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Study of the influence of technological coadjuvants on enzyme activities and phenolic and volatile compounds in virgin olive oil by a response surface methodology approach

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ABSTRACT

Although the use of technological coadjuvants during virgin olive oil extraction helps in maximising the process efficiency, it could influence the characteristics of the final product. So far, why this happens is still not understood. In this context, an influence on the biochemical activities occurring during the malaxation step was supposed, and this hypothesis was verified by a response surface methodology approach. The effect of two independent variables, namely malaxation temperature and amount of coadjuvant, on polyphenol oxidase, peroxidase and lipoxygenase activities as well as on the corresponding phenolic and volatile compounds of the oils was studied. Calcium carbonate enhanced the activities of oxidases leading to a reduction of phenolic compounds, which were negatively correlated with the coadjuvant usage. Micronized natural talc behaved the opposite but had a general weaker influence, often overcome by that of mixing temperature. A significant shift of the olive paste pH toward that optimal for oxidases enzymes was highlighted under the use of calcium carbonate. The findings evidenced a "side effect" of the use of technological coadjuvants in the context of virgin olive oil extraction.

1. Introduction

During the virgin olive oil (VOO) extraction process a certain amount of oil is lost together with the by-products; this oil could be recovered in different ways but, regardless those used, it could no longer be labelled as "virgin" (Council Regulation (EC) No 1513/2001). Thus, strategies and solutions to improve the extraction efficiency are welcomed. In this context, one possible strategy is represented by the use of technological coadjuvants.

Micronized natural talc (MNT) has been deeply studied and used in practical application and, despite it is generally considered not involved in any kind of chemical or biochemical interaction with the olive paste matrix, as a technological coadjuvant actually should do, modification of the chemical characteristics of the oils have been generally found under its application. From a literature overview (Caponio et al., 2016) it emerged that talc addition could reduce the peroxide value of the VOOs and lead to an increased phenolic content. However, few hypotheses about why this happens have been proposed. Koprivnjak, Brkić Bubola, & Kosić (2016), supposed that the partition of phenols into the oily phase was enhanced because of the absorption of surfactants by MNT, thus allowing a closer contact of oily and water phases. Such absorption mechanism was then confirmed by the work of Sadkaoui, Jimenez, Pacheco, and Beltran (2017).

Over the years, other coadjuvants have been tested such as calcium carbonate (CC) (Espínola, Moya, Fernández, & Castro, 2009; Moya et al., 2010; Squeo et al., 2016; Tamborrino et al., 2017). Respect to the MNT it has several advantages, lying principally in the cheapness and safety characteristics.

Technological choices and solutions adopted during extraction could influence the final product characteristics (Di Giovacchino, Sestili, & Di Vincenzo, 2002). However, along with the mere physical effects, much more important is the indirect effect of machines and of the whole process, including the potential use of processing aids, on the biochemical activities triggered out after crushing (Clodoveo, Hbaieb, Kotti, Mugnozza, & Gargouri, 2014). While during the mixing phase thermo-mechanical phenomenon allowed oil coalescence (Clodoveo, 2012), the concomitant biochemical processes are responsible for freeing, developing and eventually oxidizing phenols and volatile compounds (Clodoveo et al., 2014; Kalua et al., 2007; Romero-Segura, García-Rodríguez, Sánchez-Ortiz, Sanz, & Pérez, 2012). In previous

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Table 1
Experimental plan and responses for the trials carried out with micronized natural talc (MNT) and calcium carbonate (CC).

Factors		Responses						Responses						
		MNT						CC						
Percentage	Temperature	PPO	POD	TPC	HTD	TYD	LOX	∑ C6 aldehydes	PPO	POD	TPC	HTD	TYD	LOX	∑ C6 aldehydes
%	°C	U g^{-1} FW	U g^{-1} FW	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	U g^{-1} FW	mg kg^{-1}	U g^{-1} FW	U g^{-1} FW	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	U g^{-1} FW	mg kg^{-1}
0	25	429.75	9864.83	208.07	22.48	35.69	881.66	274.32	313.31	5649.37	268.99	34.18	42.07	657.33	254.03
0	30	225.50	6066.46	241.33	33.76	38.85	931.93	146.35	214.24	4412.83	244.59	32.81	44.67	790.37	162.70
0	35	198.23	6353.74	287.22	56.08	43.59	1036.22	154.88	219.84	4861.73	315.73	45.51	41.57	858.67	193.62
1	25	435.38	6208.69	167.63	15.10	31.05	1027.87	286.52	381.38	9049.99	285.78	14.88	38.20	771.32	252.96
1	30	277.78	5528.15	344.50	36.06	38.84	529.55	215.19	280.80	6086.08	256.85	19.47	40.97	769.32	165.45
1	30	266.30	5560.73	302.64	27.90	42.24	675.86	173.82	261.29	7738.11	286.67	16.90	40.31	793.88	181.37
1	35	252.75	2969.39	201.52	45.60	49.97	566.50	120.46	351.84	5723.16	252.60	23.70	42.28	846.86	70.14
2	25	264.47	5125.40	296.87	15.82	31.95	618.61	269.48	247.20	4452.18	205.72	9.90	34.75	693.26	251.85
2	30	333.75	1893.92	268.90	39.07	42.53	587.40	207.11	358.11	6421.24	236.15	23.68	41.72	757.85	103.21
2	35	254.26	4928.48	321.31	44.53	43.72	696.08	139.79	437.92	10038.51	250.66	28.22	39.73	755.00	96.35

The experiments were carried out in a random order.
PPO, polyphenol oxidase; POD, peroxidase; TPC, total phenolic content; HTD, hydroxytyrosol derivatives (3,4-DHPEA-EDA and 3,4-DHPEA-EA); TYD, tyrosol derivatives (*p*-HPEA, *p*-HPEA-EDA and *p*-HPEA-EA).
LOX, lipoxygenase; ∑ C6 aldehydes, sum of Hexanal and *(E)*-2-Hexenal.

papers, we studied the effect of CC addiction on yield, energy consumption, and quality of the resulting VOOs (Squeo et al., 2016; Tamborrino et al., 2017), observing a reduction of the phenolic content. Moving from these considerations, Caponio et al. (2018) studied the effect of CC on the activity of PPO and POD, proving that the coadjuvant addition could promote the oxidases activities. It is clear that those enzyme activities might be influenced by numerous factors. In the context of VOO extraction process mostly temperature, oxygen, and pH might be in turn determinant for these biochemical activities.

Starting from the assumption that a physical coadjuvant should not have any direct effect on oil composition, we suppose that indirect effects, or "side effects", of their usage during processing on the biochemical activities might explain the evidences previously reported. According to these considerations, this work was aimed at studying the effect of MNT and CC addition during olive processing on polyphenol oxidase (PPO, EC 1.14.18.1), peroxidase (POD, EC 1.11.1.7), and lipoxygenase (LOX, EC 1.13.11.12) activities as well as the resulting phenolic and volatile compounds in VOOs. A response surface methodology approach was followed considering two independent variables, namely mixing temperature and amount of coadjuvant.

2. Materials and methods

2.1. Experimental plan

A standard face centred Central Composite Design (CCD) was set up considering two independent variables, each studied at three levels, namely amount of coadjuvant (0, 1, and 2% w/w) and malaxation temperature (25, 30, and 35 °C) for a total of 9 experiments as reported in Table 1, to which another experiment at the centre point (1% - 30 °C) was added to estimate the pure error (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). Such design allowed to build the following model:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2$$

which takes into account the linear terms, the interaction among the factors as well as the quadratic terms. As responses, or dependent variables, the enzymatic activities (PPO, POD, and LOX) and the quality indices (phenolic and volatile compounds) were considered. Two equal designs were followed, one per each of the coadjuvants. The experiments were carried out in a random order.

2.2. Technological coadjuvants

Micronized natural talc (CAS no. 14807-96-6) and calcium carbonate (CAS no. 1317-65-3) were kindly furnished by Imerys Talc (Luzenac, France) and Omya Spa (Milan, Italy), respectively.

2.3. Plant material and oil extraction

Olive fruits were harvested from trees of Ogliarola barese cv. in January 2018 in Triggiano (Bari, Italy) at a pigmentation index of 3.4 (Squeo et al., 2016). A total of about 25 kg of olives were collected and used, within 24 h after harvesting, for the experimental trials. Oil extraction was performed at laboratory scale using 1 kg per each trial, withdrawn from the homogeneous olive mass. The extraction system was made up of a semi-industrial scale hammer crusher (RETSCH GmbH 5657, Haan, Germany) working at 2850 rpm (Caponio & Catalano, 2001). The obtained olive paste was indirectly heated according to the experimental plan (Table 1) and mixed for 20 min. When due, technological coadjuvants were added at the proper concentration at the begin of mixing. About 50 g of olive paste was withdrawn after malaxation for the enzymatic assays while the oily phase was recovered by means of a basket centrifuge (Marelli Motori S.p.A., Arzignano, VI, Italy).

Table 2
Estimated models of the studied responses for the trials carried out with micronized natural talc (MNT) and calcium carbonate (CC).

Coadjuvant	Responses	Intercept	A	B	AB	A^2	B^2
MNT	PPO	290.60	−0.17	**−70.73**	55.33	−29.54	34.90
	POD	4532.06	**−1722.87**	−1157.89	828.54	460.51	1069.36
	TPC	277.21	25.08	22.91	−13.68	24.26	−46.28
	HYD	32.00	−2.15	**15.47**	−1.22	4.40	−1.66
	TYD	41.09	0.01	**6.43**	0.97	−0.95	−1.13
	LOX	644.42	−157.95	−38.22	−19.27	73.53	111.05
	∑ C6 aldehydes	187.67	6.81	**−69.20**	−2.56	−4.11	22.65
CC	PPO	293.10	**49.31**	11.28	**71.05**	−28.97	51.47
	POD	6809.54	998.00	245.31	1593.49	−1289.95	679.58
	TPC	265.34	−22.80	9.75	−0.45	−18.55	10.27
	HYD	18.16	**−8.45**	**6.41**	1.75	**10.12**	1.16
	TYD	41.57	**−2.02**	1.43	1.37	0.69	−2.26
	LOX	807.37	**73.87**	**64.23**	−46.59	**−129.83**	−24.06
	∑ C6 aldehydes	151.16	−26.49	**−66.45**	−23.77	4.04	32.64

A, percentage of coadjuvant addition (%); B, malaxation temperature (°C); PPO, polyphenol oxidase; POD, peroxidase; TPC, total phenolic content; HTD, hydroxytyrosol derivatives (3,4-DHPEA-EDA and 3,4-DHPEA-EA); TYD, tyrosol derivatives (*p*-HPEA, *p*-HPEA-EDA and *p*-HPEA-EA); LOX, lipoxygenase; ∑ C6 aldehydes, sum of Hexanal and *(E)*-2-Hexenal.
In bold are reported the significant coefficients ($p \leq 0.05$).

2.4. Temperature and pH measurements

The temperature of the olive paste during malaxation was monitored every 5 min by means of a mercury thermometer while the pH was recorded at the end of mixing using a pHmeter Basic 20 (Crison Strumenti S.p.A., Carpi, Italy) with automatic temperature compensation.

2.5. Enzymatic activities

Enzymes activities were determined on acetone powders obtained as reported in Caponio et al. (2018). POD and PPO activities were then determined according to Peres et al. (2016). One unit of POD activity was defined as the consumption of 1 μmol guaiacol min^{-1} mL^{-1} of enzyme extract using a molar absorptivity (ξ) of 26.6 mM^{-1} cm^{-1}. PPO activity was evaluated using 30 mM catechol as substrate, following the increase in absorbance at 420 nm; one unit of PPO was defined as the amount of enzyme that caused a ΔA_{420} of 0.001 min^{-1} mL^{-1} at 25 °C. LOX activities were determined according to Theerakulkait and Barrett (1995). One unit of LOX was defined as an increase in absorbance of 0.001 at 234 nm min^{-1}. All the results were expressed as U g^{-1} FW.

2.6. Phenolic characterization of VOOs

Phenolic compounds were extracted and measured by liquid-liquid extraction and spectrophotometric assay according to previous papers (Squeo et al., 2019; Zago, Squeo, Bertoncini, Difonzo, & Caponio, 2019). Quantification was obtained by means of a calibration curve of pure gallic acid and the results expressed as gallic acid equivalent (GAE, mg kg^{-1}). For the determination of single phenols the extraction was carried out using 5 g of VOO and 2 mL of methanolic mixture plus the addition of 250 μL of a 100 mg kg^{-1} solution of gallic acid as internal standard for quantification. Then the HPLC-DAD analysis was carried out as previously reported (Paradiso, Squeo, Pasqualone, Caponio, & Summo, 2019) using an UHPLC binary system (Dionex Ultimate 3000 RSLC, Waltham, MA, USA). The identification was performed by comparing the peak retention times with those obtained by the injection of pure standards and, in absence of these, with data in literature (International Olive Council, 2009). The quantification was achieved using the gallic acid internal standard and the ratio between the response factors (RRF) of gallic acid and hydroxytyrosol in order to express the results as mg of hydroxytyrosol equivalents (HTE) per kg of oil (International Olive Council, 2009). Each analysis was performed at least in duplicate.

2.7. C6 aldehydes from LOX pathway measurement

Head-space volatile C6 aldehydes from the LOX pathway were measured by means of HS-SPME-GC-MS as reported in Caponio, Leone, Squeo, Tamborrino, and Summo (2019). An Agilent 6850 series gas chromatograph coupled to a mass spectrometer Agilent 5975 series (Agilent Technologies, Santa Clara, CA, USA) and provided with a GC Sampler 80 autosampler was used. The capillary column was an HP-Innowax (60 × 0.25 mm, 0.25 μm film thickness; Agilent Technologies, Santa Clara, USA). The compounds of interest were identified by comparison of their mass spectra with the mass spectra present in the NIST and Wiley libraries. Results were expressed as mg kg^{-1} of 1-octanol equivalents (Conte et al., 2019). Each analysis was performed at least in duplicate.

2.8. Statistical analysis

Correlation analysis and models evaluation were carried out by using CAT (Chemometric Agile Tool) R-based chemometric software (R version 3.1.0 (2014-04-10)) (Leardi, Melzi, & Polotti, 2019). The coefficients were considered significant at $\alpha = 5\%$ and the results were reported numerically in Table 2 and as contour plots by Design-Expert 11 (Stat-Ease Inc., MN, USA). Significance differences in the olive paste pH were assessed by means of two-way analysis of variance at the same significance level using Minitab 17 (Minitab Inc., State College, PA, USA).

3. Results and discussion

3.1. Model quality

The experimental plan, together with the response values, is reported in Table 1 while in Table 2 are reported the estimated quadratic models for each response. Overall, a quite higher variability has been found. Considering the pure error at the central points of each response for each coadjuvant, a relative standard deviation from 0.42% to about 18% was found. Because of this high variability, the effect of the factors studied was often masked and not significant by a statistical point of view. The high experimental variability was also confirmed considering the trials carried out without coadjuvant addition (0%) for both the designs (MNT and CC) which, actually, could be considered as replicates. In those cases, the relative standard deviation ranged from 0.95% to about 38% in the case of POD activity measurement. Anyway, despite this large variability, the outcomes provided significant insights about the effect of coadjuvants during olive oil extraction and are worthy to be discussed.

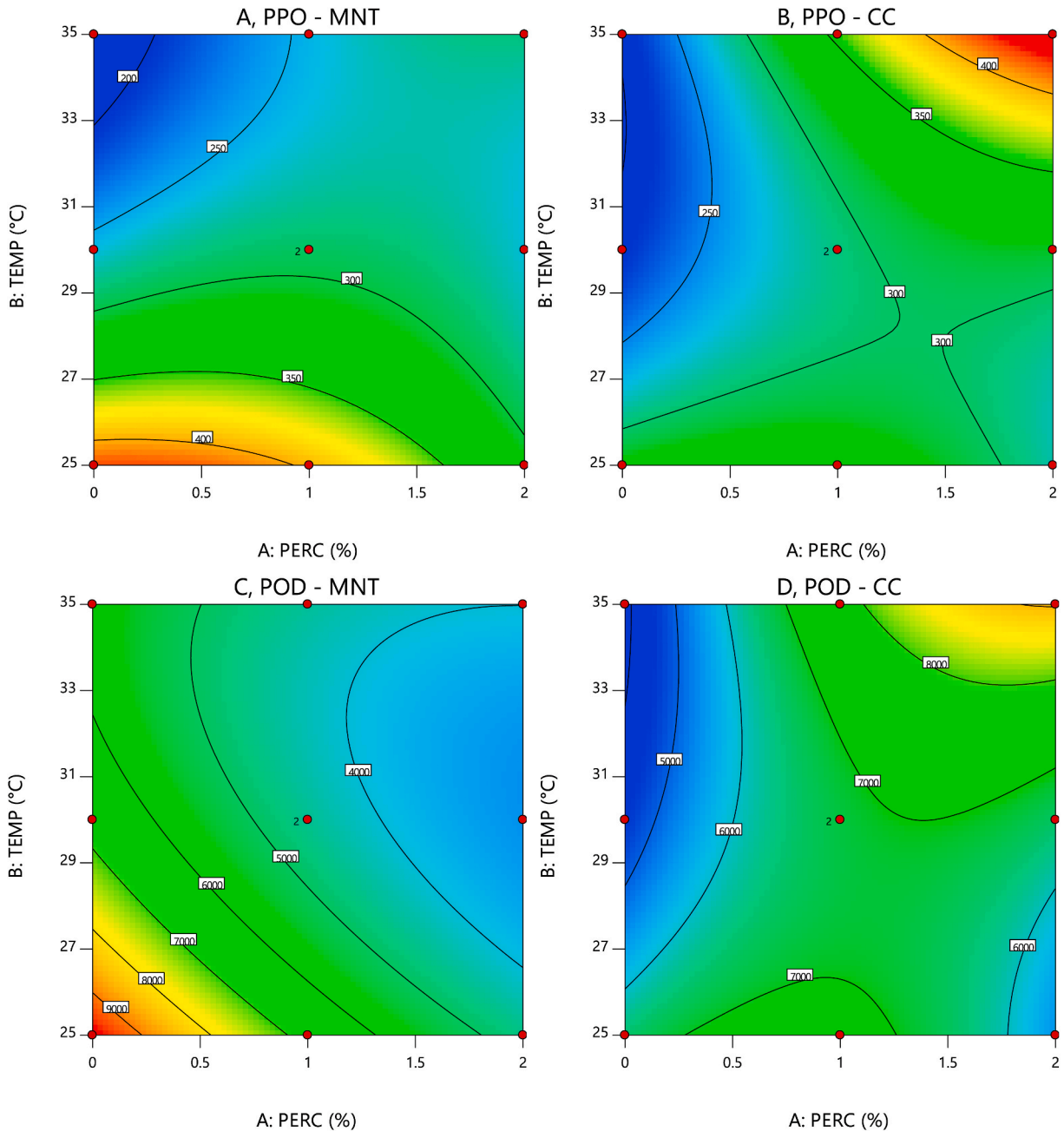

Fig. 1. Contour plots of polyphenol oxidase (PPO) and peroxidase (POD) activities using micronized natural talc (MNT, A and C, respectively) and calcium carbonate (CC, B and D, respectively). The isoresponse lines reported the results in U g^{-1} FW.

3.2. Effect on the PPO, POD and phenols

Fig. 1 reports the contour plot of the PPO activity using MNT and CC (Fig. 1A and B), respectively. The enzyme response was different comparing the two trials. In particular, when MNT was used (Fig. 1A), the coadjuvant exerted a weak and not significant activity, quite weaker than that of temperature which was found to be significant ($p = 0.037$, Table 2). The maximum activity was found at the lowest temperature and without MNT addition. On the opposite, the lowest PPO activity was found at the highest temperature and, again, without talc addition. The effect of temperature might be explained by an increased inactivation of the enzyme at 35 °C during the 20 min of malaxation (Taticchi et al., 2013). A very different pattern has been found using CC and, in this case, a significant effect of the coadjuvant ($p = 0.041$, Table 2) on the activity of PPO was observed (Fig. 1B). Indeed, increasing from 0% to 2% the addition of calcium carbonate brought to an enhanced PPO activity, being the highest at 35 °C and 2%. Caponio et al. (2018) reported similar results considering Coratina and Nociara cultivars, although the effect of the coadjuvant was observed only in stored fruits. The contour plots of the POD activity are reported in Fig. 1C and D. Consistently with the results for PPO, when MNT was used (Fig. 1C), the oxidase activity was lower, no matter the malaxation temperature used, even if a much more marked effect was reported at lower temperature. In this case, a significant negative effect of the MNT was observed ($p = 0.042$) as evidenced by the coefficient reported in Table 2. The response of POD was very similar to that of PPO in the trials with calcium carbonate (Fig. 1D), when aid addition brought to a general enhancement of the activity more marked at higher mixing temperature. The highest activity was found at 35 °C and 2% coadjuvant concentration. The enhanced effect of CC at higher temperature might be explained by the reduced viscosity of

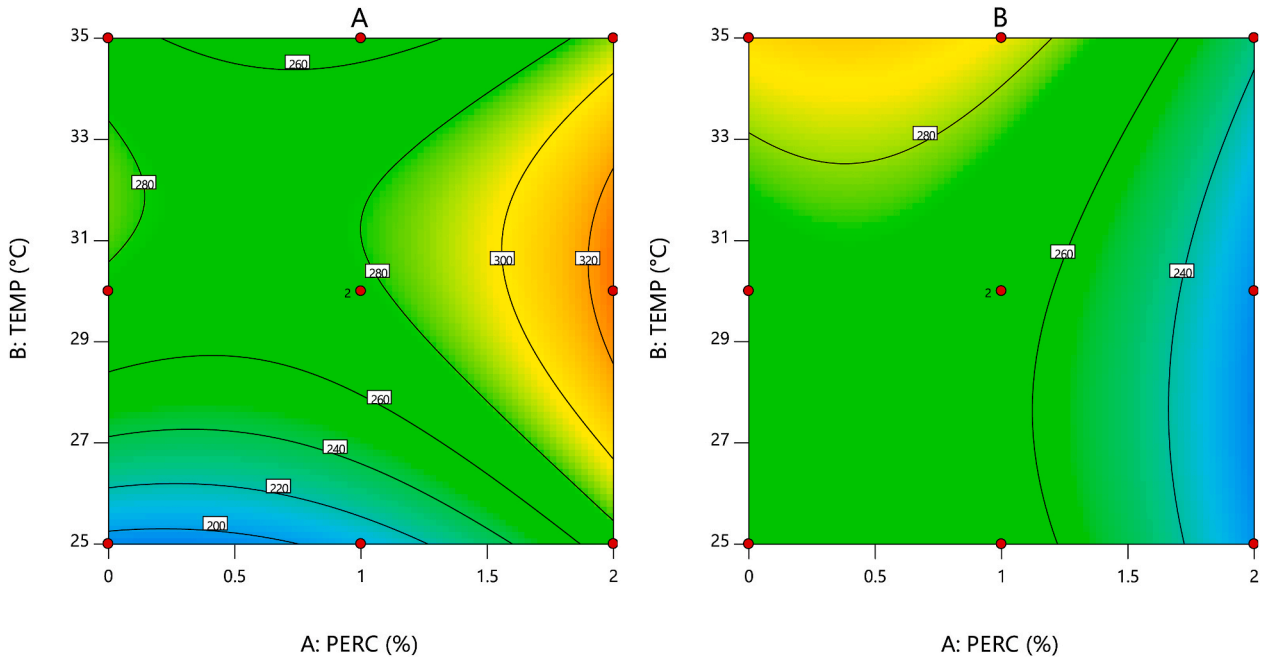

Fig. 2. Contour plots of total phenolic content (TPC) using micronized natural talc (A) and calcium carbonate (B), respectively. The isoresponse lines reported the results in mg kg^{-1}.

the matrix (olive paste) at such temperatures which likely undergoes to a closer contact with the calcium carbonate. Moreover, the solubilisation of the CC itself might be promoted by heating, overcoming the general lower water solubility of the salt (National Center for Biotechnology Information, 2019). This represents a clear example of interaction among factors, quite difficult to point out when planning the experiment in an univariate manner. The behaviour of the oxidases was confirmed by the trend of the TPC reported in Fig. 2. From Fig. 2A, considering MNT, it is evident that the lowest amount of TPC corresponded to the highest PPO and POD activities (Fig. 1A and C, respectively) which, in turn, were found at lower coadjuvant addition. This is confirmed by the correlation analysis, which showed a negative correlation among TPC and PPO ($r = -0.590$, $p = 0.072$) and TPC and POD ($r = -0.187$, $p = 0.604$). As previously stated, different authors (Aguilera, Jimenez, Sanchez-Villasclaras, Uceda, & Beltran, 2015; Caponio et al., 2014; Caponio et al., 2015; Cert, Alba, León-Camacho, Moreda, & Pérez-Camino, 1996; Espínola, Moya, de Torres, & Castro, 2015) reported an increase in the phenolic content of the oils under the usage of MNT. Our results here reported might furnish one plausible explanation about how this happens being clear that MNT addition decreased the oxidases activity.

Similar but opposite conclusions could be drawn in the case of CC (Fig. 2B) for which the statistical analysis revealed the existence of a negative correlation among TPC and coadjuvant addition ($r = -0.607$, $p = 0.063$). This negative correlation could be explained by the enhanced activity of oxidases (Fig. 1B and D).

In Fig. 3, the sum of hydroxytyrosol derivatives (HTD, namely 3,4-DHPEA-EDA and 3,4-DHPEA-EA) and the sum of tyrosol derivatives (TYD, *p*-HPEA, *p*-HPEA-EDA and *p*-HPEA-EA), were considered as a response, respectively. MNT (Fig. 3A and C), did not exert any remarkable effect on HTD nor TYD, which were much more affected by temperature (Table 2). Differently, a more marked influence could be ascribed to CC ($p = 0.002$), which usage lowered the HTD, especially at lower temperatures (Fig. 3B, Table 2). The negative effect of CC is even better highlighted considering the TYD (Fig. 3D). On the whole, these findings could give an explanation about the reduction of phenolic compounds observed as a consequence of CC usage (Squeo et al., 2016; Tamborrino et al., 2017), even if discordant results might be found in literature (Ben Brahim, Marrakchi, Gargouri, & Bouaziz, 2015; Moya et al., 2010).

3.3. Effect on LOX and volatiles

Among the oxidases, lipoxygenase is a key enzyme acting as the first of the cascade, so called lipoxygenase pathway (Kalua et al., 2007), responsible for the development of the positive fruity notes of VOOs. Fig. 4A and B report the contour plots of the LOX activity. An inverse effect of the coadjuvant was reported, as also shown by the coefficient in Table 2. More in detail, MNT (Fig. 4A) act as an inhibitor whilst CC as an enhancer (Fig. 4B). Always taking into account the great variability observed (subsection 3.1 Model quality), this last evidence confirmed the previous considerations and helps in drawing a general consideration that is, the CC addition triggered the oxidase activities. Fig. 4 (C and D), reports the sum of C6 aldehydes (*i.e.* hexanal and *(E)*-2-hexenal) for both the trials. Even though the LOX activity was lowered by the MNT usage, no effect of the coadjuvant on the LOX aldehydes has been found in the resulting VOOs. Differently, the amount of C6 aldehydes was influenced by the CC, even if in a different way respect to what expected. Indeed, one would expect a corresponding increase in LOX aldehydes (Fig. 4D) to an increased LOX activity (Fig. 4B). However, it should be considered that we focused our attention to the oxidases activity but the LOX pathway is made up of other enzymes. Particularly, after LOX, the hydroperoxide lyase (HPL) carries out the cleavage of hydroperoxides to aldehydes. So, an influence on this enzyme would explain the results reported. In fact, the general lower amount found at higher temperatures could be explained by the worsening of the environmental conditions, especially temperature, whose optimal value would have been 15 °C (Kalua et al., 2007).

3.4. Effect on olive paste pH

Although the results reported highlighted an effect of the technological coadjuvants on the enzymatic activities, and in particular the oxidases, the reason why this happened was not clear. With this regard, we suppose that coadjuvants could shift the environmental conditions of the olive paste to those optimal for oxidases activity. In these

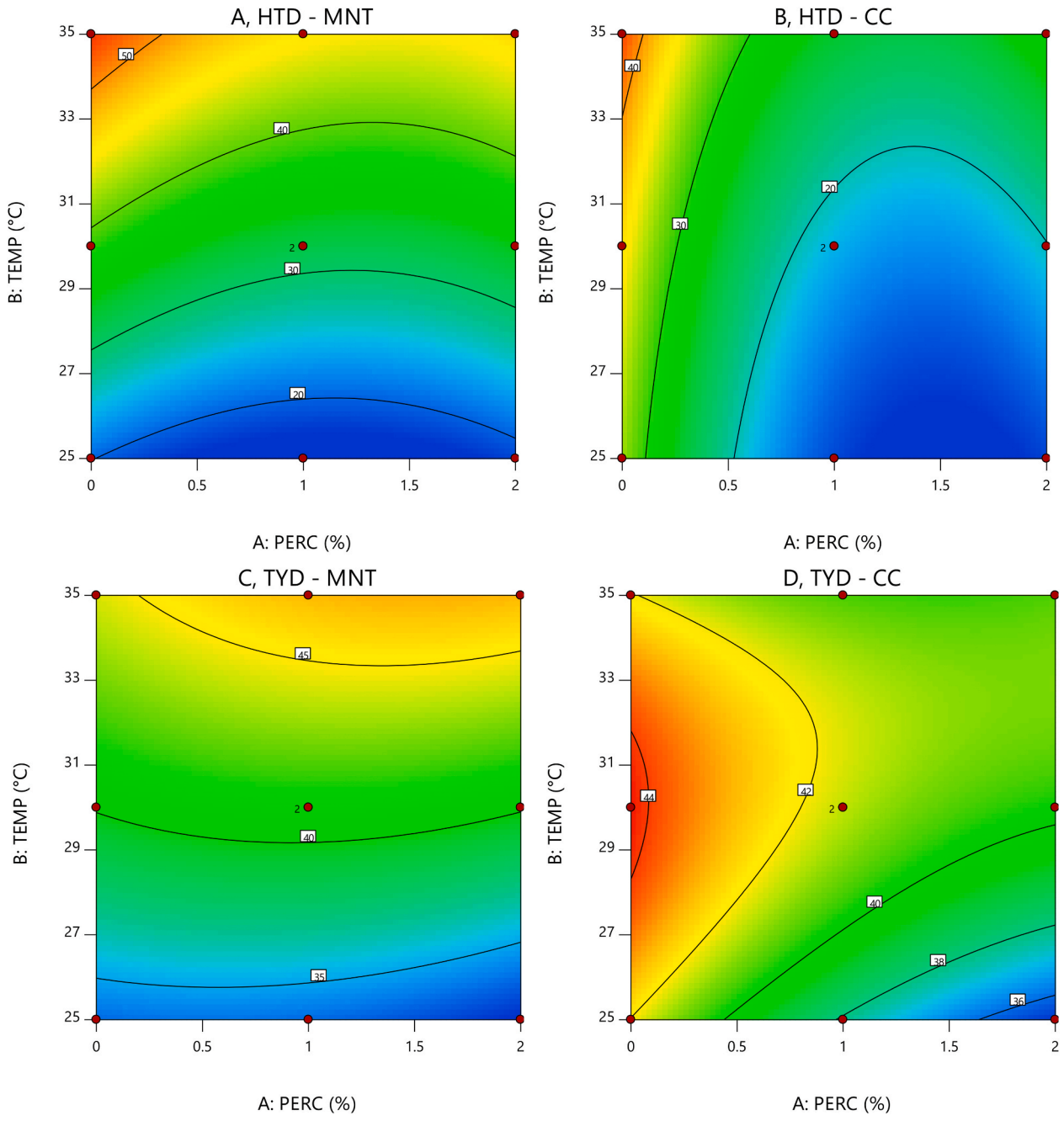

Fig. 3. Contour plots of hydroxytyrosol derivatives (HTD) and tyrosol derivatives (TYD) using micronized natural talc (MNT, A and C, respectively) and calcium carbonate (CC, B and D, respectively). The isoresponse lines reported the results in mg kg^{-1}.

experiments, temperature was under control, so we focused on modification of the pH (Table 3). A significant effect of the type of processing aid on olive paste pH was found. More in detail, a significant increase in pH was observed using CC respect to the control (no addition), reaching values close to 6, which is reported to be optimal for the activity of PPO, POD and LOX (Clodoveo et al., 2014; Kalua et al., 2007). The higher the addition the higher the pH even if without any statistical difference between 1% and 2% addition. Differently, MNT did not change significantly the olive paste pH.

4. Conclusion

The use of technological aids during VOO extraction process alters the activity of oxidase enzymes. Polyphenol oxidase and peroxidase activities, negatively correlated with the quality of the product, were enhanced by calcium carbonate addition. A "side effect" on the olive paste pH was highlighted, giving an explanation about the reported effect of calcium carbonate on the virgin olive oil quality. Micronized natural talc exerted a weaker action even if under its usage a reduction of PPO and POD activities was reported. These results, obtained at a laboratory scale, should be confirmed by industrial scale application.

CRediT authorship contribution statement

Giacomo Squeo: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Graziana Difonzo:** Investigation, Data curation, Writing - original draft. **Carmine Summo:** Conceptualization, Writing - review &

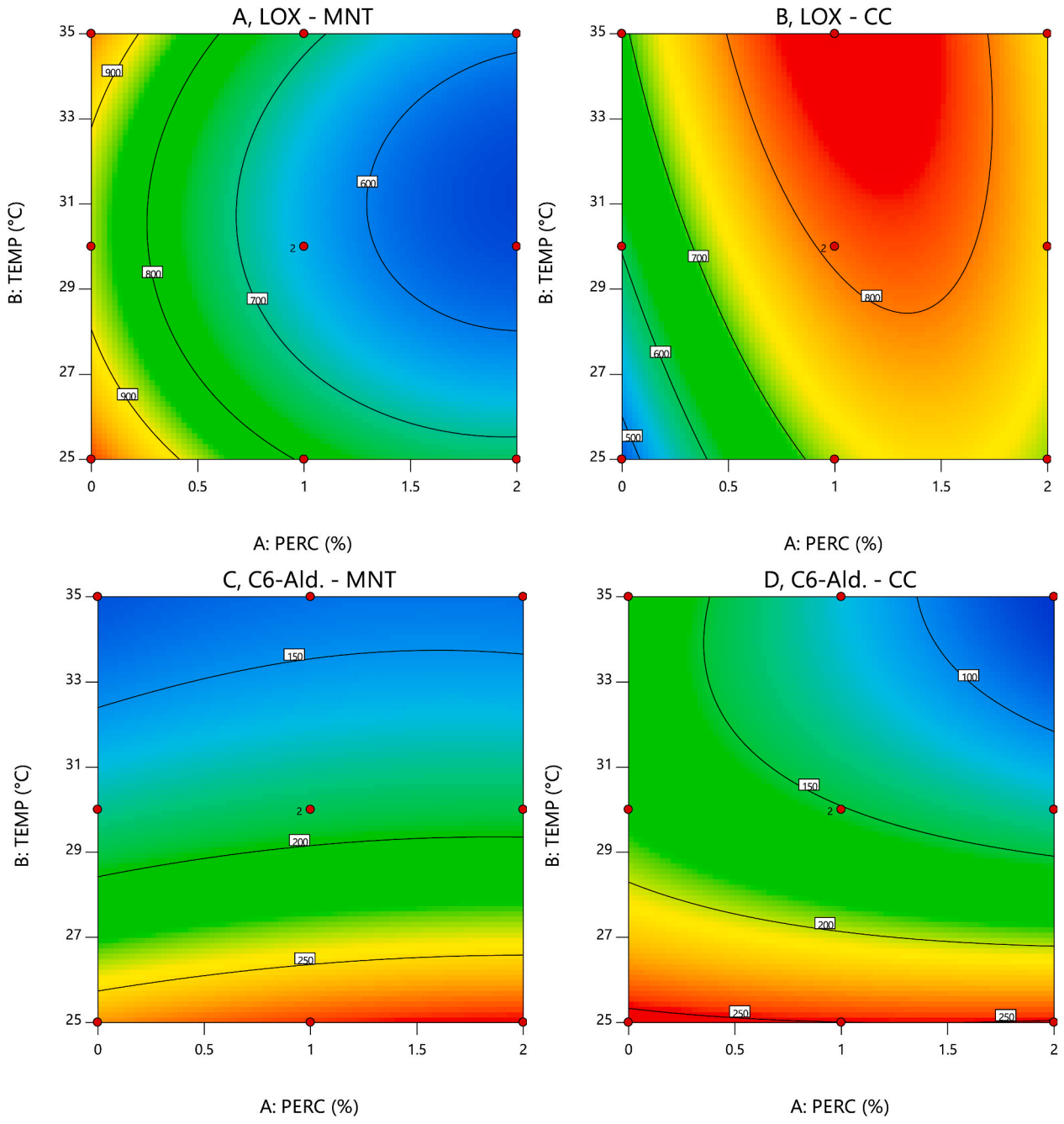

Fig. 4. Contour plots of lipoxygenase (LOX) activity and C6-aldehydes using micronized natural talc (MNT, A and C, respectively) and calcium carbonate (CC, B and D, respectively). The isoresponse lines reported the results in U g^{-1} FW (LOX) and mg kg^{-1} (C6-aldehydes).

Table 3
pH (mean ± standard deviation, $n = 3$) of the olive paste after malaxation with micronized natural talc (MNT) and calcium carbonate (CC).

Coadjuvant	Percentage	pH
MNT	0	5.57 ± 0.14 b
	1	5.38 ± 0.22 b
	2	5.45 ± 0.07 b
CC	0	5.43 ± 0.11 b
	1	5.82 ± 0.04 a
	2	5.89 ± 0.06 a

Different letters indicate significant differences at $p \leq 0.05$ according to Fisher's LSD post-hoc test.

editing. **Carmine Crecchio:** Methodology, Investigation, Data curation, Writing - review & editing. **Francesco Caponio:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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