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Optimization of the production process of dried unripe olives (*Olea europaea* L.) as a nutraceutical ingredient naturally rich in phenolic compounds

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ABSTRACT

Phenols from *Olea europaea* L. exert several beneficial effects on human health. Olive fruits, particularly the unripe ones, thanks to very high phenols contents (e.g. oleuropein, 80,000 mg/kg), can become a new source of income for olive oil producers, with only a negligible reduction of olive oil production. Aim of this research was to define the best process for obtaining dried unripe green olives very rich in phenols from *Olea europaea* L. analyzing three typical Tuscan cultivars. Four different freezing methods and different combinations of storage times and temperatures were applied to olives before lyophilization for selecting the best procedure to preserve the native phenols. Moraiolo harvested seven-ten days before complete stone lignification showed to be the most suitable cv for production of the ingredient, with oleuropein 100 g/kg and total phenols 178 g/kg. The application of liquid nitrogen immediately followed by lyophilization gave the best results, while other approaches led to losses of oleuropein of at least 68%. As far as storage before lyophilization, the best performance was for olives frozen in liquid nitrogen kept at −80 °C, with a loss of phenols less than 20% after five months, and oleuropein contents still higher than 80,000 mg/kg.

1. Introduction

In the last decades, many have been the evidences that phenolic compounds from *Olea europaea* L. exert beneficial effects for human health (Beauchamp, Keast, Morel, Lin, & Breslin, 2005; De Bock, Hodgkinson, Curtfield, & Schlothauer, 2014; Gentile et al., 2018; Giovannelli, 2013; Romani et al., 2019; Zanoni, 2014). Many of these properties have been ascribed to oleuropein (Fujiwara et al., 2017; Idrus & Saim, 2019; Liu, Dai, & Ye, 2019; Nediani, Ruzzolini, Romani, & Calorini, 2019; Omar, 2010), well-recognized as the most abundant phenol present into the whole fruit (Cecchi, Migliorini, Cherubini, Innocenti, & Mulinacci, 2015; Trapani et al., 2017). Following the many scientific evidences, food supplements based on oleuropein-rich extracts, most of them prepared from olive leaves, have been proposed with the aim of using plant resources for the protection of people's well-being (Benincasa, Santoro, Nardi, Cassano, & Sindona, 2019; Romani, Mulas, & Heimler, 2016; Somerville, Moore, & Braakhuis, 2019). Not only virgin olive oils, olive byproducts (Bellumori, Cecchi, Romani, Mulinacci, & Innocenti, 2018; Lozano-Sánchez et al., 2017) and olive leaves can be seen as a valuable source of phenols, but also the fruit, if correctly managed, becomes a very rich source of bioactive phenols. In a study of our research group (Cecchi et al., 2015), it has been demonstrated that the phenolic content in whole lyophilized olives is much higher than in fresh fruits. In fact, drying olives before crushing allows preventing, or at least minimizing, any enzymatic process affecting phenolic compounds, mainly the action of the β-glucosidase and phenoloxidases (Gutierrez-Rosales, Romero, Casanovas, Motilva, & Minguez-Mosquera, 2010 and, 2012; Rovellini & Cortesi, 2002; Kalua, Bedgood, Bishop, & Prenzler, 2006).

One of the main problems affecting the virgin olive oil producers is that olive oil is produced only in a limited period of the year, usually about 3 months, since October to January in the northern hemisphere countries. It is well-known that unripe olives are much richer in phenolic compounds than the ripe ones (Gutierrez-Rosales et al., 2012). For this reason, they can become a new source of income for the olive oil producers with a very slight reduction of olive oil production, which

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can be estimated in percentages lower than 1%. Furthermore, unripe olives harvested in the early summer are also devoid of oil and stone, thus they are free from any trace of woody material and, after lyophilization, can be easily transformed in a homogeneous dried powder. Consequently, these unripe fruits are particularly suitable as raw material to produce an ingredient for food supplements without the need of any treatment, such as the extraction processes usually applied to olive leaves for obtaining the commercial leaf extracts (Wang et al., 2019; Xie et al., 2015; Xie, Huang, Zhang, You, & Zhang, 2015; Zun-Qiu et al., 2015). Finally, it is worth underlining that the content of oleuropein detected in our previous studies (Cecchi et al., 2015) in dried olives is comparable or even higher than that in olive leaves extracts (Benincasa et al., 2019; Aouidi et al., 2012; Amidzic et al., 2018). These factors allow considering unripe dried olives as a green and environmentally friend raw material suitable for food supplement preparation only by applying physical processes.

Olives' freeze-drying process requires that olives are well frozen, in order to allow for a complete water sublimation. In this regard, immersion of olives in liquid nitrogen can be considered as an ideal freezing process, which guarantees direct contact with the freezing medium, thus rapidly reaching a very low temperature (≈ −195 °C) (Cecchi et al., 2015). The use of liquid nitrogen also requires high costs due to the high volume evaporating for freezing solid materials, and some necessary safety precautions. So far, data concerning the suitability of alternative freezing processes different from liquid nitrogen applied to this aim, are not available.

The aim of this study is to define the best process to obtain dried unripe green olives as a new ingredient very rich in phenols from *Olea europaea* L. The phenolic content of three typical Tuscan cultivars was preliminarily studied to define the suitable samples for obtaining the above-mentioned new ingredient. Four different freezing processes and two storing conditions for up to 5 months were applied to olives before lyophilization to select the best procedure to preserve the native phenolic content of fruits.

2. Material and methods

2.1. Chemicals

The Milli-Q-system (Millipore SA, Molsheim, France) was used to produce ultrapure water. Ethanol, hexane and formic acid of analytical reagent grade were from Sigma-Aldrich (Steinheim, Germany). Acetonitrile of HPLC and HPLC-MS grade were purchased from Panreac (Barcelona, Spain). Tyrosol (> 99.5%) from Sigma-Aldrich (Steinheim, Germany) and rutin (> 99%), luteolin-7-*O*-glucoside (> 98%), verbascoside (> 99%) and oleuropein (> 98%) from Extrasynthese (Genay, France) were used as standard compounds.

2.2. Samples

Samples of olive fruits (*Olea europaea* L.) from three typical Tuscan cultivars, namely Frantoio, Moraiolo and Leccino, were collected during the Summer 2019, as later as possible, before any stone lignification. For each of the three cultivars, 10 plants were selected from a farm located in Fiesole (Florence, Italy), which applied regular irrigation of orchards. Unripe olives were collected on the 41st Day After Full Blooming (DAFB), on the 51st DAFB and on the 61st DAFB, which were July 15th, July 25th and August 04th. No olive fly attack was registered in such period. On each sampling day, three samples of approx. 300 g of olives were collected for each cultivar picking the olives along the whole circumference of the selected plants at a height of about 170 cm. As soon as arrived in the laboratory, each sample was deep-frozen in liquid nitrogen and immediately freeze-dried. Table 1 reports the percentages of water removed during the lyophilization process for each sample and the weight of olives. For checking about the presence of any lignification of stone, 10 olives of each sample were crushed in the laboratory: no lignification was observed for the collected samples. Only on the third sampling day, for some olives we observed that the core of the fruit was hardened to a level that they could not bitten and broken with the teeth.

On the second harvesting day, at the 51st DAFB, 3 further batches of olives of the Moraiolo cultivar, each of approx. 3 kg, were collected and treated as described in the following paragraph 2.3.

2.3. Experimental design and treatment of olive before lyophilization

In order to test different ways to freeze the olives and the possibility to store them before lyophilization, each of the 3 batches of olives of the Moraiolo cultivar harvested on the 51st DAFB was divided in 20 aliquots. The 20 aliquots were grouped in 4 groups of 5 aliquots, for a total of 20 samples for each batch. The 5 aliquots were frozen using different freezing methods for the different groups: i) by dipping the olives in liquid N_2 for 30 s (**FN_2**), ii) into a freezer at −20 °C (samples were considered frozen after 6 h) (**F-20**), iii) into a freezer at −80 °C (samples were considered frozen after 6 h) (**F-80**), iv) into a blast chiller (Tecnodom, Padova, Italy) (samples were considered frozen after 1 cycle of 30 min) (**FBC**). For each group, one aliquot was freeze-dried immediately after freezing (**t0**), one after 40 days of storage at −20 °C (**t1-20°C**), one after 40 days of storage at −80 °C (**t1-80°C**), one after 150 days of storage at −20 °C (**t2-20°C**), and one after 150 days of storage at −80 °C (**t2-80°C**). By this way, 20 triplicates of independent samples were treated, keeping into account 4 ways to bring down the temperature, 2 storage temperatures and 2 storage times, in addition to the samples immediately lyophilized (Table s1, Supplementary material), for a total of 60 samples.

2.4. Analysis of phenolic compounds

The freeze-dried samples were minced in a laboratory miller (Zautec, Germany), thus obtaining an olive powder as homogeneous as possible, which was immediately used for extraction of phenolic compounds. The extraction was carried out following the extraction method previously reported (Cecchi et al., 2015), with some slight modifications. Briefly, 1 g olive powder was cold-extracted twice with 30 mL of EtOH:H_2O 80:20 v/v, homogenizing the mixture with Ultra-Turrax at 11.000 rpm for 4 min. The obtained solution was separated from the solid residue by cold centrifugation for 10 min at 5000 rpm, defatted twice with 30 mL of hexane and evaporated under vacuum. The residue was recovered with 8 mL of MeOH:H_2O 50:50 v/v, an aliquot of the obtained mixture was centrifuged at 14.000 rpm and the supernatant was immediately used for the chromatographic analysis. This latter analysis was carried out using an HP1100 liquid chromatograph coupled with both DAD and MS detector, the latter one equipped with HP1100 MSD API-electrospray interface (Agilent Technologies, Palo Alto, CA, USA). Separation of phenols was performed using a Poroshell 120, EC-C18 (150 mm × 3.0 mm id, 2.7 μm particle size; Agilent Technologies, Palo Alto, CA, USA) column coupled with a pre-column of the same phase, working at an oven temperature of 26 °C. The solvents for the mobile phase were (A) acetonitrile and (B) H_2O (pH 3.2 by formic acid) and elution was performed at 0.4 mL/min, with the following multistep linear gradient: solvent A varied from 5% to 40% in 40 min, stayed at 40% for 5 min, changed to 100% in 5 min, stayed at 100% for 3 min and finally returned to 5% in 2 min, for a total elution time of 55 min. Equilibration time 10 min, injection volume, 2 μL. The chromatograms were recorded at 240, 280 and 330 nm.

Five six-points calibration lines were built and used for quantitative analysis: one using tyrosol, linearity range 0–1.21 μg (R^2 = 0.9999); one using oleuropein, linearity range 0–3.16 μg (R^2 = 0.9986); one using luteolin-7-*O*-glucoside, linearity range 0–1.57 μg (R^2 = 0.9956); one using rutin, linearity range 0–1.25 μg (R^2 = 0.9975); one using verbascoside, linearity range 0–1.96 μg (R^2 = 0.9996). Using these lines, tyrosol and hydroxytyrosol were expressed as mg_{tyr}/kg, rutin as

Table 1
Moisture content of olives, measured as water lost during the lyophilization process and weight of 100 olives for samples of the Frantoio, Moraiolo and Leccino cultivars harvested on the 41st, 51st and 61st DAFB, in 2019. The value of moisture is reported as mean ± SEM calculated on three independent determinations.

Cultivar	DAFB 41		DAFB 51		DAFB 61	
	moisture (%)	weight of 100 olives (g)	moisture (%)	weight of 100 olives (g)	moisture (%)	weight of 100 olives (g)
Frantoio	67.7% ± 0.1%	43.7	62.8% ± 0.2%	66.2	60.7% ± 0.3%	76.8
Leccino	66.0% ± 1.0%	39.2	62.9% ± 0.4%	62.2	63.5% ± 0.1%	69.5
Moraiolo	67.5% ± 0.6%	44.0	63.5% ± 0.1%	63.8	57.5% ± 0.1%	73.2

mg_{rut}/kg, luteolin-7-*O*-glucoside as mg_{lut}/kg, verbascoside as mg_{ver}/kg, and the secoiridoids oleuropein, comselogoside and ligstroside as mg_{ole}/kg. Finally, the total phenolic content was evaluated in order to keep into account also the minor compounds, and it was expressed as mg_{ole}/kg, according to previous studies (Cecchi et al., 2015).

2.5. Scanning electron microscopy (SEM)

For this analysis, SEM imaging were acquired using a Gaia 3 (Tescan s.r.o, Brno, Czech Republic) focused ion beam-scanning electron microscope electron beam (voltage 15 kV), working in a high vacuum and with a secondary electron detector. Analysis was performed at the Centro di Microscopie Elettroniche (CEME) "Laura Bonzi", CNR Research Area (Florence, Italy). Samples lyophilized after different treatments were deposited on a stub, and then coated with an ultrathin coating of gold to enhance the contrast thanks to the presence of an electrically conducting material.

2.6. Statistical analysis

All the reported data are the mean of measurements from three independent samples and are reported as mean ± standard error of mean.

Two factor ANOVA was run for each phenol analyzed in olives of different cultivars harvested at different DAFB for assessing the effect of cultivar, DAFB and their interaction. Three factor ANOVA was applied to each phenol analyzed in olives lyophilized after treating and storing them with different combinations of freezing method (Freez), storage temperature (Temp) and storage time (Time): all interactions were also considered. In addition, one factor ANOVA was applied to each phenol at time 0 for evaluating the significance of the effect of the freezing method before olives' lyophilization and to water lost before lyophilization for evaluating the effect of storage; the Fisher's LSD test was applied to differentiate between mean values. All statistical analyses were run in R Studio 3.6.0 (R Studio, Boston, MA, USA).

3. Results and discussion

3.1. Characterization of the phenolic profile of unripe olives from different cultivars

The olives' weight and the moisture, evaluated as water lost during lyophilization, are reported in Table 1. The amount of the main phenolic compounds in olives is reported in Fig. 1, while Table 2 shows the significance of the effect of cultivar, harvesting day and their interaction on the variation of each phenol.

The mean weight of olives was strongly increased from the 41st to the 51st DAFB (+59% cv Leccino, +45% cv Moraiolo, +51% cv Frantoio), with a concomitant slight decrease of moisture content (Table 1). Both harvesting day (DAFB) and cultivar showed a significant effect on the content of each phenols, with only two exceptions: the DAFB for rutin and the cultivar for ligstroside (Table 2). Total phenols and oleuropein showed the highest contents for cv Leccino on the 41st DAFB and for the cv Moraiolo on the 41st and 51st DAFB, with oleuropein up to 98985.1 mg/kg and total phenols up to 178188.8 mg/kg (Fig. 1). On the 61st DAFB, a decrease of phenols content and, for some olives, a partial lignification of the stone occurred, thus making the fruits not fully suitable for the purpose of this study. Based on these data, we selected the Moraiolo at 51st DAFB as the most suitable cv and harvesting time: the sample showed the highest values of oleuropein, ligstroside, comselogoside and luteolin-7-*O*-glucoside. Thanks to the highest olives' weight and phenolic content in Moraiolo at 51st DAFB, the number of olives to be harvested can be strongly reduced, thus preserving olive oil production.

3.2. Effect of the freezing process before lyophilization on the phenolic profile of olives

The effect on the phenolic profile of the freezing process of olives immediately before lyophilization is shown in Table 3, while Table 4 (first raw) shows the effect on the percentage of water lost during lyophilization. The use of liquid nitrogen (**FN_2**) allowed for an almost complete sublimation of water, confirmed by measuring also the olives' moisture content in oven. On the contrary, the use of a blast chiller (**FBC**) or a freezer at −20 °C (**F-20**) or −80 °C (**F-80**) caused a non-complete sublimation, with 3–5% of residual water in olives. Based on the SEM analysis (Fig. 2), this behavior can be explained by the effect of the freezing process on the cellular structure of the mesocarp of the olives: when **FN_2** was used, the structure appears open and suitable for an easier sublimation of the frozen water molecules. On the contrary, when for example **F-80** was used, the internal part of the olives appears as a compact structure that reduced the amount of sublimated water (Fig. 2). The **FBC**, **F-20** and **F-80** methods cause a slower freezing process and temperatures not so low as well as using liquid nitrogen.

The use of different freezing methods before lyophilization also had a strong influence on the phenolic profile of the lyophilized olives (Table 3): the effect was very strong for the content of comselogoside and luteolin-7-*O*-glucoside (p 0.001), and particularly for oleuropein, ligstroside and total phenols ($p < 0.001$). All these molecules have a glucose moiety in their structure, thus we can assess that the observed effect is mainly due to the action of the enzyme β-glucosidase (Gutierrez-Rosales et al., 2012; Cecchi et al., 2015). This action, not observed with **FN_2**, likely took place due to the slowness of the other three freezing methods, which causes a damage of tissues and a higher amount of residual water, allowing for significant activity of the β-glucosidase. This hypothesis was confirmed by the non-significant effect ($p > 0.05$) observed for the molecules bearing either sugar moieties other than simple glucose (rutin and verbascoside) or no sugar moieties (tyrosol and hydroxytyrosol). For the molecules that showed a significant effect, the highest content was always for the olives treated with **FN_2** (Table 3) in accordance with expectations. Among the other freezing methods, we initially hypothesized that **F-80** and/or **FBC** could give better results than **F-20**. Despite these expectations, **F-20** gave the best results, followed by **FBC** and **F-80**. For example, the content of oleuropein, which reached a value of almost 100,000 mg/kg when **FN_2** was used, strongly decreased when **F-20** (31538.3, −68%), **F-80** (17712.2, −82%) and **FBC** (23146.7, −77%) were used. This datum confirms that freezing olives using **FN_2** before lyophilization is by far the most suitable method for obtaining an ingredient very rich in phenolic compounds, and particularly in oleuropein, the main bioactive

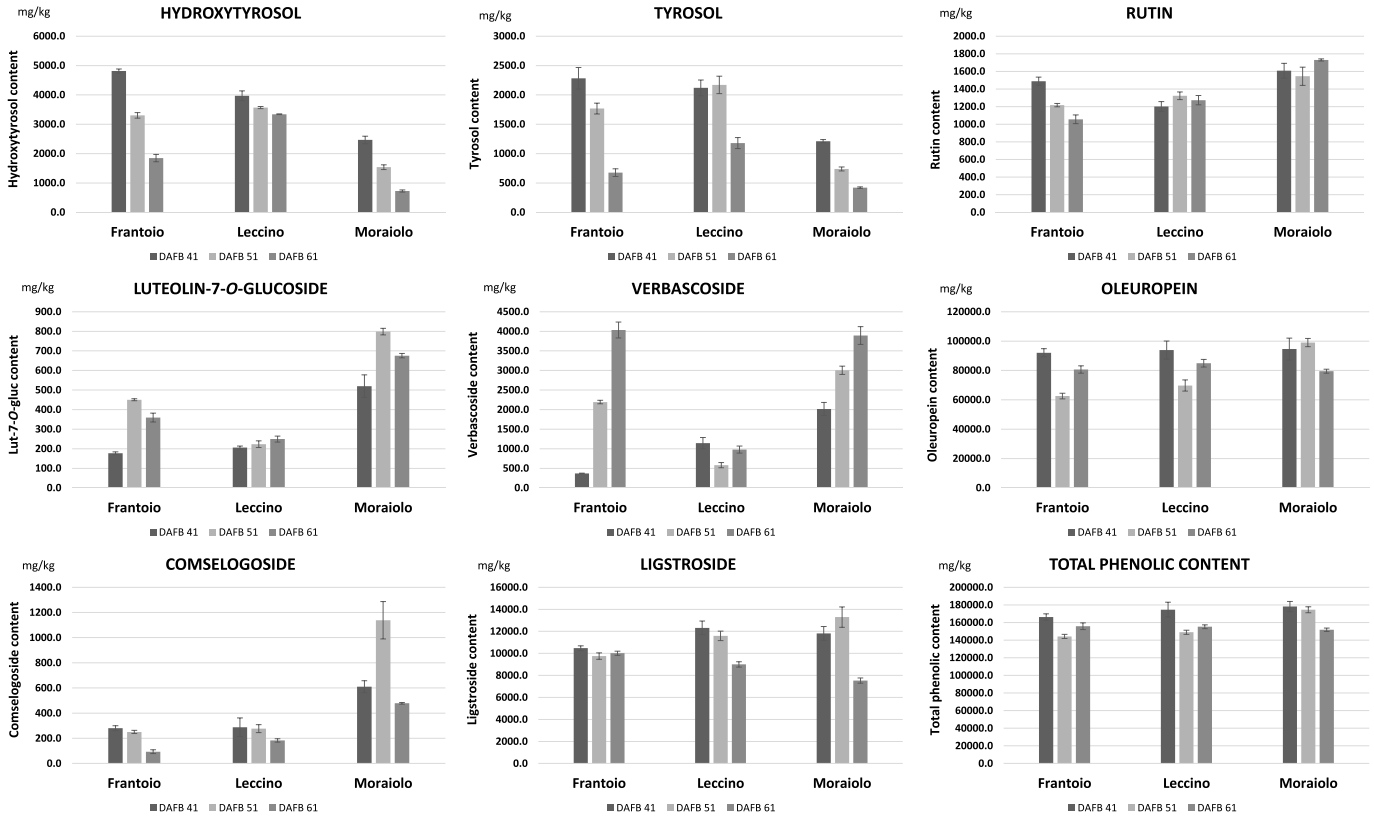

Fig. 1. Evolution of the content of the main phenolic compounds in unripe olives of Frantoio, Moraiolo and Leccino cultivars at three different harvesting times. Data are obtained from three independent batches for each sample and are reported as mean of three measurements ± standard error of mean. DAFB, Days After Full Blooming.

Table 2
p-values calculated for each molecule by two factor ANOVA, where the two factors were the harvesting day (DAFB) and the cultivar (cv). The two-way interaction DAFB × cv was also considered. Significant values ($p < 0.05$) are reported in italic.

	Hydroxytyrosol	Tyrosol	Rutin	Luteolin-7-*O*-glucoside	Verbascoside	Oleuropein	Comselogoside	Ligstroside	TPC
DAFB	*2.5E-14*	*2.8E-12*	0.21	*3.8E-08*	*1.9E-11*	*2.5E-04*	*3.1E-05*	*1.5E-06*	*5.1E-05*
cv	*5.5E-16*	*8.0E-12*	*2.6E-07*	*2.2E-14*	*1.5E-12*	*3.7E-03*	*1.9E-09*	0.07	*5.4E-03*
DAFB × cv	*8.9E-09*	*4.1E-05*	*6.9E-04*	*1.6E-04*	*1.4E-09*	*5.9E-04*	*2.9E-04*	*1.5E-04*	*6.6E-03*

Table 3
Amount of the phenolic compounds detected in olives lyophilized immediately after freezing with four different methods, namely liquid nitrogen, freezer at −20 °C, freezer at −80 °C and blast chiller. Data are mean ± SEM. For each compound, the last column reports the *p*-value obtained by the ANOVA, with the significant ones ($p < 0.05$) reported in italic; for those compounds that showed significant differences at $p < 0.05$, different letters in each raw indicate significant differences given by the treatments.

Freezing method	N_2 liq	−20°C	−80°C	blast chiller	*p* value
Compound					
Hydroxytyrosol (mg/kg)	1537.6 ± 79.9	1657.4 ± 27.6	1658.7 ± 140.7	1573.2 ± 102.0	0.758
Tyrosol (mg/kg)	653.8 ± 9.4	692.2 ± 5.8	682.4 ± 39.7	647.2 ± 26.5	0.531
Rutin (mg/kg)	1545.5 ± 103.3	1617.9 ± 17.0	1310.1 ± 119.1	1487.8 ± 77.0	0.163
Luteolin-7-*O*-glucoside (mg/kg)	798.7 ± 16.7 a	666.3 ± 27.8 b	581.9 ± 37.8 c	605.2 ± 11.1 bc	*0.001*
Verbascoside (mg/kg)	3004.7 ± 107.3	3146.7 ± 128.5	2828.6 ± 214.9	2894.6 ± 114.5	0.488
Oleuropein (mg/kg)	98985.1 ± 2842.0 a	31538.3 ± 2137.4 b	17712.2 ± 2370.4 c	23146.7 ± 6499.9 bc	*< 0.001*
Comselogoside (mg/kg)	1137.8 ± 148.8 a	733.8 ± 51.5 b	329.2 ± 10.2 c	588.1 ± 74.8 bc	*0.001*
Ligstroside (mg/kg)	13293.4 ± 926.6 a	3606.7 ± 107.5 b	2116.7 ± 155.0 b	2459.5 ± 496.9 b	*< 0.001*
Total Phenols (mg/kg)	**191784.0 ± 4705.6 a**	**156039.1 ± 3584.7 b**	**137223.7 ± 4467.2 c**	**136135.7 ± 2188.5 c**	***< 0.001***

molecule in unripe olives (Ahamad et al., 2019; Amidzic et al., 2018; Fujiwara et al., 2017; Gamli, 2016; Gutierrez-Rosales et al., 2012, 2010; Omar, 2010). The use of a freezer makes higher the amounts of olives necessary to obtain the same amount of total oleuropein in the obtained powder. On the other side, liquid nitrogen has high costs and comes safety drawbacks, thus the choose of olives refrigeration method should be carefully evaluated on case by case basis. The other molecules were present in amounts of one (ligstroside) or two orders of magnitude lower than oleuropein. For the ones bearing the glucose moiety, the effect of freezing methods different from FN_2 was similar to oleuropein,

Table 4
Water lost during the lyophilization process for samples of the Moraiolo cultivar harvested on the 51st DAFB, and lyophilized after different combinations of freezing method, time and temperature of storage before lyophilization. The value of moisture is reported as mean ± SEM calculated on three independent determinations. Different letters in each raw indicate significant differences given by the treatments at each storage time.

Freezing	Liq N_2		−20 °C		−80 °C		blast chiller	
Storing	−20 °C	−80 °C	−20 °C	−80 °C	−20 °C	−80 °C	−20 °C	−80 °C
t 0	63.5 ± 0.1 a		60.8 ± 0.8 b		57.3 ± 1.2 c		56.7 ± 0.6 c	
40 days	61.6 ± 0.3 b	63.8 ± 0.3 a	55.6 ± 0.3 c	55.2 ± 0.1 c	52.3 ± 0.5 d	52.6 ± 0.8 d	55.9 ± 1.2 c	45.5 ± 0.3 e
150 days	62.4 ± 0.2 a	63.0 ± 0.2 a	56.2 ± 2.1 b	44.8 ± 0.5 d	52.7 ± 1.7 c	42.0 ± 0.6 d	58.7 ± 0.4 b	41.8 ± 1.2 d

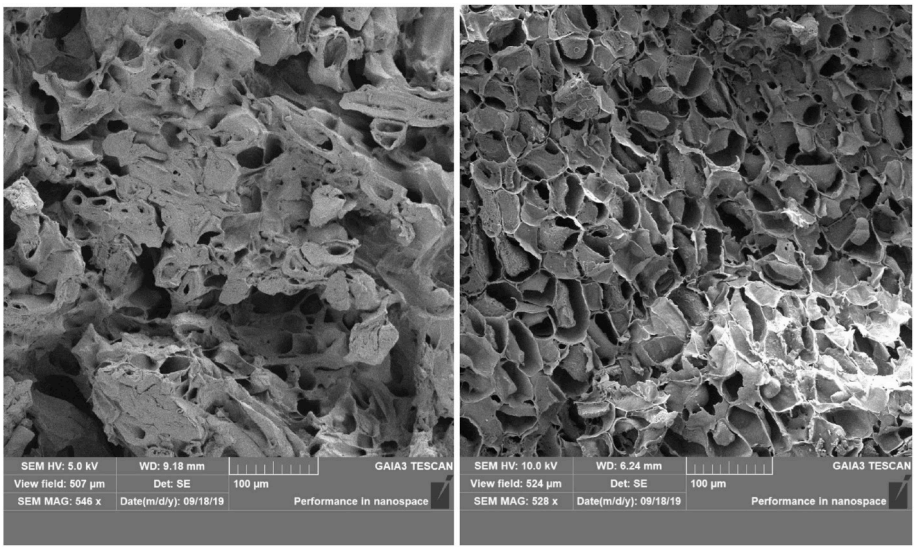

Fig. 2. SEM micrograph of (A) olives dried after freezing at −80 °C and (B) olives dried with liquid nitrogen.

with **F-80** and **F-20** that caused the highest and lowest degradation of phenols, respectively.

3.3. Effect of storage time and temperature on frozen olives and their phenolic profiles

Since a storage of fruits before freeze-drying could be necessary, this last part of the study was aimed at exploring the effect of different storage methods on olives before lyophilization. Fig. 3 shows the evolution of phenols in olives frozen with the 4 different freezing methods and stored up to 150 days at −80 °C or −20 °C. Table 5 shows the significance of each treatment (freezing method, storage time and storage temperature and their interactions) on the variation of each phenol. Oleuropein and ligstroside, the main phenols in unripe olives, showed a similar behavior: in particular, when olives were stored at −80 °C for 150 days, the content in olives frozen with **FN_2** decreased of 13% for oleuropein and 22% for ligstroside, with respect to the olives immediately lyophilized. In the case of the other three freezing methods, the decrease ranged 95–97% for oleuropein and 94–96% for ligstroside. When olives were stored at −20 °C for 150 days, the content in olives frozen with **FN_2** decreased of 32% for oleuropein and 35% for ligstroside with respect to the olives immediately lyophilized. In the case of the other freezing methods, the decrease ranged 62–87% for oleuropein and 73–88% for ligstroside. It is also worth noting that, when olives frozen by **F-20** and **FBC** were stored at −20 °C, the content of oleuropein and ligstroside only showed negligible variations.

All these data confirmed that **FN_2** guarantees the best performance also when frozen olives are stored at −20 °C or, better, at −80 °C, before lyophilization. The data also suggest that, when liquid nitrogen is not available, the freezer at −20 °C gives better results than the freezer at −80 °C both for freezing olives and for storing them.

4. Conclusions

The best conditions for the production of an ingredient in the form of dried powder obtained from unripe green olives, and naturally rich in phenols from *Olea europaea* L., were defined. The need of working with fruits harvested before any stone lignification was pointed out. We demonstrated that the use of liquid nitrogen for freezing olives immediately before lyophilization gave by far the best results in terms of phenolic content of the obtained powder. All the green fruits of the studied Tuscan varieties showed very high and comparable contents of oleuropein and total phenols. Olives frozen in liquid nitrogen can be stored at −80 °C for five months before lyophilization, with losses of phenols lower than 20%. When liquid nitrogen is not available, the use of other devices as freezer or blast chiller for freezing and storing olives before lyophilization leads to losses of more than 70% of the native content of oleuropein and ligstroside.

The very high content of phenols detected in the selected cultivars allows for preparation of the powdered ingredient without the need of any extraction process. This new ingredient from green olives is thus different from the typical commercial extracts usually obtained during the pruning period from olive leaves. The production of such ingredient will allow for a diversification of the production of virgin olive oil producers without negatively affecting the olive oil yields, thus giving them a new possibility of income.

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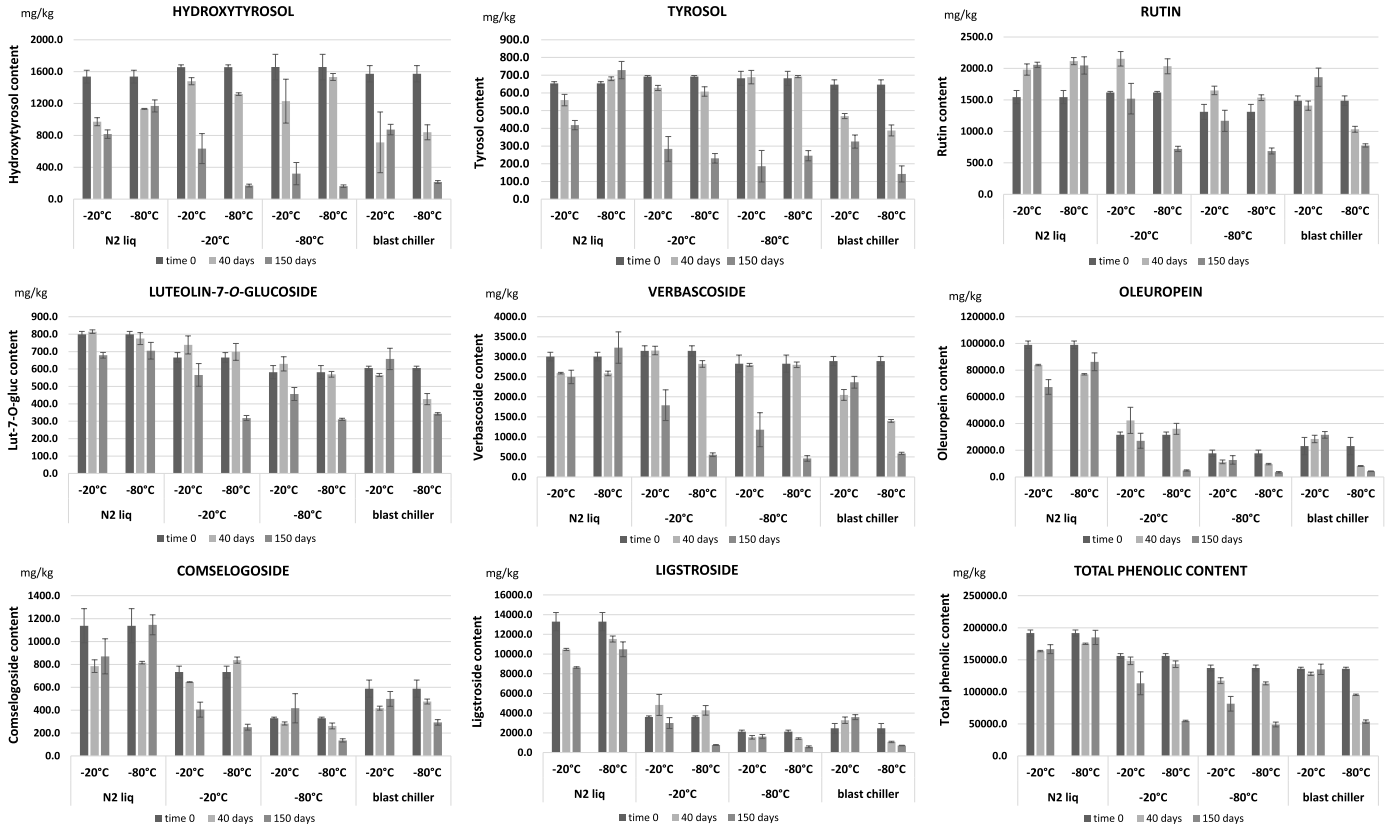

Fig. 3. Content of the main phenolic compounds in unripe olives of the Moraiolo cultivar harvested at the 51st DAFB, lyophilized after different treatments (freezing method, storage time and temperature between freezing and lyophilizing). Data are obtained from three independent batches for each sample and are reported as the mean of the three measurements ± standard error of mean. DAFB, Days After Full Blooming.

Table 5
p-values calculated for each molecule by three factor ANOVA, where the three factors were the freezing method (Freez), the storage temperature after freezing and before lyophilization (Temp) and the storage time after freezing and before lyophilization (Time). All the two-way (Freez × Temp, Freez × Time and Temp × Time) and three-way interactions were considered. Significant values ($p < 0.05$) are reported in italic.

	Hydroxytyrosol	Tyrosol	Rutin	Luteolin-7-*O*-glucoside	Verbascoside	Oleuropein	Comselogoside	Ligstroside	TPC
Freez	*0.03*	*8.4E-08*	*6.9E-11*	*2.5E-13*	*2.2E-07*	*2.0E-27*	*1.8E-34*	*6.1E-30*	*1.4E-27*
Temp	0.37	0.38	*5.6E-06*	*3.6E-06*	*1.3E-04*	*6.8E-04*	0.98	0.01	*5.0E-04*
Time	*2.7E-18*	*1.5E-19*	*2.5E-07*	*1.2E-10*	*7.4E-16*	*3.1E-06*	*8.1E-05*	*2.7E-07*	*8.3E-08*
Freez × Temp	0.05	*1.8E-04*	*4.0E-03*	*0.02*	*6.5E-04*	*4.0E-03*	0.22	*7.5E-04*	*1.6E-03*
Freez × Time	*2.3E-06*	*1.0E-08*	*2.2E-08*	*6.8E-04*	*1.1E-09*	*2.0E-03*	*6.1E-04*	*1.1E-04*	*1.5E-03*
Temp × Time	*0.03*	0.67	*1.6E-05*	*2.5E-04*	*2.1E-03*	0.06	0.17	0.12	*0.04*
3rd order	0.16	*0.02*	0.16	0.11	*6.7E-03*	*0.01*	0.23	0.10	*0.02*

CRediT authorship contribution statement

Lorenzo Cecchi: Formal analysis, Conceptualization, Methodology, Investigation, Writing - original draft. **Lorenzo Guerrini:** Data curation, Methodology, Conceptualization. **Maria Bellumori:** Formal analysis, Investigation. **Diletta Balli:** Data curation, Methodology. **Pujun Xie:** Resources, Conceptualization. **Alessandro Parenti:** Conceptualization, Funding acquisition. **Nadia Mulinacci:** Supervision, Funding acquisition, Conceptualization, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.109569.

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