

CALCIUM CARBONATE EFFECT ON ALKYL ESTERS AND ENZYMATIC ACTIVITIES DURING OLIVE PROCESSING

F. CAPONIO*, G. SQUEO, M. CURCI, R. SILLETTI, V.M. PARADISO, C. SUMMO, C. CRECCHIO and A. PASQUALONE

Department of Soil, Plant and Food Science (DISSPA), University of Bari Aldo Moro, Via Amendola 165/a, 70126 Bari, Italy

*E-mail address: francesco.caponio@uniba.it

ABSTRACT

The effect of coadjuvants during olive oil processing on the oxidative enzymes and the content of fatty acid alkyl esters (FAAE) has been investigated. Two Italian olive cultivars, at different ripening degree, were processed immediately after harvesting or after 5 and 12 days of storage. The results highlighted a general decrease of FAAE and a significant increase in the PPO and POD activities due to the coadjuvant use. The increased oxidases activity could lead to a reduction of oils phenolic compounds.

Keywords: fatty acid alkyl esters, extra virgin olive oil, oxidases, olive processing, technological coadjuvant

1. INTRODUCTION

Over the years, the goals of the oil industry have gradually changed. After the introduction of the centrifugal decanters, which made the oil extraction process continuous and led to cost decreases, solving the issues linked to the traditional production, the newest aim was to ensure the highest quality of virgin olive oils (VOO) obtained. In fact, suddenly appeared that oils had lower content of phenolic compounds compared to those obtained with the traditional method (pressure), because of the olive paste leaching by the added water, an essential step in order to ensure satisfactory extraction yields by centrifugation (RANALLI and ANGEROSA, 1996). It is common knowledge that phenolic compounds, besides affecting VOO sensory notes (bitter and pungent notes are directly related to the total phenolic content) are the main responsible of the health benefits associated to the VOO consumption (MARTÍN-PELÁEZ *et al.*, 2013). Furthermore, often happened that the olive pomace after the separation step is too much wet, with moisture content even higher than 60%, and thus not appreciated by the pomace oil factories (SÁNCHEZ MORAL *et al.*, 2006). Usually oil producers overcome this drawback submitting olive pomace to a second centrifugation step by means of three-phase decanter (CAPONIO *et al.*, 2015; PASQUALONE *et al.*, 2016). This allows even to recover an additional amount of olive oil called “ripasso” rising, however, the overall costs of production. Moreover, according to the Council Regulation 1513/2001 (OFFICIAL JOURNAL OF THE EUROPEAN COMMUNITIES, 2001), that oil has to be classified as “crude olive-pomace oil”.

Nowadays, innovative decanters, such as those equipped with variable Dn system, are available on the market. Respect to older machines, these decanters allow real-time setting of several working parameters as a function of the raw material characteristics (maturity degree), as well as of the expected virgin olive oil quality, e.g. in terms of phenolic compounds (SQUEO *et al.*, 2017a). Currently, the focus was moved towards the optimisation of the process efficiency (that is maximise the extraction yields) especially during processing of the so-called “difficult pastes” (CERT *et al.*, 1996; UCEDA *et al.*, 2006; CAPONIO *et al.*, 2014), without jeopardising VOO quality. Different approaches were tested to solve such an issue and, besides working on the malaxation parameters (time-temperature), numerous researches were recently carried out regarding the use of physic processing aids (not forbidden by the European laws). Among them, micronized natural talc (MNT) and calcium carbonate have shown a significant positive effect on the extraction process efficiency while the influence on the chemical and organoleptic features of the VOO was not univocally pointed out (BEN BRAHIM *et al.*, 2015; CAPONIO *et al.*, 2016). Besides, calcium carbonate is less expansive and does not involve any health risk for oil-mill operators than the use of MNT (ESPÍNOLA *et al.*, 2009). The employment of technological coadjuvants was even proposed in order to avoiding the need of a second centrifugation step (CAPONIO *et al.*, 2015).

The European Commission (OFFICIAL JOURNAL OF THE EUROPEAN COMMUNITIES, 2011), aiming at the protection of the highest VOO quality and in order to prevent illegal mixtures with low-quality oils, have introduced the determination of fatty acids alkyl esters (FAAE) as a quality parameter for the extra virgin olive oils (EVOO) classification. Alkyl esters originate from the esterification of fatty acids and low molecular weight alcohols, methanol and ethanol, arising from the progressive pectin degradation during the olive ripening and from the bad and/or prolonged storage of drupes, respectively (BIEDERMANN *et al.*, 2008; JABEUR *et al.*, 2015; BELTRAN *et al.*, 2016). That is, FAAE content in EVOO is strongly linked to the quality of the raw material and is considered a clear marker of the sanitary state and/or of the handling procedures of the olive fruits before processing. Moreover, due to the high stability, these compounds were even

proposed as an efficient tool for discovering mixture with mild deodorised olive oils (PÉREZ-CAMINO *et al.*, 2008). Afterwards, the focus was moved towards the content of the fatty acids ethyl esters (FAEE) only (OFFICIAL JOURNAL OF THE EUROPEAN COMMUNITIES, 2013).

In this framework, greater attention would be given to the correlation between the use of coadjuvants in olive oil mill and alkyl esters in the obtained oils. The only study present in literature (SQUEO *et al.*, 2017b) reports that the use of calcium carbonate on Coratina cv. olives processed immediately after harvesting led to a general reduction of FAEE compared to the untreated samples, evidencing a higher susceptibility of methyl esters (FAME) than ethyl esters. Furthermore, previous papers have reported as its use led to a decrease in the oil phenolic content (SQUEO *et al.*, 2016; TAMBORRINO *et al.*, 2017) but, despite the great relevance of phenolics, still today this side effect is not studied and understood. As far as we know, no information are available about the effect of the coadjuvants on the most important olive enzymatic activities and, in particular, polyphenol oxidase (PPO) and peroxidase (POD) which are responsible of the oxidation of phenolic compounds in the first stages of extraction and during the malaxation step (GARCÍA-RODRÍGUEZ *et al.*, 2011).

Hence, the aim of this research was to assess the influence of calcium carbonate on the FAEE content and enzymatic activities involved in the oxidation of the phenolic compounds, in order to reach a deeper knowledge about the possible side effects of such coadjuvant during olive processing. In particular, two Italian olive oil cultivars, having different maturation degree, were considered, both processed immediately after harvesting and after 5 and 12 days of storage, with the addition of calcium carbonate at different level and particle size.

2. MATERIALS AND METHODS

2.1. Materials and reagents

Calcium carbonate (CaCO_3) was kindly furnished by Omya Spa (Milan, Italy). Two different mean particle sizes were considered: 2.7 μm (Calcipur[®]2) and 5.7 μm (Calcipur[®]5). All the reagents used for the analytical determination were for analytical purpose or HPLC and GC grade.

2.2. Sampling

Olives of Coratina and Nociera cultivars having the following features respectively were used for the experiment: pigmentation index (defined as in SQUEO *et al.*, 2016) 2.32 and 4.40, respectively; moisture content 52.92% and 47.15%; total oil content 21.20% and 19.66% (OFFICIAL JOURNAL OF THE EUROPEAN COMMUNITIES, 1991).

For each cultivar a homogenous lot of olives were used, which was further divided in 18 batches of about 30 kg each. Five trials were carried out on olives processed immediately after harvesting: one, the control (C), without CaCO_3 addition and the others by means of coadjuvant addition (two different particle sizes: 2.7 μm and 5.7 μm) at two different percentages of use (2% and 4% respect to the olives weight), as follows:

- without calcium carbonate addition (C, control),
- with 2% of Calcipur[®]2 (Ca2-2%),
- with 4% of Calcipur[®]2 (Ca2-4%),
- with 2% of Calcipur[®]5 (Ca5-2%),

- with 4% of Calcipur[®]5 (Ca5-4%).

The remaining batches were stored in reticular plastic bins for 5 and 12 days (T5 and T12) and processed without any treatment (control, C) or by the use of 2% of CaCO₃ (2.7 μm) as follows:

- without calcium carbonate addition (C_{T5} and C_{T12}, control),
- with 2% of Calcipur[®]2 (Ca2-2% and Ca2-12-2%).

When expected, calcium carbonate was added at the beginning of the malaxation phase. Oil extraction was performed by means of a small industrial plant (Oliomio Mini 50, Mori-Tem S.r.l., Tavarnelle Val di Pesa, Florence, Italy) of a maximum capacity of 30 kg h⁻¹. After crushing by a fixed hammer crusher, olives paste was malaxed at 20±1°C for 20 min and sent to a 2-phase decanter for the oil separation. Two oil samples were collected for each extraction. The samples were then further finished by centrifugation (SL 16R model, Thermo Scientific, Waltham, MA, USA) at 8,867 × g, 5 min, 4°C. Finished oils were poured in 100 mL dark glass bottles, leaving an head-space of about 1 cm, hermetically sealed, and stored at room temperature (18-20°C) until were analyzed. All the trials were repeated twice.

2.3. Routine analyses

The determination of free fatty acids, peroxide value, spectrophotometric extinctions at 232 and 270 nm, and fatty acid composition were carried out as reported by the Official Journal of the European Communities (OFFICIAL JOURNAL OF THE EUROPEAN COMMUNITIES, 1991).

2.4. Determination of FFAE

The analyses of the methyl and ethyl esters of fatty acids were carried out according to the official method (OFFICIAL JOURNAL OF THE EUROPEAN COMMUNITIES, 2011). The gas chromatographic system was made up of a 7890B Agilent Technologies (Santa Clara, CA, USA) gas-chromatograph equipped with a flame ionization detector (FID). The column used was a capillary fused silica DB-5HT (length 15 m, i.d. 0.32 mm, film thickness 0.10 μm). The operating conditions were as follows: oven temperature, 80°C for 1 min and then increased from 20°C min⁻¹ to 140°C, then increased from 5°C min⁻¹ to 335°C and maintained for 20 min. The detector temperature was 350°C. Helium was used as the carrier gas, with a flow through the column of 2 mL min⁻¹ in on-column mode. Each sample was analysed twice.

2.5. Enzymatic activity assessment

Ten g olive pastes were homogenized with 150 mL cold acetone (-20°C) in a Waring Blender homogenizer at highest speed for 30 s. Powder extract was filtered under vacuum, then further extracted twice with 20 mL of cold acetone, finally washed with diethyl ether, dried at room temperature and finally stored at -20°C (GARCÍA-RODRÍGUEZ *et al.*, 2011). Enzymatic extracts of polyphenoloxidase (PPO, EC 1.14.18.1) and peroxidase (POD, EC 1.11.1.7) were prepared according to (PERES *et al.*, 2016): 0.4 g acetone powder were resuspended in 5 mL extraction buffer (0.05 M potassium phosphate, 1 M KCl, pH 6.2) and 2% polyvinylpyrrolidone (PVP) and stirred for 30 min at 400 rpm at 4°C; the suspension was centrifuged at 15,777 × g for 30 min at 4°C and filtered (0.45 μm).

For both enzymatic assays the reaction medium consisted of 50 mM sodium phosphate buffer pH 6.2, containing 0.5 mL of filtered crude extracts (above described) in a final volume of 2.5 mL. PPO activity was evaluated using catechol (30 mM) as substrate,

following the increase in absorbance at 420 nm, during 1 min. One unit of PPO was defined as the quantity of enzyme that causes the absorbance variation of $0.001 \text{ min}^{-1} \text{ mL}^{-1}$, at 25°C ; results were expressed as $\text{U g}^{-1} \text{ FW}$ (fresh weight). POD activity was evaluated using guaiacol (30 mM) and H_2O_2 (4 mM) as substrates. In particular, guaiacol was incubated for 5 min at 25°C , then H_2O_2 was added and the absorbance at 470 nm was measured after 2 min. One unit of POD was defined as the consumption of μmol of guaiacol $\text{min}^{-1} \text{ mL}^{-1}$, at 25°C . Results were expressed as $\text{U g}^{-1} \text{ FW}$ (fresh weight) (PERES *et al.*, 2016).

2.6. Total phenolic content

For the extraction of phenols, 1 g of oil was dissolved in 1 mL of hexane and 5 mL of methanol/water (70:30, v/v). The mixture was vortexed for 10 min and centrifuged at 6000 rpm for 10 min at 4°C (SL 16R Centrifuge, Thermo Fisher Scientific Inc., Waltham, MA, USA). The methanolic phase was recovered, centrifuged again at 9000 rpm for 5 min at 4°C , and finally filtered through 0.45mm pores filters. The quantification of the phenolic compounds was carried out by means of the Folin-Ciocalteu method. Briefly, $100\mu\text{L}$ of extract were mixed with $100\mu\text{L}$ of Folin-Ciocalteu reagent. After 4 min, $800\mu\text{L}$ of Na_2CO_3 solution 5% (w/v) was added to the mixture and heated in a water bath at 40°C for 20 min. After being cool down for 15 min, the absorbance was measured at 750 nm by an Agilent Cary 60 spectrophotometer (Agilent Technologies, Santa Clara, USA). The total phenolic content was expressed as gallic acid mg equivalents (mg kg^{-1}).

2.7. Statistical analysis

Unbalanced nested ANOVA was used to test the effects of the type of calcium carbonate and the percentages of addition on the fatty acids alkyl esters amount (Table 2). Two-way ANOVA was used to test the effects of the use of calcium carbonate and the olives days of storage on the fatty acids alkyl esters amount (Table 3) while three-way ANOVA was used for testing the influence of the experimental conditions on the enzymatic activities (Figure 1). In all cases, Tukey post-hoc test for multiple comparisons was carried out on the experimental data by means of Minitab 17 software (Minitab Inc., State College, PA, USA).

3. RESULTS AND DISCUSSION

3.1. Influence on the alkyl esters amount

Table 1 reports the characteristics of the oils extracted from the olives, without carbonate addition (C), immediately after harvesting. The samples fulfilled the European limits for the EVOO classification (OFFICIAL JOURNAL OF THE EUROPEAN COMMUNITIES, 2013).

The fatty acids profiles matched those typical for olive oils and a variability among the cultivar studied was observed confirming, as it is well known, that fatty acids composition is strongly affected by the genotype (ROTONDI *et al.*, 2010). Nociara cv. oil was richer in palmitic ($\text{C}_{16:0}$), palmitoleic ($\text{C}_{16:1}$), linoleic ($\text{C}_{18:2}$), and linolenic ($\text{C}_{18:3}$) acids than Coratina cv. oil. The latter was richer in oleic ($\text{C}_{18:1}$) and eicosenoic ($\text{C}_{20:1}$) acids. Overall, Nociara cv. oil showed a higher extent of the primary oxidative degradation, likely due to the higher pigmentation index (almost two-fold than Coratina) of the drupes and to the higher amount of polyunsaturated fatty acids. Indeed, the oxidative susceptibility of the oils rises with the increase of the fatty acids unsaturation degree (CHOE and MIN, 2006).

The mean values, standard deviations, and statistical analysis of the oils alkyl esters content obtained from non-stored olives are reported in Table 2.

Table 1. Basic analytical characteristics of the oils obtained from olives processed immediately after harvesting ($n=2$).

Parameter	Coratina		Nociara	
	Mean value	SD	Mean value	SD
FFA	0.31	0.02	0.43	0.04
PV	6.5	0.1	9.8	0.1
K232	1.829	0.016	2.215	0.023
K270	0.115	0.005	0.128	0.003
<i>Fatty acid composition (%)</i>				
< C14:0	-	-	-	-
C16:0	10.09	0.01	14.08	0.12
C16:1	0.68	0.02	1.73	0.04
C17:0	0.06	0.01	0.05	0.00
C17:1	0.08	0.01	0.10	0.01
C18:0	2.12	0.04	1.91	0.08
C18:1	78.97	0.05	67.91	0.02
C18:2	6.77	0.07	12.95	0.04
C18:3	0.55	0.01	0.67	0.03
C20:0	0.34	0.01	0.37	0.03
C20:1	0.33	0.02	0.23	0.02

FFA, free fatty acids ($\text{g } 100 \text{ g}^{-1}$); PV, peroxide value ($\text{meq O}_2 \text{ kg}^{-1}$); K_{232} , specific absorption at 232 nm; K_{270} , specific absorption at 270 nm; $C_{16:0}$, palmitic acid; $C_{16:1}$, palmitoleic acid; $C_{17:0}$, heptadecanoic acid; $C_{17:1}$, heptadecenoic acid; $C_{18:0}$, stearic acid; $C_{18:1}$, oleic acid; $C_{18:2}$, linoleic acid; $C_{18:3}$, linolenic acid; $C_{20:0}$, arachidic acid; $C_{20:1}$, eicosenoic acid.

Table 2. Mean values, standard deviations and results of unbalanced nested ANOVA followed by Tukey's HSD test of fatty acids alkyl esters determined in oils obtained from olives processed immediately after harvesting. Oils were obtained by adding or not calcium carbonate ($n=4$).

Cultivar	Trial	FAME (mg kg^{-1})		FAEE (mg kg^{-1})		FAAE (mg kg^{-1})	
Coratina	C	4.53±0.22	a	3.75±0.22	a	8.29±0.41	a
	Ca2-2%	3.90±0.16	ab	3.01±0.27	b	6.91±0.34	b
	Ca2-4%	3.94±0.17	ab	3.58±0.16	a	7.52±0.19	ab
	Ca5-2%	3.83±0.30	b	3.95±0.25	a	7.77±0.25	ab
	Ca5-4%	3.66±0.48	b	3.62±0.36	a	7.27±0.83	b
Nociara	C	3.73±0.02	a	6.31±0.31	a	10.04±0.31	a
	Ca2-2%	3.34±0.44	a	5.65±0.25	a	8.99±0.41	a
	Ca2-4%	3.46±0.34	a	6.08±0.32	a	9.53±0.36	a
	Ca5-2%	3.49±0.07	a	6.07±0.59	a	9.56±0.64	a
	Ca5-4%	3.75±0.38	a	6.00±0.48	a	9.75±0.72	a

Different letters in the same column for the same cultivar indicate significant differences ($p \leq 0.05$). C, control without calcium carbonate addition; Ca2, calcium carbonate 2.7 μm ; Ca5, calcium carbonate 5.7 μm ; FAME, fatty acids methyl esters; FAEE, fatty acids ethyl esters; FAAE, fatty acids alkyl esters.

The initial levels of FAAE in the control samples were different for Nociara or Coratina cvs.: Nociara oils were richer particularly in ethyl esters and, as a consequence, in the total amount of alkyl esters. The different starting conditions might be imputable to the different maturity degree of the drupes. By the progressive advancement of the maturity, fruits become softer and more susceptible to alterations, such as yeast growth, leading to a higher availability of ethanol by anaerobic fermentation (CONDE *et al.*, 2008; DI SERIO *et al.*, 2017). Coadjuvant use showed a different effect depending on the processed cultivar. Indeed, no statistical influence was found on the FAAE amount considering Nociara cv., while a significant effect was highlighted in the case of Coratina cv. In particular, respect to the control, FAME amount was significantly reduced by Ca5, regardless the percentage used, while FAEE were significantly lowered by Ca2-2%. As a result, the total FAAE amount was significantly lower in the case of Ca2-2% and Ca5-4%. In the light of this, it is possible to suppose a cultivar-dependent effect or, more probably, dependent on the different maturation degree of the cultivar studied. Indeed, the different amount of water or interfering compounds such as high mass polymeric substances (MAFRA *et al.*, 2001) might be determinant in modulating the action of calcium carbonate (AGUILERA *et al.*, 2010).

In order to have a deeper understanding about the coadjuvant effect on FAAE, the same olives have been processed after 5 and 12 days of storage. It is known that FAAE amount rises during olive storage as a consequence of degradation and fermentative processes (JABEUR *et al.*, 2015). Table 3 reports the mean values, the standard deviations, and the statistical analysis of the alkyl esters of oils obtained from stored olives added or not with calcium carbonate during malaxation.

Table 3. Mean values, standard deviations and results of two-way ANOVA followed by Tukey's HSD test of the fatty acids alkyl esters of oils obtained from olives stored for 5 (T5) and 12 (T12) days after harvesting. Oils were obtained by adding or not calcium carbonate ($n=4$).

Cultivar	Trial	FAME (mg kg ⁻¹)		FAEE (mg kg ⁻¹)		FAAE (mg kg ⁻¹)	
Coratina	C _{T5}	6.93±0.24	c	17.70±1.23	c	24.63±1.45	c
	Ca2 _{T5} -2%	6.89±0.18	c	10.99±0.31	d	17.88±0.22	d
	C _{T12}	17.36±0.79	a	48.74±1.64	a	66.11±1.49	a
	Ca2 _{T12} -2%	12.75±0.21	b	27.60±0.25	b	40.35±0.44	b
Nociara	C _{T5}	8.14±0.21	c	38.61±0.51	b	46.75±0.69	b
	Ca2 _{T5} -2%	7.73±0.26	c	31.00±0.47	c	38.73±0.36	c
	C _{T12}	23.55±0.96	a	174.96±2.01	a	198.51±2.82	a
	Ca2 _{T12} -2%	21.99±0.34	b	174.42±1.88	a	196.41±1.96	a

Different letters in the same column for the same cultivar indicate significant differences ($p \leq 0.05$).

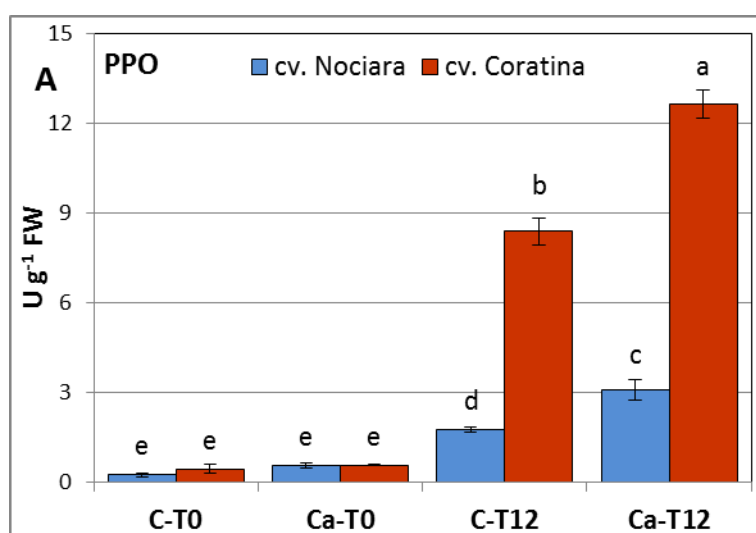
C, control without calcium carbonate addition; Ca2, calcium carbonate 2.7 μm ; Ca5, calcium carbonate 5.7 μm ; FAME, fatty acids methyl esters; FAEE, fatty acids ethyl esters; FAAE, fatty acids alkyl esters.

As expected, the variable *days of storage* had a highly significant effect on the final alkyl esters amount in both the cultivars (in all cases $p < 0.001$, data not shown). In particular, the FAAE increment was mainly due to the increase of FAEE, in accordance with the findings of (JABEUR *et al.*, 2015), confirming the role of ethyl esters of fatty acids as a marker of the raw material quality. However, the increment of these compounds was definitely more evident for Nociara cv. instead of Coratina one, probably due to the higher

maturity degree and/or greater substrate availability for the fermentative activities (GÓMEZ-COCA *et al.*, 2016), showing the higher susceptibility of the Nociara cv. olives respect to the Coratina cv. fruits. In fact, it is noteworthy to highlight that in the period from 5 to 12 days of storage, the increase of the total alkyl esters and ethyl esters content was extraordinary in the case of Nociara cv. rising of about 325% and 353%, respectively. Turning on the action of the coadjuvant, a highly significant effect of calcium carbonate addition was observed (in all cases $p < 0.01$) and, interestingly, the results were different for the cultivars studied. Similarly to what previously observed on fresh fruits, the strongest reduction caused by the coadjuvant in terms of FAAE was observed during Coratina olives processing, leading to a decrease of about 26% and 43% for FAME and FAEE, respectively, in the samples obtained after 12 days of storage. That is, the coadjuvant use at 12 days storage, in these experimental conditions, lowered the FAEE amount under the maximum EU limit for the extra virgin olive oil classification (OFFICIAL JOURNAL OF THE EUROPEAN COMMUNITIES, 2016) moving from about 50 mg kg⁻¹ to less than 30 mg kg⁻¹. After 5 days storage, it was already observed a significant reduction of FAEE and FAAE, even if sharper than what happened after 12 days. In the case of Nociara cv., a weaker effect of the calcium carbonate was observed, lowering the amount of FAEE and FAAE after 5 days of storage and the amount of FAME after 12 days. No significant effect was highlighted on the great content of FAEE and FAAE after 12 days of storage. Based on the behaviour observed in the case of Coratina cv. it seemed possible to suppose that the alkyl esters reduction by calcium carbonate was substrate dependent, i.e. the higher the content the higher the reduction. However, such hypothesis was not confirmed by the results obtained from the trials carried out on Nociara cv.

3.2. Enzymatic activity evaluation

Figure 1 depicted the enzymatic activities of polyphenol oxidase (PPO, Figure 1A) and peroxidase (POD, Figure 1B) measured on the Coratina and Nociara cvs. olive pastes added or not with calcium carbonate during malaxation.



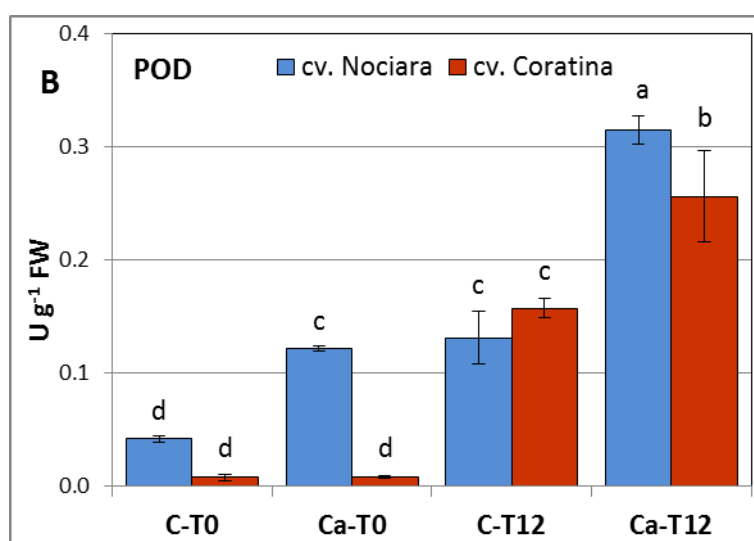


Figure 1. Polyphenol oxidase (PPO, A) and peroxidase (POD, B) activities measured on the olives pastes of Nociara and Coratina cvs. obtained from fresh olives (T0) and after 12 days of storage (T12) at ambient temperature added (Ca) or not (C) with calcium carbonate. Different letters indicate significant variations as determined by three-way ANOVA followed by Tukey's HSD test for multiple comparisons.

PPO activity (Fig. 1A) of fresh fruits was not affected by the coadjuvant use in both the cultivars. At T12, the enzyme activity was significantly higher than T0, in particular in the case of Coratina cv. The increased activity might be due to the progressive structure degradation of the fruits, due to the storage, and to the consequently increase in the substrate availability. This hypothesis seems to be confirmed by the higher enzymatic activity observed in the case of Coratina cv., which is known to be rich in phenolics (ROTONDI *et al.*, 2010). Concerning the coadjuvant treatment, it is noteworthy that the use of calcium carbonate significantly increases the PPO activity in both the varieties at T12. The most plausible explanation might be the shift of the olives paste pH to optimal values for the enzyme activity due to the basic hydrolysis of the salt. POD catalyses the oxidation of phenolic compounds using hydrogen peroxide or others organic peroxides from the medium as oxidant agents (GAJHEDE, 2001; KADER *et al.*, 2002). At T0, the enzyme activity was higher in the case of Nociara cv. respect to Coratina one, likely due to the higher value of peroxides (Table 1) necessary for the POD action, as previously stated. Calcium carbonate significantly affected the enzyme activity in the case of Nociara cv. while no statistical difference was observed for Coratina. At T12, the POD activity was significantly higher in both the cases respect to T0, and a further significant increase, due to the use of the processing aid, was highlighted. Overall, our findings might explain the reduction of the phenolic compounds of the investigated samples with the addition of calcium carbonate (Table 4), as also observed in previous studies (SQUEO *et al.*, 2016; TAMBORRINO *et al.*, 2017). Moreover, if these behaviours will be confirmed by further studies, even the action of the calcium carbonate, currently assumed as being merely physical, might be rethought.

Table 4. Mean values of phenolic compounds of the samples under investigation of oils obtained from fresh olives (T0) and after storage for 5 (T5) and 12 (T12) days after harvesting.

Trial	Coratina cv.	Nociara cv.
C _{T0}	470	188
Ca2 _{T0} -2%	463	146
Ca2 _{T0} -4%	406	127
Ca5 _{T0} -2%	316	164
Ca5 _{T0} -4%	324	121
C _{T5}	311	133
Ca2 _{T5} -2%	262	113
C _{T12}	182	127
Ca2 _{T12} -2%	139	105

C, control without calcium carbonate addition; Ca2, calcium carbonate 2.7 μm ; Ca5, calcium carbonate 5.7 μm .

4. CONCLUSIONS

The results reported highlighted that the use of calcium carbonate modifies the amount of fatty acids alkyl esters as well as the activity of selected enzymes. Coadjuvant addition during malaxation led to a general reduction of FAAE and an increase in the PPO and POD activities, the former useful for the virgin olive oil classification, the latter involved in the phenolic content of VOO. The effect of the coadjuvant seems to be cultivar dependent or, more realistically, linked to the raw material ripening degree and oxidative degradation. In the light of this, further studies are needed to confirm such results considering more thoroughly the effect of the olives maturation degree and even concerning other coadjuvants commonly used during EVOO extraction.

ACKNOWLEDGEMENTS

This work has been supported by AGER 2 Project, grant n° 2016-0105.

REFERENCES

- Aguilera M.P., Beltran G., Sanchez-Villasclaras S., Uceda M. and Jimenez A. 2010. Kneading olive paste from unripe 'Picual' fruits: I. Effect on oil process yield. *J. Food Eng.* 97:533.
- Beltran G., Sánchez R., Sánchez-Ortiz A., Aguilera M.P. Bejaoui, M.A. and Jimenez A. 2016. How 'ground-picked' olive fruits affect virgin olive oil ethanol content, ethyl esters and quality. *J. Sci. Food Agric.* 96:3801.
- Ben Brahim S., Marrakchi F., Gargouri B. and Bouaziz M. 2015. Optimization of malaxing conditions using CaCO₃ as a coadjuvant: A method to increase yield and quality of extra virgin olive oil cv. Chemlali. *LWT - Food Sci. Technol.* 63:243.
- Biedermann M., Bongartz A., Mariani C. and Grob K. 2008. Fatty acid methyl and ethyl esters as well as wax esters for evaluating the quality of olive oils. *Eur. Food Res. Technol.* 228:65.
- Caponio F., Monteleone J., Martellini G., Summo C., Paradiso V.M. and Pasqualone A. 2014. Effect of talc addition on the extraction yield and quality of extra virgin olive oils from Coratina cultivar after production and during storage. *J. Oleo Sci.* 63:1125.
- Caponio F., Squeo G., Difonzo G., Pasqualone A. and Summo C., Paradiso, V.M. 2016. Has the use of talc an effect on yield and extra virgin olive oil quality? *J. Sci. Food Agric.* 96:3292.

- Caponio F., Squeo G., Monteleone J., Paradiso V.M., Pasqualone A. and Summo C. 2015. First and second centrifugation of olive paste: Influence of talc addition on yield, chemical composition and volatile compounds of the oils. *LWT - Food Sci. Technol.* 64:439.
- Cert A., Alba J., León-Camacho M., Moreda W. and Pérez-Camino M.C. 1996. Effects of talc addition and operating mode on the quality and oxidative stability of virgin olive oils obtained by centrifugation. *J. Agric. Food Chem.* 44:3930.
- Choe E. and Min D. 2006. Mechanisms and Factors for Edible Oil Oxidation. *Compr. Rev. Food Sci. Food Saf.* 5:169.
- Conde C., Delrot S. and Gerós H. 2008. Physiological, biochemical and molecular changes occurring during olive development and ripening. *J. Plant Physiol.* 165:1545.
- Di Serio M., Giansante L., Di Loreto G., Faberi A., Ricchetti L. and Di Giacinto L. 2017. Ethyl esters versus fermentative organoleptic defects in virgin olive oil. *Food Chem.* 219:33.
- Espínola F., Moya M., Fernández D.G. and Castro E. 2009. Improved extraction of virgin olive oil using calcium carbonate as coadjuvant extractant. *J. Food Eng.* 92:112.
- Gajhede M. 2001. Plant peroxidases: substrate complexes with mechanistic implications. *Biochem. Soc. Trans.* 29:91.
- García-Rodríguez R., Romero-Segura C., Sanz C., Sánchez-Ortiz A., and Pérez A.G. 2011. Role of polyphenol oxidase and peroxidase in shaping the phenolic profile of virgin olive oil. *Food Res. Int.* 44:629.
- Gómez-Coca R.B., Fernandes G.D., Pérez-Camino M.C. and Moreda W. 2016. Fatty acid ethyl esters (FAEE) in extra virgin olive oil: A case study of a quality parameter. *LWT - Food Sci. Technol.* 66:378.
- Jabeur H., Zribi A., Abdelhedi R. and Bouaziz M. 2015. Effect of olive storage conditions on Chemlali olive oil quality and the effective role of fatty acids alkyl esters in checking olive oils authenticity. *Food Chem.* 169:289.
- Kader F., Irmouli M., Nicolas J.P. and Metche M. 2002. Involvement of blueberry peroxidase in the mechanisms of anthocyanin degradation in blueberry juice. *J. Food Sci.* 67:910.
- Mafra I., Lanza B., Reis A., Marsilio V., Campestre C., De Angelis M. and Coimbra M.A. 2001. Effect of ripening on texture, micro-structure and cell wall polysaccharide composition of olive fruit (*Olea europaea*). *Physiol. Plantarum* 111:439.
- Martín-Peláez S., Covas M.I., Fitó M., Kušar A. and Pravst I. 2013. Health effects of olive oil polyphenols: Recent advances and possibilities for the use of health claims. *Mol. Nutr. Food Res.* 57:760.
- Official Journal of the European Communities. 1991. European Community Regulation No. 2568/1991, N. L. 248 of September 5^a.
- Official Journal of the European Communities. 2001. European Community Regulation No. 1513/2001, L. 201 of July 26^a.
- Official Journal of the European Communities. 2011. European Community Regulation No. 61/2011, L. 23 of January 24^a.
- Official Journal of the European Communities. 2013. European Community Regulation No. 1348/2013, L. 338 of December 17^a.
- Official Journal of the European Communities. 2016. European Community Regulation No. 2095/2016, L. 326 of September 26^a.
- Pasqualone A., Di Rienzo V., Sabetta W., Fanelli V., Summo C., Paradiso V.M., Montemurro C. and Caponio F. 2016. Chemical and molecular characterization of crude oil obtained by olive-pomace recentrifugation. *J. Chem.* DOI: 10.1155/2016/4347207.
- Peres F., Martins L.L., Mourato M., Vitorino C., Antunes P. and Ferreira-Dias S. 2016. Phenolic compounds of 'Galega Vulgar' and 'Cobrancosa' olive oils along early ripening stages. *Food Chem.* 211:51.
- Pérez-Camino M.C., Cert A., Romero-Segura A., Cert-Trujillo R. and Moreda W. 2008. Alkyl esters of fatty acids a useful tool to detect soft deodorized olive oils. *J. Agric. Food Chem.* 56:6740.
- Ranalli A. and Angerosa F. 1996. Integral centrifuges for olive oil extraction. The qualitative characteristics of products. *JAOCS* 73: 417.
- Rotondi A., Alfei B., Magli M. and Pannelli G. 2010. Influence of genetic matrix and crop year on chemical and sensory profiles of Italian monovarietal extra-virgin olive oils. *J. Sci. Food Agric.* 90:2641.

Sánchez Moral P. and Ruiz Méndez M.V. 2006. Production of pomace olive oil. *Grasas Aceites* 57:47.

Squeo G., Silletti R., Summo C., Paradiso V.M., Pasqualone A. and Caponio F. 2017b. Fatty acids methyl and ethyl esters behaviour during olives processing by means of technological coadjuvants. *Ital. J. Food Sci.* 29:370.

Squeo G., Silletti R., Summo C., Paradiso V.M., Pasqualone A. and Caponio F. 2016. Influence of calcium carbonate on extraction yield and quality of extra virgin oil from olive (*Olea europaea* L. cv. Coratina). *Food Chem.* 209:65.

Squeo G., Tamborrino A., Pasqualone A., Leone A., Paradiso V.M., Summo C. and Caponio F. 2017a. Assessment of the influence of the decanter set-up during continuous processing of olives at different pigmentation index. *Food Bioprocess Tech.* 10:592.

Tamborrino A., Squeo G., Leone A., Paradiso V.M., Romaniello R., Summo C., Pasqualone A., Catalano P., Bianchi B. and Caponio F. 2017. Industrial trials on coadjuvants in olive oil extraction process: Effect on rheological properties, energy consumption, oil yield and olive oil characteristics. *J. Food Eng.* 205:34.

Uceda M., Beltrán G. and Jiménez A. 2006. Olive oil extraction and quality. *Grasas Aceites* 57:25.

Paper Received November 29, 2017 Accepted January 13, 2018