvesting date, yeast counts decreased by about one order of magnitude from olive paste after crushing (PY) to extracted olive oil (DY), as expected on the basis of olive oil yield. At the second harvesting date, yeast counts remained almost unchanged from olive paste (PY) to olive oil (DY), or even increased in extracted olive oil (DY), suggesting a progressive yeast colonization of the malaxation equipment and/or "decanter". Indeed, at the second harvesting date, olive paste (PY) harboured almost the same yeast concentration as that at the first harvesting date, with values ranging be-

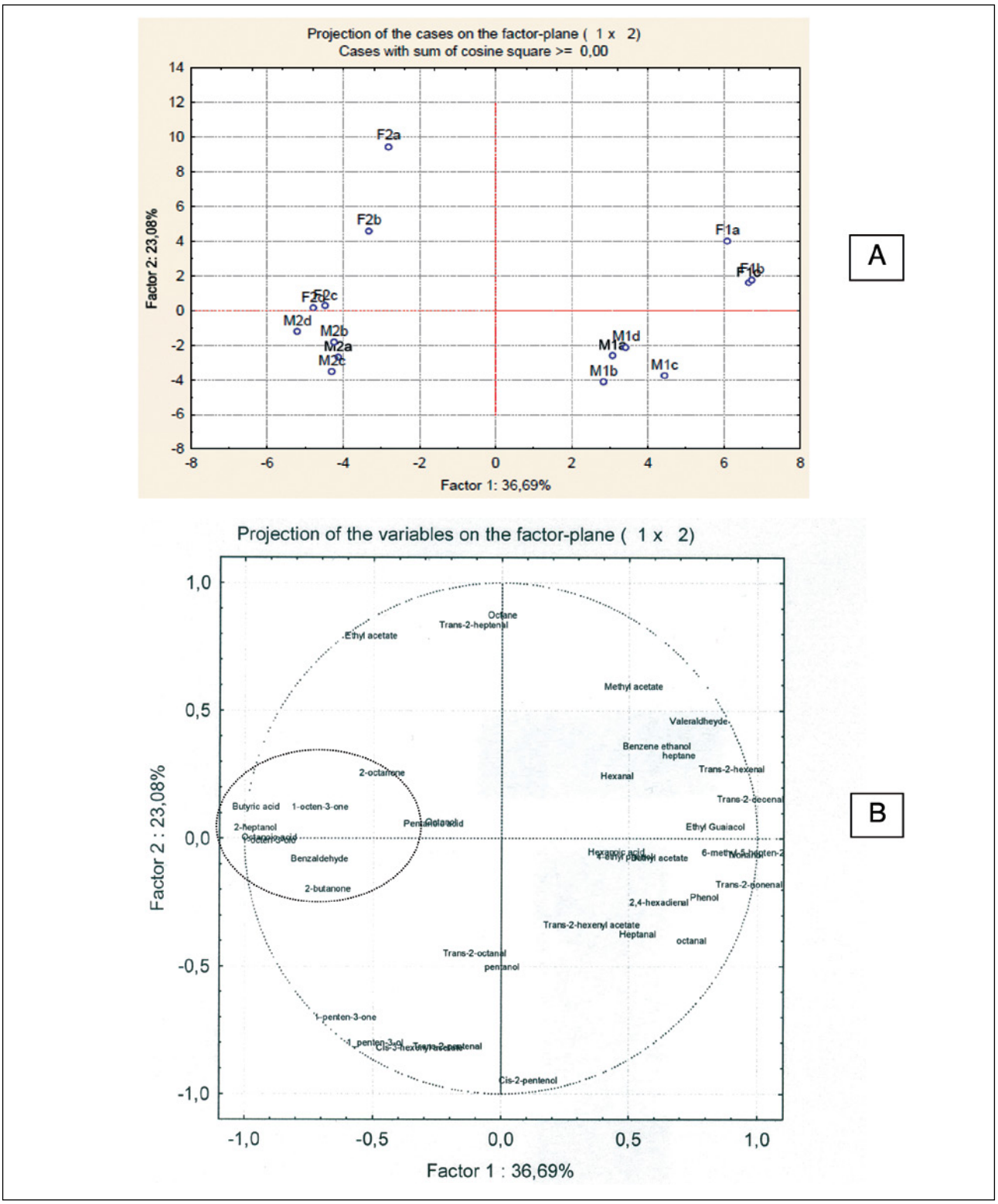

Fig. 2 - Principal Component Analysis carried out on volatile compounds content of olive oil samples. A: similarity map determined by Principal Component (Factor) 1 and 2; B: projection of the variables on the factor plane. Samples are coded as reported in Table 2.

tween 10^2 and above 10^3 CFU/g. On the contrary, the extracted olive oil of the second harvesting date (DY) harboured yeast concentrations, in most cases, of about one or two orders of magnitude higher than the extracted olive oil of the first harvesting date.

Correlation studies demonstrated that mould counts in olive paste after crushing (PM) and in extracted olive oil (DM) were positively related to each other, suggesting that mould contamination of unfiltered oil could be affected by the hygienic level of olives (Table 6). On the contrary, yeast cell densities in olive paste (PY) and in olive oil (DY) were statistically unrelated, sug-

Table 4 - Microbial cell counts at different steps of oil extraction process on two harvesting dates (HD) for *Frantoio* cultivar. P = olive paste after crushing; D = olive oil after extraction by "decanter"; O = olive oil after filtration; TMC = total microbial count; different letters indicate significant differences between different extractive steps of the same olive batch ($p < 0.05$); when no letter is reported, no significant difference was found.

HD	Batch code	Sampling point	Yeasts		Moulds		TMC	
			Mean	SD	Mean	SD	Mean	SD
1	F1a	P (CFU/g)	1.60×10^{3a}	1.40×10^{2}	1.00×10^{2}	0	1.40×10^{3a}	1.40×10^{2}
		D (CFU/mL)	4.50×10^{1b}	7.07	<10	-	4.00×10^{1b}	2.82
		O (CFU/100mL)	<1	-	<1	-	<1	-
	F1b	P (CFU/g)	8.50×10^{2a}	2.12×10^{2}	<10	-	1.60×10^{3a}	5.66×10^{2}
		D (CFU/mL)	1.00×10^{2b}	2.80×10^{1}	<10	-	4.00×10^{1b}	0
		O (CFU/100mL)	<1	-	<1	-	<1	-
	F1c	P (CFU/g)	1.10×10^{3a}	1.41×10^{2}	8.00×10^{2}	0	2.00×10^{3}	1.41×10^{3}
		D (CFU/ml)	3.25×10^{2b}	3.54×10^{1}	<10	-	5.00×10^{2}	2.83×10^{2}
		O (CFU/100mL)	<1	-	<1	-	<1	-
2	F2a	P (CFU/g)	1.00×10^{2a}	1.40×10^{1}	4.20×10^{4a}	2.82×10^{3}	4.80×10^{4a}	2.83×10^{3}
		D (CFU/mL)	3.00×10^{2b}	2.80×10^{1}	4.00×10^{1b}	2.82	3.00×10^{2b}	2.88×10^{1}
		O (CFU/100mL)	5.00×10^{1c}	1.41	<1	-	5.00×10^{1b}	2.82
	F2b	P (CFU/g)	2.70×10^{3}	1.84×10^{3}	2.85×10^{4}	2.32×10^{4}	8.75×10^{3a}	3.18×10^{3}
		D (CFU/ml)	2.92×10^{3}	1.99×10^{3}	3.33×10^{1}	3.27×10^{1}	1.00×10^{2b}	0
		O (CFU/100mL)	5.50×10^{1}	1.41	<1	-	6.50×10^{1b}	1.41
	F2c	P (CFU/g)	2.30×10^{3}	9.90×10^{2}	2.50×10^{4}	2.25×10^{4}	1.10×10^{3a}	1.41×10^{2}
		D (CFU/ml)	3.26×10^{3}	1.60×10^{3}	9.67×10^{1}	8.96×10^{1}	1.81×10^{3a}	7.66×10^{2}
		O (CFU/100mL)	5.50×10^{1}	2.82	<1	-	1.00×10^{1b}	2.82
	F2d	P (CFU/g)	4.00×10^{2a}	2.83×10^{1}	3.45×10^{4}	3.32×10^{4}	7.00×10^{3a}	1.41×10^{2}
		D (CFU/ml)	1.38×10^{4b}	6.36×10^{2}	1.20×10^{2}	2.83×10^{1}	1.35×10^{4b}	9.90×10^{2}
		O (CFU/100mL)	1.50×10^{1a}	1.40	5.00	0	4.00×10^{1c}	2.82

Table 5 - Microbial cell counts at different steps of olive oil extraction process on two harvesting dates (HD) for cultivar *Moraiolo*. P = olive paste after crushing; D = olive oil after extraction by "decanter"; O = olive oil after filtration; TMC = total microbial count; different letters indicate significant differences between different extraction steps of the same olive batch ($p < 0.05$); when no letter is reported, no significant difference was found.

HD	Batch code	Sampling point	Yeasts		Moulds		TMC	
			Mean	SD	Mean	SD	Mean	SD
1	M1a	P (CFU/g)	1.10×10^{3a}	1.41×10^{2}	4.00×10^{2a}	0	1.45×10^{3}	6.36×10^{2}
		D (CFU/mL)	4.50×10^{1b}	7.07	<10	-	4.00×10^{1}	1
		O (CFU/100mL)	<1	-	4.00×10^{1b}	1.41	1.00×10^{1}	1
	M1b	P (CFU/g)	3.75×10^{3a}	3.54×10^{2}	5.50×10^{2}	5.36×10^{2}	5.35×10^{3}	2.33×10^{3}
		D (CFU/mL)	5.00×10^{1b}	1.40	<10	-	<10	-
		O (CFU/100mL)	<1	-	2.00×10^{1}	1.00	2.00×10^{1}	1.40
	M1c	P (CFU/g)	1.10×10^{3a}	1.41×10^{2}	4.00×10^{2a}	0	2.35×10^{3a}	9.19×10^{2}
		D (CFU/mL)	6.90×10^{3b}	2.82×10^{2}	<10	-	1.50×10^{4b}	3.54×10^{3}
		O (CFU/100mL)	<1	-	1.00×10^{1b}	2.82	1.00×10^{1a}	1.40
	M1d	P (CFU/g)	1.10×10^{3}	1.41×10^{2}	4.00×10^{2a}	1.41×10^{1}	1.45×10^{3}	7.78×10^{2}
		D (CFU/ml)	3.20×10^{2}	3.11×10^{2}	<10	-	3.50×10^{1}	2.12×10^{1}
		O (CFU/100mL)	<1	-	2.00×10^{1b}	0	2.00×10^{1}	1.40
2	M2a	P (CFU/g)	1.70×10^{3}	2.83×10^{2}	2.60×10^{3a}	1.98×10^{3}	2.70×10^{3a}	4.24×10^{2}
		D (CFU/mL)	1.04×10^{3}	7.45×10^{2}	6.00×10^{1a}	5.66×10^{1}	9.73×10^{2ab}	8.60×10^{2}
		O (CFU/100mL)	<1	-	5.50×10^{1b}	2.82	5.50×10^{1a}	1.41
	M2b	P (CFU/g)	2.45×10^{3a}	7.78×10^{2}	6.00×10^{2}	5.66×10^{2}	2.35×10^{3a}	9.19×10^{2}
		D (CFU/ml)	3.27×10^{2b}	1.42×10^{2}	3.50×10^{1}	2.12×10^{1}	3.80×10^{2b}	2.31×10^{2}
		O (CFU/100mL)	1.00×10^{1c}	0	1.60×10^{2}	2.82×10^{1}	7.50×10^{1b}	3.53
	M2c	P (CFU/g)	7.45×10^{3}	2.19×10^{3}	1.00×10^{3}	2.82×10^{2}	4.15×10^{3a}	2.12×10^{2}
		D (CFU/mL)	9.72×10^{3}	5.04×10^{3}	4.00×10^{1}	3.66×10^{1}	1.16×10^{3b}	1.07×10^{3}
		O (CFU/100mL)	8.00×10^{1}	14.00	<1	-	1.65×10^{2b}	3.00
	M2d	P (CFU/g)	1.65×10^{3}	1.61×10^{3}	6.75×10^{3}	1.77×10^{3}	5.80×10^{3}	3.11×10^{3}
		D (CFU/mL)	3.08×10^{3}	3.02×10^{3}	6.00×10^{1}	5.66×10^{1}	2.53×10^{3}	2.04×10^{3}
		O (CFU/100mL)	1.10×10^{3}	2.00	<1	-	5.50×10^{1}	1.41

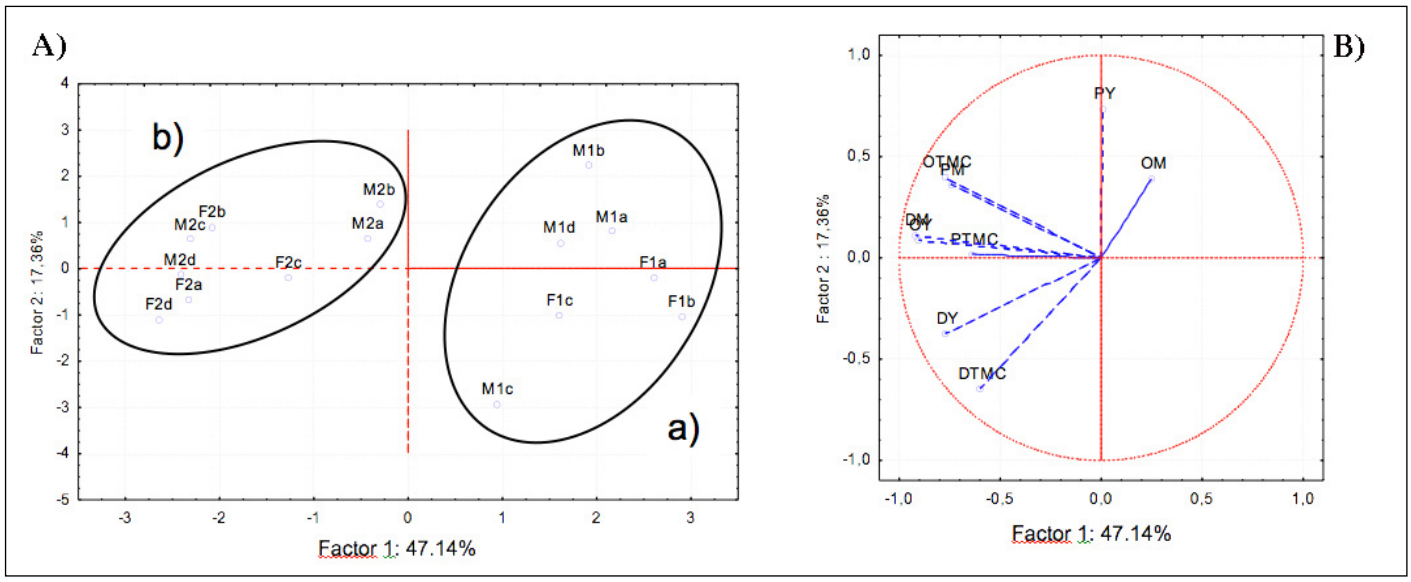

Fig. 3 - Principal Component Analysis of the various olive batches tested by considering as variables the microbial cell concentrations during various extraction process steps. Samples are coded by combination of letters which identify both samples at processing steps (P = olive paste after crushing; D = olive oil after centrifugation by "decanter"; O = olive oil after filtration) and microorganisms (TMC = total microbial count; Y = yeasts; M = moulds). A: similarity map determined by Principal Component (Factor) 1 and 2; B: projection of the variables on the factor plane.

gesting that yeast growth could be encouraged by malaxation and/or "decanting" steps. Finally, no correlation was found between yeast and mould concentrations in both olive paste (PY and PM, respectively) and filtered oil (OY and OM, respectively).

According to PCA of all microbiological data (Fig. 3), processed olive batches clustered into two different groups, independently of the olive cultivar: The samples of the first harvesting date, harboring the lowest microbial cell densities, clustered in group a), while all batches of the second harvesting date resulted to be included in group b). It is worth noting that both the PCA resulting from all microbiological data (Fig. 3) and the PCA resulting from volatile compounds (Fig. 2) are in full agreement, as olive batches from both statistical analyses are clustered in the same way.

Finally, some statistically significant correlations were found between microbial cell densities at the different steps of oil processing and some volatile compounds of olive oil. The significant correlations between yeast (Y) and mould (M) counts, in both extracted (D) and filtered olive oil (O), and volatile compounds content of the final olive oil samples are reported in Table 7. In particular, correlation coefficients (i.e. Pearson and Spearman) agreed on indicating significant positive correlations between yeast and mould counts in olive oil, both before and after filtration, and some volatile compounds; among the latter, the highest significance was related to ethyl acetate, 2-butanone, butyric acid, pentanol, 2-heptanol, octanoic acid and 1-octen-3-ol contents.

Since most of these compounds were identical to those correlated to olive oil batches with sensory defects, as described in the previous paragraph, yeast and mould contamination may have been responsible for those sensory defects. Which specific sensory defects were associated with the above-mentioned compounds could not be explained, as in the literature "rancid", "fusty", "winey–vinegary" and "musty" defects have been associated with both yeasts and moulds. As an example, a recent study demonstrated the capability of some oil born strains of *Candida*

Table 6 - Correlation coefficients calculated between microbial contaminations (Y = yeasts; M = moulds) of olive paste after crushing (P) and microbial contaminations of extracted (D) and filtered olive oil (O). Statistically significant correlations ($p<0.05$) are underlined.

	DY		DM		OY		OM	
	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r
PM			<u>0.8304</u>	<u>0.7347</u>			-0.1575	-0.2485
PY	0.08641	0.05563			0.2841	0.1241		

Table 7 - Correlation coefficients calculated between yeast (Y) and mould (M) counts of extracted and filtered olive oil (D) and volatile compounds of the final olive oil samples (O). Statistically significant correlations ($p<0.05$) are underlined.

	DY		DM		OY		OM	
	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r
Esters, acids and hydrocarbons								
Methyl acetate	-0.006737	-0.3253	-0.08564	-0.4042	0.01485	-0.2926	-0.8317	-0.5387
Ethyl acetate	0.4464	0.2619	0.6978	0.5603	0.7348	0.6665	-0.4953	-0.4413
Butyl acetate	-0.3046	-0.27	-0.5824	-0.5091	-0.3481	-0.3366	-0.1901	-0.1524
Cis-3-hexenil acetate	0.3194	0.325	0.1589	0.2075	-0.0114	0.01915	0.652	0.6335
Trans-2-hexenil acetate	-0.5621	-0.4382	-0.862	-0.4494	-0.7775	-0.3811	0.243	0.2902
Butyric acid	0.5532	0.5918	0.8694	0.9727	0.7434	0.8125	0.03821	-0.0028
Pentanoic acid	-0.0685	-0.1613	-0.1662	-0.1045	-0.3671	-0.331	0.5344	0.5813
Hexanoic acid	-0.1944	-0.3239	-0.337	-0.3943	-0.4902	-0.5172	-0.01623	-
Octanoic acid	0.4624	0.4945	0.8818	0.9251	0.6282	0.6242	0.1469	0.1633
Heptan	-0.6824	-0.5102	-0.5535	-0.4601	-0.5756	-0.4022	-0.0887	-0.218
Octan	-0.1092	-0.2505	0.0174	-0.04	0.205	0.0699	-0.6035	-0.5001
Aldehydes								
Valeraldheyde	-0.5583	-0.4607	-0.708	-0.7013	-0.5681	-0.5144	-0.291	-0.388
Hexanal	-0.3341	-0.3271	-0.3477	-0.3659	-0.4185	-0.4153	-0.3585	-0.315
Trans-2-Pentenal	0.3599	0.3423	0.2134	0.1599	0.06842	0.02669	0.5254	0.464
Cis-3-Hexenal	-0.2487	0.1017	-0.59	-0.1577	-0.4412	0.03461	0.3088	0.07831
Heptanal	-0.3023	-0.3369	-0.4689	-0.5529	-0.7091	-0.7582	0.2273	0.2932
Trans-2-Hexenal	-0.6784	-0.6681	-0.8182	-0.8242	-0.6991	-0.7192	-0.2628	-0.1959
Octanal	-0.405	-0.4349	-0.6645	-0.7347	-0.7142	-0.7745	0.1745	0.2041
Trans-2-Heptenal	-0.0552	-0.01303	0.1559	0.1205	0.2061	0.1596	-0.6411	-0.5767
2.4 Hexadienal	-0.4503	-0.3867	-0.7002	-0.6627	-0.4882	-0.42	-0.0957	-0.0823
Trans-2-Octanal	-0.0244	0.1057	-0.0918	-0.1199	0.08817	0.08237	-0.0718	-0.1306
Benzaldehyde	-0.5041	0.3715	0.5785	0.4958	0.4283	0.3689	-0.1509	0.1584
Trans-2-Nonenal	-0.6304	-0.6281	-0.8605	-0.9855	-0.7762	-0.8358	0.07103	0.04037
Trans-2-Decenal	-0.5946	-0.615	-0.8119	-0.895	-0.6682	-0.7092	-0.2806	-0.2243
Alcohols, ketones and phenols								
1-Penten-3-ol	0.6681	0.4847	0.5822	0.4303	0.3147	0.1095	0.5055	0.5417
2-Heptanol	0.6178	0.6498	0.8857	0.9774	0.7328	0.7846	0.06237	0.0213
Pentanol	0.4111	0.4182	0.8213	0.8118	0.7221	0.7422	-0.1372	-0.2154
Cis-3-Hexenol	0.3252	0.2959	0.2486	0.216	0.07623	0.05016	0.6277	0.5872
Trans-3-Hexenol	0.3032	0.2647	0.2875	0.1636	0.0247	-	0.5309	0.4904
1-Octen-3-ol	0.6212	0.6381	0.9304	0.9199	0.7286	0.7176	-0.0491	-0.0339
Cis-2-Pentenol	0.0486	0.08362	-0.1142	-0.16	-0.2467	-0.2997	0.685	0.618
2-Butanone	0.5204	0.5477	0.782	0.7111	0.5529	0.5283	0.00517	-0.1297
1-Penten-3-one	0.6461	0.5539	0.6247	0.5717	0.412	0.3304	0.3666	0.4029
2-Octanone	0.264	0.4949	0.5565	0.5658	0.4097	0.3878	-0.06146	-0.0623
1-Octen-3-one	0.2545	0.3958	0.5142	0.5573	0.5459	0.5804	0.1314	0.0085
6-methyl-5-Hepten-2-one	-0.5882	-0.5785	-0.8678	-0.9186	-0.7828	-0.779	-0.1013	-0.1062
Guaiacol	-0.0316	-0.1852	0.08201	-0.1711	-0.2404	-0.3518	-0.1757	-0.1935
Phenol	-0.4724	-0.258	-0.8245	-0.8028	-0.8142	-0.7034	0.1395	0.0637
Ethyl-guaiacol	-0.3957	-0.4097	-0.6943	-0.7573	-0.6262	-0.6423	-0.3215	-0.2771
4-Ethyl-phenol	-0.0820	-0.1033	-0.4472	-0.4672	-0.3997	-0.3962	-0.1999	-0.2132

spp. to induce defects such as "musty" and/or "rancid" in oil (ZULLO *et al.*, 2013).

CONCLUSIONS

This study was carried out on several olive oil samples extracted by olive batches from *Frantoio* and *Moraiolo* cultivars, harvested on two different dates. All extracted olive oil samples from the second olive harvesting date were classified as "non extra virgin", as they were affected by sensory defects.

By combining chemical, sensory, and microbiological data, it can be assumed that the olive oil samples with sensory defects were significantly correlated with specific volatile compounds (i.e., 2-butanone, butyric acid, 2-heptanol, octanoic acid, 1-octen-3-ol). The same volatile compounds were correlated to both yeast and mould counts. It could not be evidenced whether a specific sensory defect might result from specific volatile compounds, which in turn can be produced by specific yeasts and moulds.

Different processing steps were also identified, which resulted to be the most critical steps to cause the measured sensory defects: (i) the

mould contamination of olives; (ii) the two central steps of olive oil processing (i.e. malaxation and extraction by "decanter"), which were likely to have enabled some yeast species to grow. A study on identification of yeast isolates and determination of their enzymatic properties is being carried out to further investigate the incidence of yeast populations during olive oil extraction process.

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